THE PROKARYOTES

Third Edition

A Handbook on the Biology of Bacteria:

Proteobacteria: Alpha and Beta Subclasses

Edited by
MARTIN DWORKIN (EDITOR-IN-CHIEF)
STANLEY FALKOW
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Volume 5

Springer

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Martin Dworkin (Editor-in-Chief), Stanley Falkow, Eugene Rosenberg,
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Springer

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Library of Congress Control Number: 91017256

Volume 5
ISBN-10: 0-387-25495-1
ISBN-13: 978-0387-25495-1
e-ISBN: 0-387-30745-1
Print + e-ISBN: 0-387-33492-0
DOI: 10.1007/0-387-30745-1

Volumes 1–7 (Set)
ISBN-10: 0-387-25499-4
ISBN-13: 978-0387-25499-9
e-ISBN: 0-387-30740-0
Print + e-ISBN: 0-387-33488-2

Printed on acid-free paper.

Printed in Singapore. (BS/KYO)

9 8 7 6 5 4 3 2 1

springer.com

Preface

Each of the first two editions of *The Prokaryotes* took a bold step. The first edition, published in 1981, set out to be an encyclopedic, synoptic account of the world of the prokaryotes—a collection of monographic descriptions of the genera of bacteria. The Archaea had not yet been formalized as a group. For the second edition in 1992, the editors made the decision to organize the chapters on the basis of the molecular phylogeny championed by Carl Woese, which increasingly provided a rational, evolutionary basis for the taxonomy of the prokaryotes. In addition, the archaea had by then been recognized as a phylogenetically separate and distinguishable group of the prokaryotes. The two volumes of the first edition had by then expanded to four. The third edition was arguably the boldest step of all. We decided that the material would only be presented electronically. The advantages were obvious and persuasive. There would be essentially unlimited space. There would be no restrictions on the use of color illustrations. Film and animated descriptions could be made available. The text would be hyperlinked to external sources. Publication of chapters would be seriati—the edition would no longer have to delay publication until the last tardy author had submitted his or her chapter. Updates and modifications could be made continuously. And, most attractively, a library could place its subscribed copy on its server and make it available easily and cheaply to all in its community. One hundred and seventy chapters have thus far been presented in 16 releases over a six-year period. The virtues and advantages of the online edition have been borne out. But we failed to predict the affection that many have for holding a bound, print version of a book in their hands. Thus, this print version of the third edition shall accompany the online version.

We are now four years into the 21st century. Indulge us then while we comment on the challenges, problems and opportunities for microbiology that confront us.

Moselio Schaechter has referred to the present era of microbiology as its third golden age—the era of "integrative microbiology." Essentially all microbiologists now speak a common language. So that the boundaries that previously separated subdisciplines from each other have faded: physiology has become indistinguishable from pathogenesis; ecologists and molecular geneticists speak to each other; biochemistry is spoken by all; and—mirabile dictu!—molecular biologists are collaborating with taxonomists.

But before these molecular dissections of complex processes can be effective there must be a clear view of the organism being studied. And it is our goal that these chapters in *The Prokaryotes* provide that opportunity.

There is also yet a larger issue. Microbiology is now confronted with the need to understand increasingly complex processes. And the modus operandi that has served us so successfully for 150 years—that of the pure culture studied under standard laboratory conditions—is inadequate. We are now challenged to solve problems of multimembered populations interacting with each other and with their environment under constantly variable conditions. Carl Woese has pointed out a useful and important distinction between empirical, methodological reductionism and fundamentalist reductionism. The former has served us well; the latter stands in the way of our further understanding of complex, interacting systems. But no matter what kind of synoptic systems analysis emerges as our way of understanding host–parasite relations, ecology, or multicellular behavior, the understanding of the organism as such is sine qua non. And in that context, we are pleased to present to you the third edition of *The Prokaryotes*.

Martin Dworkin
Editor-in-Chief

Foreword

The purpose of this brief foreword is unchanged from the first edition; it is simply to make you, the reader, hungry for the scientific feast that follows. These four volumes on the prokaryotes offer an expanded scientific menu that displays the biochemical depth and remarkable physiological and morphological diversity of prokaryote life. The size of the volumes might initially discourage the unprepared mind from being attracted to the study of prokaryote life, for this landmark assemblage thoroughly documents the wealth of present knowledge. But in confronting the reader with the state of the art, the Handbook also defines where more work needs to be done on well-studied bacteria as well as on unusual or poorly studied organisms.

This edition of *The Prokaryotes* recognizes the almost unbelievable impact that the work of Carl Woese has had in defining a phylogenetic basis for the microbial world. The concept that the ribosome is a highly conserved structure in all cells and that its nucleic acid components may serve as a convenient reference point for relating all living things is now generally accepted. At last, the phylogeny of prokaryotes has a scientific basis, and this is the first serious attempt to present a comprehensive treatise on prokaryotes along recently defined phylogenetic lines. Although evidence is incomplete for many microbial groups, these volumes make a statement that clearly illuminates the path to follow.

There are basically two ways of doing research with microbes. A classical approach is first to define the phenomenon to be studied and then to select the organism accordingly. Another way is to choose a specific organism and go where it leads. The pursuit of an unusual microbe brings out the latent hunter in all of us. The intellectual challenges of the chase frequently test our ingenuity to the limit. Sometimes the quarry repeatedly escapes, but the final capture is indeed a wonderful experience. For many of us, these simple rewards are sufficiently gratifying so that we have chosen to spend our scientific lives studying these unusual creatures. In these endeavors many of the strategies and tools as well as much of the philosophy may be traced to the Delft School, passed on to us by our teachers, Martinus Beijerinck, A. J. Kluyver, and C. B. van Niel, and in turn passed on by us to our students.

In this school, the principles of the selective, enrichment culture technique have been developed and diversified; they have been a major force in designing and applying new principles for the capture and isolation of microbes from nature. For me, the "organism approach" has provided rewarding adventures. The organism continually challenges and literally drags the investigator into new areas where unfamiliar tools may be needed. I believe that organism-oriented research is an important alternative to problem-oriented research, for new concepts of the future very likely lie in a study of the breadth of microbial life. The physiology, biochemistry, and ecology of the microbe remain the most powerful attractions. Studies based on classical methods as well as modern genetic techniques will result in new insights and concepts.

To some readers, this edition of the *The Prokaryotes* may indicate that the field is now mature, that from here on it is a matter of filling in details. I suspect that this is not the case. Perhaps we have assumed prematurely that we fully understand microbial life. Van Niel pointed out to his students that—after a lifetime of study—it was a very humbling experience to view in the microscope a sample of microbes from nature and recognize only a few. Recent evidence suggests that microbes have been evolving for nearly 4 billion years. Most certainly those microbes now domesticated and kept in captivity in culture collections represent only a minor portion of the species that have evolved in this time span. Sometimes we must remind ourselves that evolution is actively taking place at the present moment. That the eukaryote cell evolved as a chimera of certain prokaryote parts is a generally accepted concept today. Higher as well as lower eukaryotes evolved in contact with prokaryotes, and evidence surrounds us of the complex interactions between eukaryotes and

prokaryotes as well as among prokaryotes. We have so far only scratched the surface of these biochemical interrelationships. Perhaps the legume nodule is a pertinent example of nature caught in the act of evolving the "nitrosome," a unique nitrogen-fixing organelle. Study of prokaryotes is proceeding at such a fast pace that major advances are occurring yearly. The increase of this edition to four volumes documents the exciting pace of discoveries.

To prepare a treatise such as *The Prokaryotes* requires dedicated editors and authors; the task has been enormous. I predict that the scientific community of microbiologists will again show its appreciation through use of these volumes—such that the pages will become "dog-eared" and worn as students seek basic information for the hunt. These volumes belong in the laboratory, not in the library. I believe that a most effective way to introduce students to microbiology is for them to isolate microbes from nature, i.e., from their habitats in soil, water, clinical specimens, or plants. *The Prokaryotes* enormously simplifies this process and should encourage the construction of courses that contain a wide spectrum of diverse topics. For the student as well as the advanced investigator these volumes should generate excitement.

Happy hunting!

Ralph S. Wolfe
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Prokaryotes (2006) 5:3–37
DOI: 10.1007/0-387-30745-1_1

Introduction to the Proteobacteria

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Introduction

Within the domain Bacteria, the phylum Proteobacteria constitutes at present the largest and phenotypically most diverse phylogenetic lineage. In 1988, Stackebrandt et al. named the Proteobacteria after the Greek god Proteus, who could assume many different shapes, to reflect the enormous diversity of morphologies and physiologies observed within this bacterial phylum. In 2002, the Proteobacteria consist of more than 460 genera and more than 1600 species, scattered over 5 major phylogenetic lines of descent known as the classes "Alphaproteobacteria," "Betaproteobacteria," "Gammaproteobacteria," "Deltaproteobacteria" and "Epsilonproteobacteria." The Proteobacteria account for more than 40% of all validly published prokaryotic genera and encompass a major proportion of the traditional Gram-negative bacteria, show extreme metabolic diversity, and are of great biological importance, as they include the majority of the known Gram-negatives of medical, veterinary, industrial and agricultural interest. Moreover, not only can the origin of mitochondria be traced back to the "Alphaproteobacteria," but several representatives are ecologically important because they play key roles in the carbon, sulfur and nitrogen cycles on our planet. In this context, the purple nonsulfur bacteria and the rhizobia (both members of the "Alphaproteobacteria") are two of the most apparent and well-known examples, because the former are purple-colored photosynthetic prokaryotes using light as energy source, and the latter are able to reduce atmospheric nitrogen gas when living in symbiosis with leguminous plants. Agriculture and life on earth would be very different in the absence of these nitrogen-fixing rhizobia!

The Proteobacteria, formerly known as "purple bacteria and relatives," are characterized by a bewildering diversity of morphological and physiological types: besides rods and cocci, curved, spiral, ring-shaped, appendaged, filamentous and sheathed bacteria occur among this phylum. Most Proteobacteria are mesophilic, but some thermophilic (e.g., *Thiomonas thermosulfata* and *Tepidomonas*) and psychrophilic (e.g., *Polaromonas*) representatives have been described. A great number of Proteobacteria are motile by means of polar or peritrichous flagella, whereas the myxobacteria (belonging to the "Deltaproteobacteria") display a gliding type of motility and show highly complex developmental lifestyles, whereby often remarkable multicellular and macroscopic structures (so-called "fruiting bodies") are formed. Most of the known Proteobacteria are free-living; some (such as the rhizobia) enter in symbiotic associations with specific leguminous plants, where they fix nitrogen in root or stem nodules. Others live as intracellular endosymbionts of protozoa and invertebrates (mussels, insects and nematodes), whereas the rickettsiae are obligate intracellular parasites of humans or mammals. The extreme diversity of energy-generating mechanisms is a unique biochemical characteristic of the Proteobacteria: some are chemoorganotrophs (e.g., *Escherichia coli*), others are chemolithotrophs (e.g., the sulfur-oxidizing bacteria such as the thiobacilli and the ammonia-oxidizing bacteria such as *Nitrosomonas*) or phototrophs (e.g., the purple colored *Chromatium*, *Rhodospirillum* and many others). Concerning their relationship towards oxygen, the Proteobacteria include strictly aerobic and anaerobic species as well as facultative aerobes and microaerophiles. Denitrifiers are reported among the "Alphaproteobacteria," "Betaproteobacteria," "Gammaproteobacteria," and the "Epsilonproteobacteria." To reveal the extreme phenotypic biodiversity among the Proteobacteria, Table 1 gives an overview of the distribution of the five phylogenetic proteobacterial groups among 13 major phenotypic groups as defined in the 9th edition of *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994). Table 1 clearly shows that only three of these 13 phenotypic groups are unique to any one of the current proteobacterial classes. Representatives of some of these 13 phenotypic groups occur also among several other prokaryotic phyla.

Table 1. The occurrence of major phenotypic groups among the five classes of the Proteobacteria.

Phenotypic group[a]	Group nr.[a]	Occurring in proteobacterial class:
Aerobic/microaerophilic, motile, helical/vibrioid Gram-negative bacteria	2	α, β, γ, δ and ε
Gram-negative aerobic/microaerophilic rods and cocci	4	α, β, γ, δ and ε
Facultatively anaerobic Gram-negative rods	5	α, β, γ, δ and ε
Anaerobic straight, curved, and helical Gram-negative rods	6	β, γ and δ
Symbiotic and parasitic bacteria of vertebrate and invertebrate species	9	α and γ
Anoxygenic phototrophic bacteria	10	α, β and γ
Aerobic chemolithotrophic bacteria and associated genera	12	β, γ and ε
Budding and/or appendaged nonphototrophic bacteria	13	α and γ
Nonphotosynthetic, nonfruiting gliding bacteria	15	β and γ
Nonmotile or rarely motile, curved Gram-negative bacteria	3	α and ε
Dissimilatory sulfate- or sulfur-reducing bacteria	7	δ
Sheathed bacteria	14	β
Fruiting gliding bacteria	16	δ

[a]Phenotypic groups and group numbers are according to *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994). See also Garrity and Holt (2001).

This chapter sketches how and why the Proteobacteria were recognized as a coherent but diverse phylogenetic group and summarizes its phylogenetic structure as well as its phenotypic and ecological diversity.

Towards a Phylogenetic Definition of the "Purple Bacteria and Their Nonphototrophic Relatives"

In the context of the above mentioned extreme biodiversity of the Proteobacteria, it is not surprising that some 50 years ago—when only phenotypic techniques were available for characterization and comparison of prokaryotes—it was impossible to foresee that morphologically and physiologically highly different bacterial groups such as the Enterobacteriaceae and the photosynthetic purple sulfur bacteria (e.g., *Chromatium*) would be phylogenetically more closely related to each other, whereas morphologically similar bacteria such as the Enterobacteriaceae and the flavobacteria would harbor widely different genomes. Microbiologists possessed in those years only tools to look at the tips of the evolutionary branches of the tree of life. About 15 years prior to the introduction of ribosomal RNA analysis as a suitable method for determining prokaryote evolution, two different approaches were followed aiming at the same goal. The physiologists used the deductive strategy, trying to establish the phylogeny of prokaryotes from selected metabolic properties (Broda, 1970; Broda, 1975), while geneticists and biochemists followed the inductive strategy, originating from the pioneering publication by Zuckerkandl and Pauling (1965).

The question about the origin of bacterial respiration placed the photosynthetic bacteria, especially the anoxygenic "purple bacteria," in the center of discussion. To be more precise, the problem was how respiration could evolve in parallel in so many groups of organisms and independently from that evolved in cyanobacteria. The similarity of the electron-flow chains in photosynthesis and respiration as an ordered assembly of flavoproteins, cytochromes, quinones and non-heme Fe-S proteins in connection with membranes has been noted (Olson, 1970), and it was proposed that the photosynthetic chain could have been modified and adapted to respiration. It was Broda (1970) who concluded that respiration evolved independently from different kinds of photosynthetic apparatus, and if photosynthetic bacteria themselves evolved in different lines of descent, so would the aerobic respiring bacteria. This "conversion" hypothesis placed significant emphasis on the presence of phototrophic bacteria in various lines of descent and focused on the evolution of electron chains from photosynthetic anaerobes, anaerobic respirers, and aerobes ranging from phototrophic aerobes to strictly aerobic respirers. Broda's hypothesis, later supported (though refined) by the general phylogenetic frame of ribosomal RNA/DNA sequences, is illustrated by two examples. Firstly, he postulated the existence of a Gram-positive phototroph, later proven by the isolation of the phylogenetically Gram-positive *Heliobacterium chlorum* (Gest and Favinger, 1983; Woese et al., 1985a), branching deeply in the *Clostridium* lineage. Secondly, early 16S rRNA cataloguing data indicated the high phylogenetic similarity between the nonsulfur purple bacterium *Rhodopseudomonas palustris* and the aerobic *Nitrobacter winogradskyi* (Seewaldt et al., 1982). However, Broda (1975) was not in a position to predict the specific phylogenetic clustering of the purple sulfur and the purple nonsulfur bacteria as opposed to the lin-

eages of green sulfur bacteria, the green nonsulfur bacteria, and the cyanobacteria. Also, as they had not been described in the 1970s, the aerobic bacteriochlorophyll *a*-containing bacteria (α-3 and α-4 groups and a few in the "Betaproteobacteria") are not mentioned in the conversion hypothesis. Modifications to the outline of biochemical pathways given by Broda have been made by Schwemmler (1989), combining rRNA-based phylogenetic relatedness between species and the biochemistry of components of electron chains involved in aerobic respiration and anaerobic and aerobic photosynthesis.

Zuckerkandl and Pauling (1965) highlighted the importance of using semantides, such as genomic DNA, its primary transcript RNA, and the translation product, the proteins, as evolutionary markers. They postulated that the path of evolution is laid down in the primary structure of nucleic acids and determination of the blueprint of evolutionary conservative genes would necessarily unravel the evolutionary history of organisms. Owing to methodological restrictions, proteins rather than DNA or RNA were accessible to sequence analyses, restricting early molecular studies to those proteins easily isolated and sequenced. Besides histones and fibrinopeptides, used in the determination of the rate of gene evolution in eukaryotes, primarily proteins such as ferredoxin and cytochrome *c* allowed the first glimpse of the evolution of prokaryotic genes. While the ferredoxin data were obtained for mainly Gram-positive anaerobic bacteria and cyanobacteria, cytochrome *c* sequences were generated for respiring organisms as well as for anaerobic and aerobic photosynthetic bacteria (Schwartz and Dayhoff, 1978; Ambler et al., 1979) as well as for mitochondria, the origin of which could be traced to members of the "Alphaproteobacteria." Surprising to taxonomists, the clustering of species according to the primary and tertiary structure of proteins (Dickerson, 1980) did not correlate with the traditional systematic groupings such as those laid down in *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons, 1974). It was especially the phylogenetic closeness between Gram-negative, nonphototrophic organisms and phototrophs that shed doubts on the correctness of the sequence-based findings. However, shortly afterwards, results from the 16S rRNA cataloguing approach, fully supporting the protein-based studies, demonstrated the obvious failure of the phenotype to mirror phylogenetic relatedness. Some 30 years ago, the pioneering studies on 16S rRNA oligonucleotide cataloguing by Woese and coworkers (Fox et al., 1977; Woese and Fox, 1977) revolutionized the insights in microbial evolution, in particular, and evolution of life on earth, in general. The purple bacteria (nowadays named Proteobacteria) were first recognized and circumscribed as a distinct division (phylum) of the eubacteria by Woese and coworkers on the basis of signature and sequence analyses of the 16S rRNA/rDNA (Gibson et al., 1979; Fox et al., 1980; Woese et al., 1985b; Woese, 1987). Because the purple photosynthetic phenotype was distributed throughout the group the trivial name "purple bacteria" seemed to be justified for this phylogenetic group of Gram-negatives (Woese, 1987), although many nonphotosynthetic species (such as the enterics, the pseudomonads, rhizobia, rickettsiae, etc.) grouped among the photosynthetic representatives. The oligonucleotide signature analysis of 16S rRNAs revealed already in the 1980s that the purple bacteria comprised at least three major subdivisions, arbitrarily designated as α, β and γ. The purple nonsulfur (PNS) bacteria (e.g., *Rhodospirillum*, *Rhodobacter* and *Rhodocyclus*) were found in two subdivisions (α and β; Woese et al., 1984a; Woese et al., 1984b), whereas the purple sulfur (PS) bacteria (e.g., *Chromatium* and *Ectothiorhodospira*) belonged to the γ-subdivision (Woese et al., 1985c). When more Gram-negatives were investigated by rRNA-cataloguing, rDNA-sequencing and also by the DNA/rRNA hybridization technique, it became clear that the majority of the so-called purple bacteria were in fact nonphotosynthetic prokaryotes. Without being able to trace the deeper phylogenetic relationships, De Ley and coworkers (reviewed by De Ley, 1992) studied hundreds of Gram-negatives by the DNA/rRNA hybridization technique, which allowed their relative phylogenetic position within the various rRNA-branches to be determined. The α, β and γ groups corresponded to the rRNA superfamilies IV, III and I+II, respectively, as defined by De Ley and coworkers (De Vos and De Ley, 1983; De Vos et al., 1985; De Vos et al., 1989; De Ley et al., 1986; Jarvis et al., 1986; Rossau et al., 1986; Willems et al., 1991a; De Ley, 1992). The DNA/rRNA hybridization approach together with the application of various genomic and phenotypic techniques (i.e., the polyphasic approach; Colwell, 1970; Vandamme et al., 1996) allowed improvement of the classification of various Gram-negative taxa, such as the pseudomonads (De Vos and De Ley, 1983; De Vos et al., 1985; De Vos et al., 1989; Willems et al., 1991a; De Ley, 1992; Kersters et al., 1996), *Alcaligenes* and *Bordetella* (Kersters and De Ley, 1984; De Ley et al., 1986), the Pasteurellaceae (De Ley et al., 1990), the Neisseriaceae and Moraxellaceae (Rossau et al., 1986; Rossau et al., 1991), the Acetobacteraceae (Gillis and De Ley, 1980; De Ley and Gillis, 1984; Swings, 1992), the Campylobacteraceae (Vandamme and De Ley, 1991) and many others.

During the last 15 years, the knowledge concerning the phylogeny of the Gram-negatives gradually became more comprehensive by the availability of an increasing number of nearly complete 16S rRNA sequences, indicating that the Gram-negative sulfur- and sulfate-reducing bacteria form a fourth group or δ-subdivision among the purple bacteria together with the myxobacteria and the bdellovibrios (Oyaizu and Woese, 1985; Woese, 1987). Because the majority of the genera belonging to the purple bacteria are not purple and not photosynthetic, Stackebrandt et al. proposed in 1988 the name "Proteobacteria" for a new higher taxon (at the level of class), including these purple bacteria and their relatives; the α to δ groups were temporarily considered as subclasses, pending further studies and nomenclatural proposals. Later some microaerophilic and helical-shaped bacteria such as the campylobacters were placed in the fifth or ε-subclass, corresponding to rRNA-superfamily VI of De Ley's research group (Vandamme and De Ley, 1991; Stackebrandt, 1992).

Each new issue of the *International Journal of Systematic and Evolutionary Microbiology* adds new genera and species to the Proteobacteria, entailing a more bush-like rRNA/rDNA-tree topology. Yet, the distinctness of the subclasses seems to be maintained, although the differentiation between the β- and the γ-group is becoming less clear, and some rDNA-based trees indicate that *Desulfurella* and allied bacteria of the δ-group may constitute a sixth subdivision (Rainey et al., 1993). In the second edition of *Bergey's Manual of Systematic Bacteriology* (Garrity, 2001a), the Proteobacteria have been elevated to the rank of phylum and the subclasses α to ε have been elevated to the rank of classes, corresponding to the names "Alphaproteobacteria," "Betaproteobacteria," "Gammaproteobacteria," "Deltaproteobacteria," and "Epsilonproteobacteria," respectively (see Table 2). In a recently revised megaclassification of the prokaryotes, Cavalier-Smith (2002) proposes a new classification and nomenclature for the five major subgroups of the Proteobacteria (see Table 2).

In the present chapter, we follow the classification of the major reference work, *Bergey's Manual of Systematic Bacteriology* (Garrity, 2001), and will use ranks and names as listed in Table 2 (2nd column).

Is the 16S-rRNA-based Phylogeny of the Proteobacteria Confirmed by Other Experimental Approaches?

The first indications that the traditional groupings of the Gram-negative bacteria according to the previous editions of *Bergey's Manual of Systematic Bacteriology* (Buchanan and Gibbons, 1974; Krieg and Holt, 1984) and *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994) were not phylogenetic came from comparative sequence analysis of some redox proteins, such as cytochrome *c* [Introduction]. Also studies by Byng et al. (1983) on the regulation of multibranched pathways for the biosynthesis of aromatic amino acids indicated that some members of the genus *Pseudomonas*, such as *P. testosteroni* and *P. solanacearum*, were more similar to other genera than to the group of species around the type species *Pseudomonas aeruginosa* (belonging to the "Gammaproteobacteria"), which is in perfect agreement with rRNA/rDNA data. Later, *Pseudomonas testosteroni* and *P. solanacearum* (both belonging to the "Betaproteobacteria") were indeed transferred to the genera *Comamonas* and *Ralstonia*, respectively (Tamaoka et al., 1987; Yabuuchi et al., 1995).

The definition of the Proteobacteria as a separate evolutionary lineage and the overall picture of the grouping in five classes are confirmed by comparative analysis of 23S rRNA-genes and alternative phylogenetic markers such as the genes coding for elongation factor Tu and

Table 2. Three systems of Proteobacteria classification: correspondence between the names of taxa above the rank of order.

Classification		
1[a]	2[b]	3[c]
Class Proteobacteria	Phylum Proteobacteria	Division Proteobacteria
		Subdivision Rhodobacteria
Subclass alpha	Class "Alphaproteobacteria"[d]	Class Alphabacteria
Subclass beta	Class "Betaproteobacteria"	Class Chromatibacteria
Subclass gamma	Class "Gammaproteobacteria"	
		Subdivision Thiobacteria
Subclass delta	Class "Deltaproteobacteria"	Class Deltabacteria
Subclass epsilon	Class "Epsilonproteobacteria"	Class Epsilobacteria

[a]From Stackebrandt et al. (1988b).
[b]From *Bergey's Manual of Systematic Bacteriology* (Garrity, 2001).
[c]From Cavalier-Smith (2002).
[d]Quotation marks are used for names that have not yet been validated.

ATPase (Ludwig et al., 1995; Ludwig and Schleifer, 1999; Ludwig and Klenk, 2001). Moreover, the comparative analysis of conserved insertions and deletions (so-called "signature sequences") found in different bacterial proteins yielded molecular means to define the phylum Proteobacteria and its various classes and improved the understanding of their evolutionary relationships to other groups of Bacteria and Eukarya (Gupta, 2000; Gupta, 2002). Proteins studied were, e.g., alanyl-tRNA synthetase, succinyl-CoA synthetase, Hsp 60 (GroEL), Hsp70 heat shock protein, and *recA* protein. The listing of extensive literature on this subject would be unjustifiable in the context of this chapter (see Gupta, 2000). The proteobacterial classes emerged in the following order during evolution (Gupta, 2000): epsilon and delta → alpha → beta → gamma, which is similar to the order proposed on the basis of RNA polymerase β and β′ subunits (Klenk et al., 1999). Indeed, the ribosomal DNA tree does not clearly resolve the order in which the majority of main lineages emerged during evolution. Proteobacteria, Gram-positive bacteria, *Cyanobacteria*, Cytophaga/Flavobacteria and some other minor lineages appear to evolve at the same node, giving the rDNA-tree a fork-like appearance. The branching order of the various main lines of descent as depicted by Gupta (2000) is different. Gupta places the low G+C Gram-positive bacteria (clostridia) at the base of bacterial evolution, while those organisms constituting the base of the bacterial 16S rDNA tree (*Aquifex*, *Deinococcus-Thermus*, and green sulfur bacteria) are described as having evolved later. Proteobacteria appear as the last evolutionary group. The importance of the proteobacterial cell is highlighted not only as the donor of the mitochondria (Margulis, 1993; Falah and Gupta, 1994), but also as one of the fusion partners (the other being a archaeal cell) giving rise to the ancestral eukaryotic cell (see Gupta [2000] for an extensive list of references). More genes and complete genomes will have to be sequenced to determine the extent and relative time of lateral gene transfer and to highlight those genes that represent the core of genes transmitted vertically, hence representing the evolutionarily stable component of the cell.

So far, no reliable phenotypic features were found that would be characteristic for the whole phylum of the Proteobacteria and differentiate it from other phyla of the domain Bacteria. It seems also very difficult to find stable phenotypic features for the differentiation of the five classes, which is not surprising in view of the enormous variety of morphological and physiological types existing in each of these five lineages. Phototrophy cannot be used as phylogenetic marker, except that PNS- and PS-bacteria are only reported so far among the Proteobacteria, which could indicate that this type of photosynthesis was common in the proteobacterial ancestors and was lost during evolution in a great number of sublineages. Support for the validity of some of the results of nucleic acid and protein sequencing came from chemotaxonomy. Within the "Alphaproteobacteria," *Rhodopseudomonas palustris*, *Nitrobacter winogradskyi*, *Blastochloris (Rhodopseudomonas) viridis*, *Phenylobacterium immobile*, certain thiobacilli, *Brucella melitensis* and *Brevundimonas (Pseudomonas) diminuta* exhibited an unusual lipid A, which lacked glucosamine but contained instead a 2,3-diamino-2,3-dideoxy-D-glucose (Stackebrandt et al., 1988a). This structure was not uniformly distributed among all members of the "Alphaproteobacteria" but was shared among closely related species. Lipid A composition also confirmed the phylogenetic distinctness of species hitherto affiliated to the same genus on the basis of their phenotype. For example, *Rhodocyclus tenue* and *Rhodocyclus gelatinosa* (members of the "Betaproteobacteria"; Imhoff et al., 1984) differed significantly in the chemistry of their lipid A, a finding that supported the reclassification of the latter species as *Rubrivivax gelatinosus* (Willems et al., 1991b). As far as we could determine from the literature, quinones and polyamines seem to be good marker molecules to differentiate the major proteobacterial lineages (Collins and Jones, 1981; Collins and Widdel, 1986; Busse and Auling, 1988; Auling, 1992; Hamana and Matsuzaki, 1993; Busse et al., 1996; Table 3; Directory, Databases and Dictionaries Compiled by WDCM website (http://www.wdcm.nig.ac.jp/cgi-bin/search.cgi)). Ubiquinones are typical for the α, β and γ lineages, whereas menaquinones are characteristic for the "Deltaproteobacteria" and "Epsilonproteobacteria," although some photosynthetic "Alphaproteobacteria" and "Betaproteobacteria" contain also MK-8, MK-9 or MK-10 (Hiraishi et al., 1984). Some members of the Pasteurellaceae ("Gammaproteobacteria") are characterized by demethylmenaquinones. Gas chromatographic analysis of the methylesters of cellular fatty acids (FAME) does not allow differentiation of the five major lineages. Of course, phenotypic and chemotaxonomic features (e.g., FAME) are very useful for the description and differentiation of species, genera and families, and the polyphasic taxonomic approach integrating all available data (Colwell, 1970; Vandamme et al., 1996) has been applied during the last decade to improve the classification of previously polyphyletic proteobacterial genera, such as *Erwinia* (Hauben et al., 1998), *Pseudomonas* (Kersters et al., 1996; Anzai et al., 2000), *Rhizobium* and allies (de Lajudie et al., 1998), *Thiobacillus* (Kelly and

Table 3. Some selected key genera, general characteristics, and differentiating features of the five classes of the Proteobacteria.

	Proteobacterial class				
	Alpha	Beta	Gamma	Delta	Epsilon
Important genera	*Acetobacter* *Agrobacterium*[a] *Bartonella*[a] *Bradyrhizobium* *Brucella*[a] *Caulobacter*[a] *Ehrlichia* *Gluconobacter* *Hyphomicrobium* *Mesorhizobium*[a] *Methylobacterium*[b] *Nitrobacter* *Rhizobium* *Rhodobacter*[b] *Rhodospirillum* *Sinorhizobium*[a] *Sphingomonas*[b] *Rickettsia*[a,b] *Wolbachia*[b]	*Alcaligenes* *Bordetella*[a,b] *Burkholderia*[b] *Comamonas* *Neisseria*[a,b] *Nitrosomonas*[b] *Ralstonia*[b] *Rhodocyclus* *Sphaerotilus* *Spirillum* *Thiobacillus*	*Actinobacillus*[b] *Azotobacter* *Buchnera*[a] *Chromatium* *Coxiella*[b] *Erwinia*[b] *Escherichia*[a,b] *Francisella*[b] *Haemophilus*[a,b] *Legionella*[b] *Methylococcus*[b] *Pasteurella*[a] *Pectobacterium* *Pseudomonas*[a,b] *Salmonella*[a,b] *Shewanella*[b] *Shigella*[a,b] *Stenotrophomonas* *Vibrio*[a,b] *Xanthomonas*[a,b] *Xylella*[a,b] *Yersinia*[a,b]	*Bdellovibrio* *Chondromyces* *Desulfobacter* *Desulfovibrio*[b] *Geobacter*[b] *Myxococcus*[b] *Polyangium* *Syntrophus*	*Campylobacter*[a] *Helicobacter*[a] *Sulfurospirillum* *Wolinella*
Number of genera/number of species[c]	140/425	76/225	181/755	57/165	6/49
Major ubiquinone type[d]	Q-10	Q-8	Q-8, Q-9, or Q-10 to Q-14	—	—
Major menaquinone type[d]	Some contain also MK-9 or MK-10	Some contain also MK-8	Some contain also MK-8 or MK-7	MK-6, MK-6(H_2), MK-7, MK-7(H_2) or MK-8[e]	MK-6, methyl-substituted MK-6[f]
Characteristic polyamines[g]	Most contain a triamine (*sym*-homospermidine or spermidine)	2-Hydroxy-putrescine	Spermidine and/or putrescine or cadaverine; or 1,3-diamino-propane	Most contain a triamine (*sym*-homospermidine or spermidine)	Spermidine

Symbols and abbreviations: —, absent; DMK, demethylmenaquinone; and MK-6(H_2), hydrogenated menaquinone-6.
[a]The genome of at least one representative strain has been sequenced (as of mid 2002).
[b]Sequencing of the genome of at least one representative strain is in progress (as of mid 2002).
[c]Only validly published names (situation as of mid 2002).
[d]Collins and Jones (1981), Hiraishi et al. (1984), (http://www.wdcm.nig.ac.jp/cgi-bin/search.cgi), and H.J. Busse, personal communication.
[e]Collins and Widdel (1986).
[f]Moss et al. (1990).
[g]Auling (1992), Busse and Auling (1988), and Hamana and Matsuzaki (1993).

Wood, 2000), *Leptothrix* (Spring et al., 1996) and *Oceanospirillum* (Satomi et al., 2002).

No single nucleotide signature is found in the 16S rRNA that could serve unambiguously to define the proteobacterial phylum (sensu *Bergey's Manual*). However, specific 16S rRNA sequence signatures for the various classes of the Proteobacteria have been described and used for the construction of DNA probes (Woese, 1987; Stackebrandt et al., 1988b; Manz et al., 1992; Ludwig et al., 1998). Such probes were extensively applied for the detection and visualization of Proteobacteria and other prokaryotes in activated sludge (Wagner et al., 1993; Snaidr et al.,

Table 4. Some rRNA-targeted oligonucleotide probes for fluorescent in-situ hybridization.

Probe	Position	Probe sequence (5′ → 3′)	Specificity	Reference
ALF1b	16S rRNA 19–35	CGTTCG(C/T)TCTGAGCCAG	"Alphaproteobacteria," but not exclusive	Manz et al., 1992
BET42a	23S rRNA 1027–1043	GCCTTCCCACTTCGTTT	"Betaproteobacteria"	Manz et al., 1992
GAM42a	23S rRNA 1027–1043	GCCTTCCCACATCGTTT	"Gammaproteobacteria," but not the deeply branching taxa	Manz et al., 1992
Delta 385	16S rDNA 385–402	CGGCGT(C/T)GCTGCGTCAGG	"Deltaproteobacteria" sulfate-reducers, but not exclusive	Rabus et al., 1996

1997; Juretschko et al., 2002). Table 4 lists the sequence of some representative rRNA-targeted oligonucleotide probes.

The Proteobacterial Classes: Morphological, Physiological, Ecological and Phylogenetic Diversity

Figure 1 is a simplified phylogenetic tree of the Proteobacteria based on the nearly complete 16S rDNA sequences of the type strains of the type species of the majority of proteobacterial genera. Only the names of the families and major groups are indicated. The "Deltaproteobacteria" and "Epsilonproteobacteria" form the deeper branches of the phylum; the "Alphaproteobacteria" are also clearly separated, whereas the closer relationship between the β and γ lineages may indicate the common origin of the latter groups (Ludwig and Klenk, 2001).

Phototrophic bacteria occur only in the "Alphaproteobacteria," "Betaproteobacteria" and "Gammaproteobacteria" (purple colored triangles in Fig. 1); these anoxygenic phototrophic purple bacteria can be subdivided into the purple sulfur (PS; e.g., *Chromatium* and *Ectothiorhodospira*) and purple nonsulfur (PNS) bacteria (e.g., *Rhodospirillum*). All known PS bacteria belong to the "Gammaproteobacteria" and use H_2S or S° as sole electron donor. The PNS bacteria occur among the "Alphaproteobacteria" and "Betaproteobacteria." A great number of phototrophic Proteobacteria are versatile and can easily switch from a phototrophic to a heterotrophic lifestyle in the absence of light. The results of comparative 16S rDNA sequence analysis have led to extensive taxonomic rearrangements within previously defined taxa of proteobacterial phototrophs (Imhoff, 2001a).

In this chapter, we briefly touch on the morphological, physiological, ecological and phylogenetic diversity of the major proteobacterial groups. The reader will find more detailed information in the chapters dealing with the individual families and genera of the Proteobacteria.

An incomplete survey of disease-causing Proteobacteria is given in Table 5 for the bacteria of clinical and veterinary interest and in Table 6 for the phytopathogens. Some examples of industrial and biotechnological applications are listed in Table 7.

The "Alphaproteobacteria"

The 16S rDNA-tree separates the α-class clearly from the other proteobacterial classes (Fig. 1). The bacterial taxa belonging to the "Alphaproteobacteria" (some140 genera and 425 species at present) are morphologically and metabolically extremely diverse. More detailed information can be found in the chapters dealing with the individual families and genera of this class. Table 8 provides an overview of the major phylogenetic groups (see also Fig. 1) and the names of the orders and families of the "Alphaproteobacteria" as they are listed in the 2nd edition of *Bergey's Manual of Systematic Bacteriology* (Garrity, 2001a).

The majority of the "Alphaproteobacteria" are rod-shaped, but cocci and curved, spiral, stalked, budding and prosthecate forms do also occur. Some are phototrophic purple nonsulfur (PNS) bacteria (such as *Rhodospirillum* and *Rhodobacter*), whereas others are chemolithotrophs (e.g., the nitrite-oxidizing *Nitrobacter*) or chemoorganotrophs (e.g., *Sphingomonas* and *Brucella*). Marine and halophilic phototrophic PNS bacteria seem to be restricted to the "Alphaproteobacteria" (Imhoff, 2001b), whereas a physiologically remarkable group of bacteria, containing bacteriochlorophyll (BChl) but unable to grow phototrophically under anaerobic conditions, belongs to various lineages of the "Alphaproteobacteria" (Yurkov and Beatty, 1998; Yurkov, 2001). Except for *Roseateles*, all aerobic BChl-containing bacteria investigated so far are exclusively found within the "Alphaproteobacteria" and appear to be related phylogenetically to aerobic purely chemoorganotrophic bacteria. This could indicate that the presence of

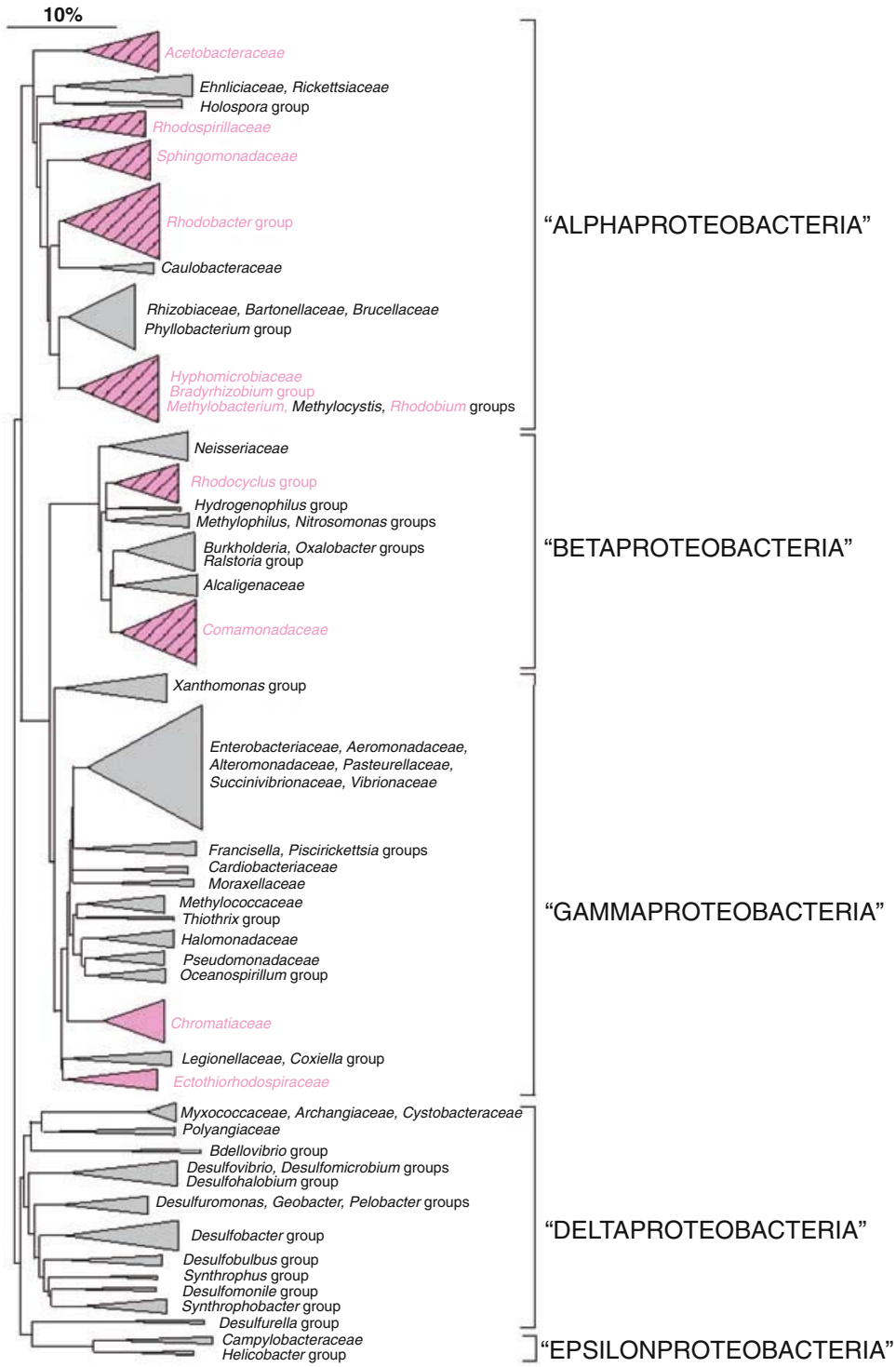

Fig. 1. Simplified neigbor-joining phylogenetic tree of the Proteobacteria based on the 16S rDNA sequences of the type strains of the proteobacterial genera. Distances were calculated using the substitution rate calibration method in TREECON 3.1 (Van de Peer and De Wachter, 1997). The bar indicates 10% estimated sequence divergence. *Bacillus subtilis* was used as outgroup (not shown). The width of the triangles is proportional to the number of genera within each cluster. The purple color indicates clusters where phototrophic bacteria occur: purple sulfur photosynthetics (Chromatiaceae and Ectothiorhodospiraceae) in the "Gammaproteobacteria," purple nonsulfur photosynthetics and aerobic phototrophs in the "Alphaproteobacteria" and "Betaproteobacteria." Shaded purple triangles indicate that most representatives of phototrophic "Alphaproteobacteria" and "Betaproteobacteria" are phylogenetically related to nonphototrophic, strictly chemotrophic bacteria belonging to the respective families or groups. Aerobic phototrophic Proteobacteria were reported among the Acetobacteraceae (sensu lato), the Sphingomonadaceae, the *Rhodobacter*, *Methylobacterium* and *Bradyrhizobium* groups (all "Alphaproteobacteria"), and in the genus *Roseateles* ("Betaproteobacteria," family Comamonadaceae).

Table 5. Some Proteobacteria involved in human and animal disease.

Proteobacterial class and species	Family[a]	Disease (site of action)	Risk group[b]
"Alphaproteobacteria"			
Bartonella henselae	Bartonellaceae	Cat-scratch disease, and bacillary angiomatosis	2
Bartonella quintata	Bartonellaceae	Trench fever	2
Brucella melitensis	Brucellaceae	Abortion (genital tract of animals), and brucellosis in man	3
Ehrlichia chaffeensis	Ehrlichiaceae	Human ehrlichiosis	2
Orientia tsutsugamushi	Rickettsiaceae	Scrub typhus	3
Rickettsia rickettsii	Rickettsiaceae	Rocky Mountain spotted fever	3
Rickettsia prowazekii	Rickettsiaceae	Typhus fever	3
"Betaproteobacteria"			
Bordetella pertussis	Alcaligenaceae	Whooping cough (respiratory tract)	2
Burkholderia mallei	"Burkholderiaceae"	Glander disease in equines	3
Burkholderia pseudomallei	"Burkholderiaceae"	Melioidosis	3
Neisseria gonorrhoeae	Neisseriaceae	Gonorrhoea (genital tract)	2
Neisseria meningitidis	Neisseriaceae	Meningitis (central nervous system)	2
Taylorella equigenitalis	Alcaligenaceae	Endometritis in mares	2
"Gammaproteobacteria"			
Coxiella burnetii	"Coxiellaceae"	Q-fever	3
Escherichia coli	Enterobacteriaceae		
intestinal variants		Diarrhea	2
uropathogenic variants		Urinary tract infections	2
verocytotoxigenic strains		e.g., O157:H7, causing hemorrhagic diarrhea and kidney failure	3
Francisella tularensis	"Francisellaceae"	Tularemia (skin and lymph nodes)	3
Haemophilus influenzae	Pasteurellaceae	Meningitis, pericarditis, and pneumonia	2
Legionella pneumophila	Legionellaceae	Legionnaires' disease (respiratory tract)	2
Pseudomonas aeruginosa	Pseudomonadaceae	Nosocomial infections (skin and respiratory tract, urinary tract)	2
Salmonella typhi	Enterobacteriaceae	Typhoid fever (gastrointestinal tract)	3
Shigella dysenteriae	Enterobacteriaceae	Dysenteria (gastrointestinal tract)	3
Vibrio cholerae	Vibrionaceae	Cholera (gastrointestinal tract)	2
Yersinia pestis	Enterobacteriaceae	Plague (blood)	3
"Epsilonproteobacteria"			
Campylobacter coli, *C. jejuni*	Campylobacteraceae	Diarrhea	2 2
Helicobacter pylori	"Helicobacteraceae"	Duodenal and gastric ulcers, and gastric carcinoma	2

[a]According to *Bergey's Manual of Systematic Bacteriology* (Garrity and Holt, 2001). See also Fig. 1. Quotation marks are used for names which have not yet been validated (as of mid 2002).
[b]According to EU-directive 2000/54/EG; see also (http://www.dsmz.de/).

Table 6. Some selected plant diseases caused by Proteobacteria.

Proteobacterial class and species	Family[a]	Disease (symptoms)
"Alphaproteobacteria"		
Agrobacterium rhizogenes	Rhizobiaceae	Hairy root
Agrobacterium tumefaciens	Rhizobiaceae	Crown gall
"*Candidatus* Liberibacter asiaticus"	in cluster of Rhizobiaceae, Bartonellaceae, etc.	Greening disease on citrus (a phloem-restricted disease)
"Betaproteobacteria"		
Acidovorax anthurii	Comamonadaceae	Leaf-spot on *Anthurium*
Burkholderia cepacia	"Burkholderiaceae"	Soft rot (sour skin on onion)
Burkholderia glumae	"Burkholderiaceae"	Sheath necrosis on rice
Ralstonia solanacearum	"Ralstoniaceae"	Moko disease on banana (vascular wilt)
Xylophilus ampelinus	Comamonadaceae	Necrosis and canker on grapevine
"Gammaproteobacteria"		
Brenneria (Erwinia) salicis	Enterobacteriaceae	Watermark disease on willow
Brenneria nigrifluens	Enterobacteriaceae	Bark canker on Persian walnut (*Juglans regia*)
Erwinia amylovora	Enterobacteriaceae	Fire blight on pome fruit (vascular wilt)
Erwinia stewartii	Enterobacteriaceae	Stewart's wilt on corn (vascular wilt)
Pectobacterium (Erwinia) carotovorum	Enterobacteriaceae	Soft rot
Pseudomonas agarici	Pseudomonadaceae	Spots on mushrooms
Pseudomonas marginalis	Pseudomonadaceae	Soft rot (pink eye) on potato
Pseudomonas savastanoi	Pseudomonadaceae	Galls on olive trees
Pseudomonas syringae	Pseudomonadaceae	Wildfire on tobacco, haloblight on beans, spots on tomato and pepper (blights and spots)

Table 6. *Continued*

Proteobacterial class and species	Family[a]	Disease (symptoms)
Pseudomonas syringae	Pseudomonadaceae	Canker on stone fruit
Xanthomonas campestris	"Xanthomonadaceae"	Black rot on crucifers (vascular wilt)
Xanthomonas citri	"Xanthomonadaceae"	Canker on citrus
Xanthomonas oryzae	"Xanthomonadaceae"	Blight on rice
Xanthomonas populi	"Xanthomonadaceae"	Canker on poplar trees
Xanthomonas translucens	"Xanthomonadaceae"	Blight on cereals
Xanthomonas vesicatoria	"Xanthomonadaceae"	Spots on tomato and pepper
Xylella fastidiosa	"Xanthomonadaceae"	Pierce's disease (e.g., on grapevine)

[a]According to *Bergey's Manual of Systematic Bacteriology* (Garrity and Holt, 2001). See also Fig. 1. Quotation marks are used for names which have not yet been validated (as of mid 2002).

Table 7. Some Proteobacteria involved in industrial and biotechnological processes.

Proteobacterial class, genus or species	Family[a]	Industrial product or process
"Alphaproteobacteria"		
Acetobacter aceti	Acetobacteraceae	Vinegar
Acetobacter xylinus	Acetobacteraceae	Cellulose membranes
Agrobacterium	Rhizobiaceae	Plant engineering (Ti-plasmid)
Gluconobacter oxydans	Acetobacteraceae	Oxidation of sorbitol (for vitamin C production)
Rhizobium	Rhizobiaceae	Inoculants for nodule formation on leguminous plants (N_2-fixation)
Rhodobacter capsulatus	"Rhodobacteraceae"	Production of hydrogen gas
Zymomonas mobilis	Sphingomonadaceae	Ethanol
"Betaproteobacteria"		
Ralstonia eutropha	"Ralstoniaceae"	Poly-β-hydroxybutyrate (bioplastics) and single-cell protein
"Gammaproteobacteria"		
Azotobacter	Pseudomonadaceae	Alginates (polysaccharide) and poly-β-hydroxybutyrate (bioplastics)
Chromatium	Chromatiaceae	Production of hydrogen gas
Erwinia herbicola	Enterobacteriaceae	Biological control of frost damage
Escherichia coli	Enterobacteriaceae	Production of heterologous proteins (e.g., insulin, interferon, and antiviral vaccines)
Photobacterium	Vibrionaceae	Luciferase (*lux*-genes)
Pseudomonas	Pseudomonadaceae	Oxidation of aliphatic and aromatic compounds
Acidithiobacillus ferrooxidans	Acidithiobacillus group	Active metal mining (bioleaching)
Xanthomonas campestris	"Xanthomonadaceae"	Xanthan (polysaccharide)

[a]According to *Bergey's Manual of Systematic Bacteriology* (Garrity and Holt, 2001). See also Fig. 1. Quotation marks are used for names which have not yet been validated (as of mid 2002).

Table 8. The "Alphaproteobacteria": orders, families and number of genera.[a]

Order	Family	Number of genera
Rhodospirillales	Rhodospirillaceae	10
	Acetobacteraceae	12
Rickettsiales	Rickettsiaceae	3
	Ehrlichiaceae	5
	"Holosporaceae"	7
"Rhodobacterales"	"Rhodobacteraceae"	20
"Sphingomonadales"	Sphingomonadaceae	9
Caulobacterales	Caulobacteraceae	4
"Rhizobiales"	Rhizobiaceae	7
	Bartonellaceae	1
	Brucellaceae	3
	"Phyllobacteriaceae"	6
	"Methylocystaceae"	3
	"Beijerinckiaceae"	3
	"Bradyrhizobiaceae"	8
	Hyphomicrobiaceae	19
	"Methylobacteriaceae"	3
	"Rhodobiaceae"	1

[a]According to the second edition of *Bergey's Manual of Systematic Bacteriology* (Garrity and Holt, 2001). Quotation marks are used for names which have not yet been validated (as of mid 2002).

BChl in some members of the "Alphaproteobacteria" (e.g., *Erythrobacter* and *Roseobacter*) is an atavistic trait that remained functional after the aerobic bacteria evolved from their anaerobic phototrophic ancestors (Stackebrandt et al., 1996; Yurkov, 2001). Thus, aerobic phototrophic bacteria could represent an intermediate phase of evolution from anaerobic purple phototrophs to nonphotosynthetic aerobic chemotrophs. During the last decade, the classification of a number of classical phototrophic genera, such as *Rhodospirillum* and *Rhodopseudomonas*, underwent considerable changes, which are in agreement with 16S rDNA sequence data, morphological parameters, internal membrane structures as well as important chemotaxonomic parameters, such as the composition of cellular fatty acids, ubiquinones and cytochrome *c*-type structures. Most of the photosynthetic PNS bacteria are also capable of nitrogen fixation.

Classical chemoorganotrophs (such as *Sphingomonas*), as well as typical acidophiles (e.g., *Acetobacter*) and methylotrophs (e.g., *Methylobacterium*) belong to the "Alphaproteobacteria." A great number of α-class members live in association with eukaryotes: some are indeed pathogenic for humans and animals (e.g., *Brucella*) or plants (e.g., *Agrobacterium*), and others display an obligate parasitic lifestyle, cause diseases in humans and mammals, are transmitted by insect or tick bites (e.g., the Rickettsiaceae), or live symbiotically in the roots of leguminous plants (e.g., *Rhizobium* and *Bradyrhizobium*) and play a key role in atmospheric nitrogen fixation. Hence, these "Alphaproteobacteria" thrive in widely divergent habitats and exert a significant impact in the biosphere of our planet. The evolutionary roots of the mitochondrion are within the α-class (see Symbiotic, Parasitic and Not-Yet Cultured Proteobacteria, and the Alphaproteobacterial Origin of Mitochondria), and the understanding of the molecular aspects of plant tumor induction by *Agrobacterium tumefaciens* led to revolutionary applications in plant agriculture, where plant genetic engineers have used the natural transformation system of *Agrobacterium* as a vector for the introduction of foreign DNA into plants (Birch, 1997).

The Acetobacteraceae and the Rhodospirillaceae appear to be the deeper branching lineages among the "Alphaproteobacteria" (Ludwig and Klenk, 2001). In our analysis (Fig. 1), the Ehrlichiaceae and the Rickettsiaceae form also one of the deeper branches.

Acetobacteraceae and Related Groups The Acetobacteraceae form a clearly separate lineage of acidophilic bacteria encompassing the classical vinegar-producing *Acetobacter*, together with *Gluconobacter*, *Gluconacetobacter*, *Acidimonas*, *Acidiphilium*, *Asaia* and some other genera, as well as the phototrophic PNS bacterium *Rhodopila*. Some of the *Gluconacetobacter* species are able to fix nitrogen and live in association with sugar cane (*G. diazotrophicus*) or coffee plants (*G. azotocaptans*; Fuentes-Ramirez et al., 2001).

Rickettsiaceae, Ehrlichiaceae and the Holospora Group The Rickettsiaceae, the Anaplasmataceae and the Ehrlichiaceae form a distinct lineage among the "Alphaproteobacteria" and consist of small intracellular parasitic bacteria, such as *Rickettsia* (causing typhus and Rocky Mountain spotted fever), *Ehrlichia* (causing human granulocytic ehrlichiosis and Potomac fever in horses), *Anaplasma* (infecting erythrocytes of ruminants), and the remarkable *Wolbachia*, involved in parthenogenesis in various arthropods. Although these bacteria can infect various vertebrate hosts, their vectors and reservoirs are predominantly ticks and trematodes, except for the wolbachiae, which are highly promiscuous for diverse invertebrate hosts and are also found in a variety of helminths. On the basis of 16S rDNA gene and *groESL* operon sequence results, Dumler et al. (2001) proposed a reorganization of the genera into the families Rickettsiaceae and Anaplasmataceae: members of the Rickettsiaceae grow in the cytoplasm or nucleus of their eukaryotic host cells and the family is restricted to the genera *Rickettsia* and *Orientia*, whereas members of the Anaplasmataceae replicate while enclosed in a eukaryotic host cell membrane-derived vacuole, and the family was broadened to include all the alphaproteobacterial species of the genera *Ehrlichia*, *Anaplasma*, *Cowdria*, *Wolbachia* and *Neorickettsia* (Dumler et al., 2001). Various endosymbiotic bacteria of protozoa, such as *Paramecium*, were allocated to the genera *Holospora*, *Caedibacter* and *Lyticum* forming the *Holospora* rDNA lineage, which is distantly related to the group formed by the rickettsiae (Fig. 1). The evolutionary roots of the eukaryotic mitochondrion are among the rickettsiae (Symbiotic, Parasitic and Not-Yet Cultured Proteobacteria, and the Alphaproteobacterial Origin of Mitochondria).

Rhodospirillaceae The Rhodospirillaceae lineage corresponds to the α-1 subgroup of Woese et al. (1984a) and encompasses at least seven genera of spiral-shaped phototrophic PNS bacteria (e.g., *Rhodospirillum*, *Phaeospirillum*, *Rhodospira* and *Rhodovibrio*). Previously, these genera (except *Rhodospira*) were species assigned to the broad and heterogeneous genus *Rhodospirillum* (Imhoff et al., 1998). The phototrophic rhodospirillae practice a photoorganotrophic type of metabolism, where a simple fatty

acid or amino acid is the carbon source and light the energy source. Most of the phototrophic rhodospirillae can also fix nitrogen gas, a property that is also characteristic for members of the genus *Azospirillum*, belonging to the Rhodospirillaceae rDNA-lineage. The azospirillae are nonphototrophic N_2-fixing bacteria, widely distributed in the rhizosphere of tropical and subtropical grasses where they seem to enhance the growth of these plants. Magnetotactic bacteria belonging to the genus *Magnetospirillum* are also members of this phylogenetic lineage, together with some former *Aquaspirillum* species.

Sphingomonadaceae Proteobacteria containing glycosphingolipids in their cell envelopes belong to the phylogenetic lineage of the sphingomonads, forming a versatile group of aerobic bacteria occurring in various environments such as soil, water and clinical specimens. This lineage corresponds to the α-4 subgroup. The genus *Sphingomonas* contained at least 20 species and was recently split on the basis of phylogenetic and chemotaxonomic analyses into four different genera (*Sphingomonas* sensu stricto, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*; Takeuchi et al., 2001). Some of the species belonging to these genera can metabolize various aromatic compounds and possess promising biotechnological properties. The Sphingomonadaceae comprise also the genera *Erythrobacter*, *Erythromicrobium* and *Porphyrobacter*, which are aerobic chemoorganotrophs, although their cells contain bacteriochlorophyll *a* and carotenoids. The genus *Zymomonas* is related to the sphingomonads. *Zymomonas* strains occur in palm sap and pulque, where they ferment sugars to high concentrations of ethanol by using the Entner-Doudoroff pathway, leading to the alcoholic beverages palm wine and tequila, respectively (Swings and De Ley, 1977; Sahm et al., 2001).

Rhodobacter Group The *Rhodobacter* group is a heterogeneous phylogenetic lineage among the "Alphaproteobacteria," including a number of photosynthetic rod-shaped PNS bacteria as well as numerous chemoorganotrophs. It corresponds to the α-3 group (Woese et al., 1984a). Typical photosynthetic PNS bacteria of this lineage belong to the genera *Rhodobacter* and *Rhodovulum*, containing freshwater and marine species, respectively. *Rhodobacter sphaeroides* and *R. capsulatus* were extensively used for genetic studies of bacterial photosynthesis. The facultative chemolithotroph *Paracoccus denitrificans* belongs also here; it is a remarkable and versatile bacterium, being able to grow chemolithoautotrophically at the expense of hydrogen gas or reduced inorganic sulfur compounds as electron donors, carbon dioxide and oxygen, but it can also grow chemoorganotrophically with various organic compounds as sole carbon source. Some hyphal, budding and prosthecate aerobic bacteria such as *Hyphomonas*, *Hirschia* and *Gemmobacter* belong to this lineage, as well as the budding *Staleya* and *Antarctobacter*, which were isolated from a hypersaline, heliothermal antarctic meromictic lake (Labrenz et al., 1998; Labrenz et al., 2000). Gram-negative cocci typically occurring in regular packages of tetrads and isolated from activated sludge biomass were assigned to the genus *Amaricoccus*, belonging to the *Rhodobacter* lineage (Maszenan et al., 1997). The *Rhodobacter* group is peripherally related to some of the most abundant cultured marine species and strains from some terrestrial halophilic lake environments. These organisms, frequently referred to as the *Roseobacter* group, include in addition to the aerobic bacteriochlorophyll-containing genera (such as *Roseobacter*, *Roseovarius* and *Rubrimonas*) the genera *Antarctobacter*, *Ketogulonicigenium*, *Methylarcula*, *Octadecabacter*, *Sagitulla*, *Silicibacter*, and *Sulfitobacter*, as well as the phylogenetically heterogeneous genus *Ruegeria*.

Caulobacteraceae The Caulobacteraceae are aquatic chemoorganotrophic and aerobic bacteria often attaching to surfaces with a stalk at one end and forming polarly flagellated swarming cells at the other end. Stalks of several cells may form rosettes. Caulobacters occur typically in freshwater and marine habitats with low nutrient levels. The rod-shaped *Brevundimonas* strains, which were previously allocated to the genus *Pseudomonas*, belong also to the Caulobacteraceae.

Rhizobiaceae, Bartonellaceae, Brucellaceae and Phyllobacterium Group This large and complex rDNA-cluster contains at present at least 15 genera and 70 species and corresponds to the α-2 subgroup of Woese et al. (1984a), together with the adjacent lineage formed by the Hyphomicrobiaceae and the *Bradyrhizobium*, *Methylobacterium* and *Methylocystis* rDNA groups (Fig. 1). One common trait of bacteria belonging to this cluster is that most of them interact with eukaryotes: agrobacteria are plant pathogens causing crown-gall or hairy-root disease (tumors) on various dicotyledonous plants; the rhizobia induce nodules on roots or stems of leguminous plants and live symbiotically in these nodules where they reduce atmospheric nitrogen; and the bartonellae and brucellae are pathogenic for humans or animals. The fast growing rhizobia classified in the genera *Rhizobium*, *Allorhizobium*, *Mesorhizobium* and *Sinorhizobium* form a major rDNA cluster, together with

the plant pathogenic genus *Agrobacterium* and the genus *Phyllobacterium*, whose members were isolated from leaf nodules of various plants belonging to the Myrsinaceae and Rubiaceae. The genus *Mesorhizobium* (more related to *Phyllobacterium*) and members of the genus *Sinorhizobium* form also a distinct clade among the rhizobia. According to 16S rDNA analysis, various *Rhizobium* species as well as *Allorhizobium* are highly related to the plant pathogenic agrobacteria. Recently, Young et al. (2001) proposed inclusion of all the species of *Agrobacterium* and *Allorhizobium* in the genus *Rhizobium*. The rRNA-based classification of the rhizobia is generally supported by sequence analysis of *atpD* and *recA* genes (Gaunt et al., 2001). The genes for nodule formation (*nod* genes), nitrogen fixation (*nif* genes) and tumor induction (in the case of agrobacteria) are localized on large plasmids. Species of the genus *Bartonella* occur in the blood of man and mammals; they are often vector borne, but can also be transmitted by animal scratches or bites. Bartonellae are considered to be emerging human pathogens. The type species *Bartonella bacilliformis* is a fastidious hemophilic organism that invades and destroys human red blood cells and is transmitted by a sandfly. *Bartonella quintana* (Brenner et al., 1993) was previously classified in the genus *Rochalimaea* and causes trench fever, a disease transmitted by lice and afflicting, e.g., soldiers during the First World War (1914–1918). *Bartonella henselae* (formerly *Rochalimaea henselae*) is nowadays recognized as the causative agent of cat scratch disease (Table 5). Brucellae develop intracellularly and cause worldwide infections (brucellosis) in humans and a great number of animals such as cattle, pigs, dogs, and even marine mammals.

Hyphomicrobiaceae, the Bradyrhizobium, Methylobacterium, Methylocystis and Related Groups The family Hyphomicrobiaceae contains hyphal, prosthecate and budding bacteria; most of them (e.g., *Hyphomicrobium* and *Pedomicrobium*) are chemoorganotrophs and prefer to grow on one-carbon compounds such as methanol, whereas others are phototrophic (e.g., *Rhodomicrobium*). *Hyphomicrobium* is well adapted to grow in oligotrophic freshwater habitats. Other members of the family are *Xanthobacter*, a versatile soil bacterium capable of nitrogen fixation and autotrophic growth in an atmosphere of hydrogen, oxygen and carbon dioxide, and *Azorhizobium caulinodans*, a nitrogen-fixing bacterium living symbiotically in the stem nodules of some leguminous plants such as *Sesbania*.

A large subcluster within this complex lineage is formed by the *Bradyrhizobium* group, including the slow-growing rhizobia nodulating soy bean and other leguminous plants and the stem-nodulating phototrophic bradyrhizobia (Molouba et al., 1999; Giraud et al., 2002), the photosynthetic PNS *Rhodopseudomonas*, the ecologically important chemolithotrophic nitrobacters oxidizing nitrite to nitrate, as well as the opportunistic *Afipia* species. The *Methylobacterium* and *Methylocystis* groups contain various methanotrophic and methylotrophic bacteria, mostly utilizing the serine pathway for the assimilation of one-carbon intermediates. Some of these methanotrophs may have biotechnological applications because they can utilize chloromethanes from polluted environments, whereas others such as *Methylocapsa* are acidophilic and can fix atmospheric nitrogen (Dedysh et al., 2002). A subgroup of the methylotrophic methylobacteria has been shown to nodulate *Crotolaria* legumes and fix nitrogen (Sy et al., 2001). Other members of this rDNA lineage are the genera *Beijerinckia*, consisting of aerobic, acid-tolerant free-living nitrogen-fixing rods occurring mainly in tropical acidic soils, and *Rhodobium*, which contains marine budding phototrophic PNS bacteria.

The "Betaproteobacteria"

The "Betaproteobacteria" (at least 75 genera and 220 species) clearly represent a monophyletic group within the larger phylogenetic lineage composed of the β-γ proteobacterial complex, named *Chromatibacteria* by Cavalier-Smith (Cavalier-Smith, 2002; Table 2). From the metabolic, morphological and ecological viewpoint, the "Betaproteobacteria" are very heterogeneous. They contain some purple nonsulfur (PNS) phototrophs (Fig. 1, purple triangles), various chemolithotrophs, some methylotrophs, a great number of chemoorganotrophs, some nitrogen-fixing bacteria, and some important plant-, human- and animal pathogens (see Tables 5 and 6). Their morphologies can vary from rods or cocci to spiral and sheathed cells. Some members of the "Betaproteobacteria" are of biotechnological interest owing to their biodegradation properties. Recently nitrogen-fixing "Betaproteobacteria" were described that nodulate the roots of legumes (Chen et al., 2001; Moulin et al., 2001). More detailed information concerning the group can be found in the chapters dealing with the individual families and genera of the "Betaproteobacteria." Table 9 gives an overview of the major phylogenetic groups (see also Fig. 1) and the names of the six orders and 12 families of the "Betaproteobacteria" as they are listed in the 2nd edition of *Bergey's Manual of Systematic Bacteriology* (Garrity, 2001a).

Table 9. The "Betaproteobacteria": orders, families and number of genera.[a]

Order	Family	Number of genera
"Burkholderiales"	"Burkholderiaceae"	4
	"Ralstoniaceae"	1
	"Oxalobacteraceae"	5
	Alcaligenaceae	6
	Comamonadaceae	15
"Hydrogenophilales"	"Hydrogenophilaceae"	2
"Methylophilales"	"Methylophilaceae"	3
"Neisseriales"	Neisseriaceae	14
"Nitrosomonadales"	"Nitrosomonadaceae"	2
	Spirillaceae	1
	Gallionellaceae	1
"Rhodocyclales"	"Rhodocyclaceae"	6

[a]According to the second edition of *Bergey's Manual of Systematic Bacteriology* (Garrity and Holt, 2001). Quotation marks are used for names which have not yet been validated (as of mid 2002).

Although the majority of the phototrophic PNS bacteria belong to the "Alphaproteobacteria," some PNS species are members of the *Rhodocyclus* group or the family Comamonadaceae of the "Betaproteobacteria." Similarly to the previously mentioned PNS "Alphaproteobacteria," the PNS betaproteobacterial phototrophs are phylogenetically intermingled with nonphototrophs (Fig. 1, visualized by purple shaded areas) and can be clearly differentiated from the PNS "Alphaproteobacteria" (e.g., *Rhodobacter*, *Rhodobium*, etc.) on the basis of fatty acid and quinone composition as well as cytochrome *c* sequences (Imhoff, 2001a).

Alcaligenaceae, Comamonadaceae, Burkholderia, Oxalobacter and Related Groups

The Alcaligenaceae, Comamonadaceae, and the *Burkholderia*-, *Ralstonia*- and *Oxalobacter*-groups are phylogenetically more closely related to each other than to other 16S rDNA branches of the "Betaproteobacteria" (Fig. 1). A number of clinically important taxa belong to this lineage: members of the genus *Alcaligenes* are mostly saprophytic, but behave often as nosocomial bacteria, whereas the closely related *Bordetella pertussis* (Table 5) causes whooping cough in humans (other *Bordetella* species are pathogenic for animals). *Taylorella* is responsible for endometritis in mares, *Pelistega* is associated with a respiratory disease in pigeons (Vandamme et al., 1998), and *Brackiella oedipodis* (Willems et al., 2002) was recently reported as the causal agent of endocarditis in a small neotropical primate.

The former "acidovorans" and "solanacearum" rRNA groups of the genus *Pseudomonas* sensu lato (i.e., respectively, rRNA groups III and II of Palleroni [1984]) belong to the "Betaproteobacteria." The "acidovorans" group has at present the status of the family Comamonadaceae, a phylogenetically coherent but physiologically heterogeneous group of prokaryotes encompassing genera such as *Acidovorax*, *Comamonas*, *Delftia*, *Hydrogenophaga*, *Variovorax*, *Xylophilus*, *Rhodoferax*, *Roseateles*, *Rubrivivax*, *Leptothrix* and *Sphaerotilus* (Willems et al., 1991a; Willems et al., 1991b). The latter two genera are heterotrophic sheathed bacteria, which may appear yellow or dark brown owing to the deposition of iron and manganese oxides, whereas *Rhodoferax* and *Rubrivivax* are typical PNS photosynthetic bacteria. Some Comamonadaceae, such as *Acidovorax facilis*, *Hydrogenophaga flava* and *Variovorax paradoxus*, are able to grow chemolithotrophically at the expense of hydrogen gas oxidation. *Xylophilus ampelinus* causes necrosis and canker on grapevines. Other representatives, e.g., belonging to *Comamonas* or *Delftia*, can degrade aromatic compounds (via the *meta*-cleavage of the aromatic ring) and are important in the biodegradation of toxic wastes. *Roseateles* was described as the first obligate aerobic betaproteobacterium containing bacteriochlorophyll *a* (Suyama et al., 1999). However, light does not support growth of *Roseateles* strains under anaerobic conditions and in this sense *Roseateles* resembles physiologically the aerobic bacteriochlorophyll *a*-containing "Alphaproteobacteria," such as *Erythrobacter* and *Erythromicrobium* (Sphingomonadaceae).

The former "*Pseudomonas solanacearum* group" is composed of the genera *Burkholderia*, *Ralstonia* and a few others. Some of these bacteria are typical plant pathogens (e.g., *Ralstonia solanacearum*, causing wilt on many cultivated plants; Table 6), whereas others are important animal and human pathogens. *Burkholderia mallei* and *B. pseudomallei* have been classified as risk group 3 organisms (Table 5) because they cause respectively glanders disease in horses and melioidosis, a disease endemic in animals and humans in Southeast Asia. *Burkholderia cepacia* seems ubiquitous and is both friend and foe to humans (Govan et al., 2000): it is associated with plants (the original isolate was reported by Burkholder [1950] as the causative agent of bacterial rot of onion bulbs), occurs in soil and water, protects crops from bacterial and fungal infections, and is also frequently reported in the environment of patients, particularly cystic fibrosis patients, where *B. cepacia* infections have a considerable impact on their morbidity and mortality, as well as on their social life. The remarkable genomic heterogeneity among the *B. cepacia* strains isolated from various ecological niches makes their correct identification problematic (Coenye et al., 2001). It was demonstrated that presumed *B. cepacia* strains isolated

from cystic fibrosis patients and other sources belong to at least nine distinct genomic species or genomovars (Vandamme et al., 1997; Coenye et al., 2001). At least two of these genomovars are often implicated in the transmission of *B. cepacia* between cystic fibrosis patients (Mahenthiralingam et al., 2000; LiPuma et al., 2001). The extreme metabolic versatilty of *B. cepacia* could be applied in the bioremediation of recalcitrant xenobiotics (Parke and Gurian-Sherman, 2001). However, more caution is undoubtedly needed in this area, because many strains used or under development for biocontrol or bioremediation purposes are taxonomically poorly characterized, causing a potential hazard to the cystic fibrosis community worldwide (Parke and Gurian-Sherman, 2001). The related genus *Ralstonia* is also unusual as it harbors important plant pathogens, opportunistic human pathogens, as well as organisms of considerable biotechnological interest because of their potential for biodegradation of xenobiotics and recalcitrant compounds. Moreover, Chen et al. (2001) described *Ralstonia taiwanensis* as a betaproteobacterium capable of root nodule formation and nitrogen fixation in a leguminous plant (*Mimosa*). Together with the *Burkholderia*-like strains described by Moulin et al. (2001), these are the first betaproteobacteria known to be capable of root nodule formation and nitrogen fixation. *Ralstonia eutropha* (previously classified as *Alcaligenes eutrophus*) is a well-known and intensely studied facultative chemolithotroph (a typical "Knallgas" bacterium) that can grow at the expense of hydrogen gas, CO_2 and O_2 (Aragno and Schlegel, 1992). When grown under appropriate conditions, *R. eutropha* is also an excellent producer of the bioplastic poly-β-hydroxybutyrate and similar polyhydroxyalkanoates (Table 7). Some toxic-metal-resistant bacteria isolated from industrial biotopes were recently allocated to the genus *Ralstonia* (Goris et al., 2001). The latter organisms can be exploited to recycle soils polluted by toxic metals, such as mercury, cadmium or nickel.

The *Oxalobacter* group is composed of *Herbaspirillum* (a diazotroph colonizing endophytically roots, stems or leaves of graminaceous plants), *Janthinobacterium* (one of the known violacein-producing organisms), *Oxalobacter* and a few other genera. *Oxalobacter formigenes* occurs in the rumen or the large bowel of humans and animals; it performs a unique fermentation of oxalate to formate and CO_2, whereby ATP is generated via a membrane-linked proton-translocating ATPase.

Hydrogenophilus Group The genus *Hydrogenophilus* harbors thermophilic (optimum growth temperature 50–60°C), facultatively chemolithoautotrophic hydrogen-oxidizing rods that form a distinct lineage among the "Betaproteobacteria" (Hayashi et al., 1999). The classification of the colorless chemolithotrophic S-oxidizing *Thiobacillus* species has been thoroughly revised by Kelly and Wood (2000). The type species *T. thioparus* together with a few other *Thiobacillus* species belongs to the "Betaproteobacteria" and is distantly related to the *Hydrogenophilus* group. The majority of the other *Thiobacillus* species belong to the "Gammaproteobacteria" and were reassigned to three other genera (see The "Gammaproteobacteria"). Because they are able to leach metals from ore, Thiobacilli (e.g., *Acidithiobacillus* species) are used in processing low-grade metal ores.

Methylophilus, Nitrosomonas and Related Groups The majority of the one-carbon utilizers (methanotrophs) belong either to the "Alphaproteobacteria" or to the "Gammaproteobacteria." However, three methylotrophic genera (*Methylobacillus*, *Methylophilus* and *Methylovorus*) group in the *Methylophilus* rRNA lineage of the "Betaproteobacteria." These are phylogenetically distantly related to the *Nitrosomonas* lineage. The aerobic chemolithoautotrophic ammonia-oxidizing bacteria such as the rod-shaped *Nitrosomonas* and the spiral-shaped *Nitrosospira* constitute a monophyletic lineage among the "Betaproteobacteria." *Spirillum volutans* is distantly related to this group, as well as the Fe^{2+}-oxidizing chemolithotrophic *Gallionella ferruginea*, already known since 1836 for its peculiar bean-shaped cells forming very long spiral stalks composed of ferric hydroxide (Ehrenberg, 1836).

Rhodocyclus Group The *Rhodocyclus* lineage harbors besides PNS bacteria (e.g., *Rhodocyclus purpureus*) also diazotrophic rhizosphere bacteria such as *Azoarcus* and *Azospira*. Several strains of *Azoarcus* and the related *Thauera* can degrade aromatic compounds including chlorobenzoates. *Thauera selenatis* is capable of nitrate and selenate respiration and another member of this group, *Zoogloea ramigera* (Rossellomora et al., 1993), is considered to be a typical floc-forming prokaryote during the activated sludge process in sewage treatment plants (Juretschko et al., 2002). Also *Quadricoccus* (a large coccus occurring in tetrads; Maszenan et al., 2002) is found in the activated sludge biomass where it synthesizes polyphosphate and polyhydroxyalkanoates.

Dechloromonas and *Dechlorosoma* are newly described genera of perchlorate-reducing bacteria, associated, e.g., with the manufacture and dismantling of ammunition (Achenbach et al., 2001).

Neisseriaceae The most prominent human pathogens of the "Betaproteobacteria" belong to the genus *Neisseria*, containing the two well-known species *N. gonorrhoeae* and *N. meningitidis*. Strains of these organisms have been extensively studied by multi-locus-sequencing-typing (MLST; Achtman, 1998; Maiden et al., 1998).

Other clinical bacteria, such as *Kingella* and *Eikenella*, belong also to this phylogenetic group together with *Chromobacterium*, which produces the purple pigment violacein. The mammalian oral commensals *Alysiella* and *Simonsiella* belong also to Neisseriaceae and are unique because their filament-forming cells show a dorsal-ventral asymmetry and display a gliding motility on the epithelial cells in the oral cavity and upper respiratory tract of their host. These bacteria might have coevolved with their mammalian hosts during the past 100 million years (Hedlund and Staley, 2002).

The "Gammaproteobacteria"

Most 16S rDNA trees show that the members of the "Gammaproteobacteria" represent a monophyletic group which includes in fact also the "Betaproteobacteria" as a major line of descent (see Fig. 1). The "Gammaproteobacteria" form the largest proteobacterial group (at least 180 genera and 750 species), including the phytopathogenic *Xanthomonas* group as a border-line member. Depending upon the rDNA-treeing method used, the *Xanthomonas* rDNA-group appears peripherally linked to either the β- or the γ-class and can be considered as a sister group of the "Betaproteobacteria" (Ludwig and Klenk, 2001). The major phylogenetic groups (Fig. 1) and the names of the 13 orders and 20 families of the "Gammaproteobacteria" as listed in the 2nd edition of *Bergey's Manual of Systematic Bacteriology* (Garrity, 2001a) are given in Table 10. The individual chapters on the families and genera of this class can be consulted for more detailed information.

The "Gammaproteobacteria" contain the photosynthetic purple sulfur (PS) bacteria (Chromatiaceae and Ectothiorhodospiraceae; Table 10; Fig. 1) together with a great number of familiar chemoorganotrophic bacterial groups, such as the Enterobacteriaceae, Legionellaceae, Pasteurellaceae, Pseudomonadaceae, Vibrionaceae, and also some chemolithotrophic mostly sulfur- or iron-oxidizing prokaryotes. The class harbors some important human and animal pathogens (Table 5). It is noteworthy that the family Enterobacteriaceae is known since 1937 as a classical phenotypic group (Rahn, 1937), which remains fully supported by modern molecular taxonomy. On the other hand, the tradional group of the pseudomonads turned out to be phylogenetically extremely heterogeneous, because its members are scattered over the "Alphaproteobacteria," "Betaproteobacteria" and "Gammaproteobacteria." The genus *Pseudomonas* is at present restricted to all species phylogenetically related to its type species *Pseudomonas aeruginosa*, a member of the "Gammaproteobacteria," whereas all the other pseudomonads belonging to the α- and β-classes have been allocated to new genera such as *Brevundimonas*, *Sphingomonas*, *Comamonas*, *Burkholderia*, *Ralstonia*, etc. (see The "Alphaproteobacteria," The "Betaproteobacteria"). An analogous taxonomic history concerns the genus *Thiobacillus*, whose members are chemolithotrophs oxidizing various inorganic sulfur-compounds. Its classification was recently clarified by Kelly and Wood (2000): thiobacilli related to the type species *T. thioparus* belong to the "Betaproteobacteria," whereas the *Thiobacillus* species belonging to the "Gammaproteobacteria" were reclassified in the genera *Acidithiobacillus*, *Halothiobacillus* and *Thermithiobacillus*.

Table 10. The "Gammaproteobacteria": orders, families and number of genera.[a]

Order	Family	Number of genera
"Chromatiales"	Chromatiaceae	22
	Ectothiorhodospiraceae	5
"Xanthomonadales"	"Xanthomonadaceae"	8
"Cardiobacteriales"	Cardiobacteriaceae	3
"Thiotrichales"	"Thiotrichaceae"	9
	"Piscirickettsiaceae"	5
	"Francisellaceae"	1
"Legionellales"	Legionellaceae	1
	"Coxiellaceae"	2
"Methylococcales"	Methylococcaceae	6
"Oceanospirillales"	"Oceanospirillaceae"	6
	Halomonadaceae	6
Pseudomonadales	Pseudomonadaceae	15
	Moraxellaceae	3
"Alteromonadales"	Alteromonadaceae	11
"Vibrionales"	Vibrionaceae	6
"Aeromonadales"	Aeromonadaceae	2
	Succinivibrionaceae	4
"Enterobacteriales"	Enterobacteriaceae	41
"Pasteurellales"	Pasteurellaceae	6

[a]According to the second edition of *Bergey's Manual of Systematic Bacteriology* (Garrity and Holt, 2001). Quotation marks are used for names which have not yet been validated (as of mid 2002).

Chromatiaceae and Ectothiorhodospiraceae The distribution of the anoxygenic photosynthetic purple sulfur (PS) bacteria is at present restricted to two clearly distinct phylogenetic lineages of the "Gammaproteobacteria" (Fig. 1): the Chromatiaceae (25 genera), which accumulate elemental sulfur globules inside their cells,

and the Ectothiorhodospiraceae (7 genera), which deposit sulfur outside their cells. These prokaryotes are mainly found in illuminated anoxic zones of lakes (particularly meromictic lakes) where H_2S accumulates and also in "sulfur springs," where biologically or geochemically produced H_2S can trigger the formation of massive blooms of purple sulfur bacteria (Imhoff, 2001a). All PS bacteria can utilize H_2S as an electron donor for CO_2 reduction, but many are also able to use other reduced sulfur compounds (e.g., thiosulfate) as photosynthetic electron donors. Ectothiorhodospiraceae are haloalkaliphilic sulfur bacteria possessing lamellar intracellular photosynthetic membrane structures, whereas most Chromatiaceae possess a vesicular type of intracellular membrane. Some nonphototrophic and alkaliphilic taxa belong also to the phylogenetic lineage of the Ectothiorhodospiraceae: for example, *Nitrococcus* is a halophilic nitrite-oxidizing chemolithotroph, *Thioalkalivibrio* (Sorokin et al., 2001) contains obligately chemolithotrophic sulfur-oxidizing bacteria, and *Alcalilimnicola* (Yakimov et al., 2001) is a halotolerant aerobic heterotroph.

Enterobacteriaceae, Aeromonadaceae, Alteromonadaceae, Pasteurellaceae, Succinivibrionaceae and Vibrionaceae The core of the γ-class is composed of the well-known enterics (see Introduction to the Family Enterobacteriaceae in the second edition), Vibrionaceae (see The Family Vibrionaceae in Volume 6), Aeromonadaceae (see The Genera Aeromonas and Plesiomonas in Volume 6), Pasteurellaceae (see The Genus Pasteurella in Volume 6) and the Alteromonadaceae (see The Genus Alteromonas and Related Proteobacteria in Volume 6), each including a large number of genera and species. Some representatives of these families are known as pathogens of humans and animals (Table 5). The Enterobacteriaceae include the well-known and thoroughly studied *Escherichia coli*, together with numerous inhabitants of the intestinal tract of warm-blooded animals (e.g., *Salmonella* and *Shigella*, the etiological agents of salmonellosis and shigellosis, respectively; see Table 5). *Escherichia coli* is an excellent indicator organism for water quality (fecal contamination). The Enterobacteriaceae comprise a relatively homogeneous phylogenetic group within this large cluster; they are mostly facultative anaerobic carbohydrate-degrading microorganisms; some perform a mixed acid fermentation, whereas others carry out the butanediol fermentation. The enterics comprise also plant pathogenic bacteria (e.g., *Erwinia*, *Pectobacterium* and *Brenneria*; see Table 6), the plague-causing *Yersinia pestis*, as well as *Xenorhabdus* and *Photorhabdus*, which are symbionts of entomopathogenic nematodes, where they live in the intestinal lumen. An interesting and somewhat distant member of the enterics is *Buchnera aphidicola*, a not-yet-cultured endosymbiont of aphids (Baumann et al., 1995). The symbiotic association between aphids and *Buchnera* seems to be obligate and mutualistic: *Buchnera* synthesizes tryptophan, cysteine and methionine and supplies these essential amino acids to the aphid host. A parallel evolution of *Buchnera* and aphids seems to have occurred, and Baumann et al. (1998) estimated the origin of this symbiotic association as 200–250 million years ago. The correlation of sequence diversity of 16S rDNA of symbionts with the age of their hosts has led to the calibration of the molecular clock of 16S rDNA in recent organisms (Moran et al., 1993), assumed to generate 1% sequence divergence within 25–50 million years (see also Stackebrandt, 1995). Also the endosymbionts of carpenter ants (*Camponotus* spp.) constitute a distinct taxonomic group within the "Gammaproteobacteria" and are phylogenetically closely related to *Buchnera* and symbionts of tsetse flies. Comparison of the phylogenetic trees of the bacterial endosymbionts and their host species suggests a highly synchronous cospeciation process of both partners (Sauer et al., 2000).

Vibrionaceae are facultative anaerobic inhabitants of brackish, estuarine and pelagic waters and sediments and form the dominant culturable microflora in the gut of molluscs, shrimps and fish. The family harbors several pathogens (e.g., *Vibrio cholerae*, the causal agent of cholera) as well as luminous bacteria (e.g., *Photobacterium* and several *Vibrio* species), occurring free-living in seawater, as well as being symbionts in the light organs of many fish and invertebrates (see Dunlap and Kita-Tsukamoto, 2001). Bioluminescent bacteria occur also among the genera *Photorhabdus* (Enterobacteriaceae) and *Shewanella*. The latter genus belongs to the Alteromonadaceae, which are strictly aerobic chemoorganotrophs requiring seawater for growth. Quorum-sensing plays an important role in the phenomenon of bioluminescence. Several representatives of the Pasteurellaceae are the etiological agents of various infectious diseases in humans and other vertebrates (cattle, sheep, goats and fowl). They encompass the following major genera: *Pasteurella*, *Haemophilus*, *Actinobacillus* and *Mannheimia. Haemophilus influenzae* was the first free-living organism whose entire genome (ca. 1.8×10^9 bp) was sequenced (Fleischmann et al., 1995). The Aeromonadaceae include the fish pathogen *Aeromonas salmonicida*. The Succinivibrionaceae (Hippe et al., 1999) are strict anaerobes fermenting glucose and other carbohydrates to succinate and acetate. They occur in the rumen of sheep and

cattle (*Ruminobacter*, *Succinomonas* and *Succinivibrio*) or in the feces or colon of dogs and humans (*Anaerobiospirillum*).

Francisella and Piscirickettsia Groups The *Francisella* and *Piscirickettsia* groups form a deeply branching lineage among the "Gammaproteobacteria." *Francisella tularensis* is the causal agent of tularemia, a plague-like zoonosis, spread to humans from rodents via direct contact or biting arthropods, whereas *Piscirickettsia salmonis* causes an epizootic disease in salmonid fishes. Members of the *Piscirickettsia* group display a great morphological and metabolic diversity: *Thiomicrospira* contains spiral-shaped chemolithotrophic sulfur-oxidizing bacteria, which are closely related to the alkaliphilic chemolithotrophic *Thioalkalimicrobium* (Sorokin et al., 2001). *Hydrogenovibrio* is a marine obligately chemolithoautotrophic hydrogen-oxidizing bacterium and *Cycloclasticus* harbors rod-shaped bacteria degrading aromatic hydrocarbons and occurring in marine sediments.

Cardiobacteriaceae The Cardiobacteriaceae are an example of a family created (Dewhirst et al., 1990) on the basis of 16S rDNA comparisons of three taxa, whose phylogenetic relationship was at first quite unexpected. *Cardiobacterium hominis* is an occasional resident of the human respiratory tract and has been recovered from blood samples of patients suffering from endocarditis. *Suttonella indologenes* (previously classified as *Kingella indologenes*) has been reported in human eye infections and blood of patients with endocarditis, whereas *Dichelobacter nodosus* (formerly *Bacteroides nodosus*) is the causative agent of footrot in ruminants. Authentic kingellae belong to the "Betaproteobacteria" and authentic bacteroideae belong to the Cytophaga-Flavobacterium-Bacteroides phylum (the so-called "Bacteroidetes" according to Garrity, 2001).

Moraxellaceae The Moraxellaceae encompass the genera *Moraxella*, *Acinetobacter* and *Psychrobacter* forming a separate lineage among the "Gammaproteobacteria" (Rossau et al., 1991). Moraxellae are commonly isolated from animals or humans and some of them are pathogenic, whereas the psychrophilic *Psychrobacter* species occur in the marine and antarctic environment (e.g., in antarctic ornithogenic soils; Bowman et al., 1996) and also in clinical material. The oxidase-negative acinetobacters are saprophytes occurring in soil, water and sewage, but also typically on the skin of healthy humans. Moreover, some acinetobacters can also cause nosocomial infections.

Methylococcaceae The Methylococcaceae form a distinct lineage (Bowman et al., 1995) encompassing methylotrophic bacteria such as the genera *Methylobacter*, *Methylococcus* and *Methylomonas*, which share the ribulose monophosphate cycle for the assimilation of reduced C1-compounds. Some of these methylotrophs form cysts as resting stages and possess internal membrane systems arranged as bundles of disc-shaped vesicles distributed throughout the cell, in contrast to some methylotrophs of the "Alphaproteobacteria," whose membrane systems run along the periphery of the cells. The moderately halophilic *Methylophaga* belong phylogenetically to the *Piscirickettsia* group.

Thiothrix Group The Thiothrix group contains a number of filamentous and gliding sulfur-oxidizing chemolithotrophs, such as *Thiothrix*, *Beggiatoa* and *Thioploca*. *Thiothrix* forms sheathed filaments, whereas *Beggiatoa* forms long filaments lacking a sheath. Sulfur granules generally accumulate in the cells. The not yet axenic *Thiomargarita* ("sulfur pearl") is related to *Thioploca* and is a real giant among the prokaryotic life forms (its spherical cells are about 500 μm wide!). *Thiomargarita* cells form thick mats near the coast of Namibia, where they perform an anoxic oxidation of H_2S coupled to the reduction of nitrate (Schulz et al., 1999; Schulz, 2002). *Leucothrix* can be considered as a chemoorganotrophic counterpart of *Thiothrix*. Some not yet cultured sulfur-oxidizing symbiotic bacteria of bivalves are closely related to the *Thiothrix* group and play an important role in the nutrition of such animals, living near hydrothermal vents.

Halomonadaceae The family Halomonadaceae is a large and complex group of moderately halophilic and marine bacteria encompassing the genera *Halomonas*, *Chromohalobacter*, *Zymobacter* and *Carnimonas*. Recent comparative sequence analysis of 16S and 23S rDNA of representative strains indicated (Arahal et al., 2002) that the genus *Chromohalobacter* forms a monophyletic group, whereas the genus *Halomonas* is clearly polyphyletic and needs a taxonomic re-evaluation based on a polyphasic taxonomic approach (Romanenko et al., 2002 [doi 10.1099/ijs.0.02240-0]).

Pseudomonadaceae In the past (Palleroni, 1984), the family Pseudomonadaceae contained an extremely heterogeneous group of aerobic, rod-shaped and mostly polarly flagellated bacteria, phylogenetically spread over the "Alphaproteobacteria," "Betaproteobacteria" and "Gammaproteobacteria." Nowadays the family is restricted to some 90 *Pseudomonas* species around the type species *Pseudomonas aeruginosa* and some related genera which form a

separate lineage within the "Gammaproteobacteria." The pseudomonads of the "Alphaproteobacteria" and "Betaproteobacteria" have been transferred to other genera (Willems et al., 1991a; Kersters et al., 1996; Anzai et al., 2000; Caulobacteraceae, Alcaligenaceae, Comamonadaceae, *Burkholderia*, *Oxalobacter* and Related Groups). Some authentic *Pseudomonas* species (e.g., *P. fluorescens*) produce fluorescent pigments. Most pseudomonads are versatile and can grow on a great variety of organic compounds, including aromatic hydrocarbons, because some of them possess efficient oxygenases. Such bacteria play key roles in the purification of wastewater and clean-up of oil spills. Some of the *Pseudomonas* species (e.g., *P. syringae*) are plant pathogens (Table 6), whereas *P. aeruginosa* and some other fluorescent pseudomonads can be involved in serious nosocomial infections (Table 5). The free-living nitrogen fixers of the genera *Azotobacter* and *Azomonas* belong also to this phylogenetic lineage, together with the cellulose-degrading *Cellvibrio* and a few other related genera.

Oceanospirillum Group The *Oceanospirillum* group encompasses six genera whose members are aerobic, are moderately halophilic and generally possess curved cells. Some representatives (e.g., *Neptunomonas*) can degrade polycyclic aromatic hydrocarbons or long chain alkanes. The taxonomy of the genus *Oceanospirillum* has recently been revised (Satomi et al., 2002). *Balneatrix alpica* was reported as a bacterium associated with a case of pneumonia and meningitis in a spa therapy center in France (Dauga et al., 1993).

Legionellaceae and Coxiella Group The clinically important legionellae occur in surface water, mud, thermally polluted lakes and streams and some (e.g., *Legionella pneumophila*) may enter the human respiratory tract when water is aerosolized in showers and air-conditioning systems. *Legionella pneumophila* is a pathogen for humans causing pneumonia (Legionnaires' disease). An obligate intracellular bacterial parasite of small free-living amoebae (previously classified as *Sarcobium lyticum*; Drozanski, 1991) belongs also to the genus *Legionella* (Hookey et al., 1996). The genera *Coxiella* and *Rickettsiella* belong to the same phylogenetic lineage as the legionellae. Although phylogenetically distinct, *Coxiella* and *Rickettsia* (the latter belonging to the "Alphaproteobacteria") share similarities in their parasitic lifestyle. *Coxiella burnetii*, an obligate parasitic bacterium growing preferentially in the vacuoles of the host cells, causes Q-fever, a pneumonia-like infection, transmitted among animals by insect bites (e.g., ticks) and occasionally causes disease in humans (originally called "abattoir fever"). Rickettsiellae are widely distributed intracellular pathogens of invertebrates, including insects, crustaceans and arachnids. They have not yet been cultivated in cell-free media and grow and multiply in cell vacuoles of the fat body and hepatopancreas of their invertebrate hosts.

Xanthomonas Group The plant pathogenic *Xanthomonas* species, *Frateuria* (phenotypically resembling the acetic acid bacteria of the α-class), *Xylella* (a phytopathogen living in the xylem of various plants) and *Stenotrophomonas*, together with some yellow-pigmented N_2O-producing bacteria isolated from ammonia-supplied biofilters (Finkmann et al., 2000), constitute a clearly separated phylogenetic lineage among the *Chromatibacteria* sensu Cavalier-Smith (2002), i.e., the large complex formed by the "Betaproteobacteria" and the "Gammaproteobacteria"; see Table 2). The ubiquitous *Stenotrophomonas maltophilia* is also involved in important nosocomial infections (Van Couwenberghe et al., 1997; Khan and Mehta, 2002). Depending on the treeing algorithms used and the number of rDNA-sequences included, the xanthomonads cluster peripherally linked either to the "Betaproteobacteria" or to the "Gammaproteobacteria" (Fig. 1). The first complete genome sequence published of a plant pathogenic bacterium (Simpson et al., 2000) was that of *Xylella fastidiosa*, a pathogen causing important diseases in citrus trees, grapevines and other plants.

The "Deltaproteobacteria"

From the viewpoint of lifestyle and morphology, the δ-class is most peculiar because it contains bacteria that are typical predators on other prokaryotes (bdellovibrios), whereas other "Deltaproteobacteria" belonging to the myxobacteria display complex developmental life cycles, forming multicellular structures called "fruiting bodies." Up to now, photosynthetic bacteria have not been reported among the δ-class. Menaquinones are the major electron carriers in the respiratory chain (Table 3). The major subgroups of the "Deltaproteobacteria" are: 1) the myxobacteria displaying gliding motility and forming multicellular fruiting bodies, which are often large enough to be observed with the aid of a hand lens or even by the naked eye (e.g., *Chondromyces*); 2) the bdellovibrios living as predators on other Gram-negative bacteria; 3) the dissimilatory sulfate- and sulfur-reducing bacteria (their genus names are mostly prefixed by *Desulfo-*); and 4) some synthrophic bacteria which ferment propionate (e.g., *Syntropho-*

bacter) or benzoate (e.g., *Syntrophus*) to acetate, CO_2 and hydrogen syntrophically in coculture with hydrogen-consuming methanogens.

Bdellovibrios and myxobacteria are strict aerobes, whereas the sulfate- and sulfur-reducers and the syntrophic bacteria are strictly anaerobic. One may speculate whether the bdellovibrios and the myxobacteria represent aerobic descendants of the sulfate- and sulfur-reducing bacteria. We consider here briefly the major phylogenetic groups of the "Deltaproteobacteria," containing at present some 60 different genera and 160 species. Table 11 summarizes the names of taxa above the rank of genus within the "Deltaproteobacteria" according to the new edition of *Bergey's Manual of Systematic Bacteriology* (Garrity, 2001).

Myxobacteria The myxobacteria constitute a fascinating group of strictly aerobic, chemoorganotrophic, gliding, generally pigmented rod-shaped cells, which under starvation conditions aggregate, forming multicellular and macroscopic fruiting bodies (see Dworkin and Kaiser [1993] and Dawid [2000]). Some of the cells within these fruiting bodies are often converted to resting stages called myxospores. The peculiar gliding movement of the vegetative cells is not restricted to myxobacteria as it occurs also amongst members of the genera *Cytophaga* and *Flexibacter*, both belonging to the Cytophaga-Bacteroides-Flavobacterium phylum (now called "Bacteroidetes"; Garrity, 2001). New molecular and genetic insights concerning gliding motility have been gathered from studies on *Myxococcus xanthus* (Spormann, 1999). The myxobacteria are grouped in the order Myxococcales, which is composed of the following four families: Myxococcaceae, Archangiaceae, Cystobacteraceae and the Polyangiaceae. Those fruiting myxobacteria whose rDNA sequence could be examined form a phylogenetically coherent group (Spröer et al., 1999; Fig. 1), consisting of two major phylogenetic lineages. One fairly homogeneous group is composed of the genera *Myxococcus*, *Angiococcus*, *Archangium*, *Cystobacter*, *Melittangium* and *Stigmatella*, whereas the second lineage is much more heterogeneous and is composed of three genera belonging to the family Polyangiaceae. Within the latter group, the genus *Nannocystis* seems to occupy the most separate position. The classification of the myxobacteria relies heavily on morphological features whose phylogenetic significance has been confirmed (Spröer et al., 1999). The myxobacteria occur in various habitats, such as soils and particularly on decaying organic material, including dung of herbivorous animals, rotting wood, and bark of living and dead trees. They obtain their nutrients primarily by causing lysis of other bacteria. The myxobacteria are mostly mesophilic, but some psychrophilic, acidophilic and alkaliphilic species have been described (Dawid, 2000). The sporangia of *Chondromyces crocatus* have been found to harbor a sphingobacterium-like organism which was described as "*Candidatus* comitans" by Jacobi et al. (1997).

Several myxobacteria produce potentially useful secondary compounds, such as antibiotics and cytostatic peptides (Reichenbach and Höfle, 1993; Reichenbach, 2001). The fruiting myxobacteria possess the most complex behavioral patterns and life cycles of all prokaryotes known so far and are therefore considered as a very interesting study object. They typically display social behavior expressed by collective food

Table 11. The "Deltaproteobacteria": orders, families and number of genera.[a]

Order	Family	Number of genera
"Desulfurellales"	"Desulfurellaceae"	2
"Desulfovibrionales"	"Desulfovibrionaceae"	3
	"Desulfomicrobiaceae"	1
	"Desulfohalobiaceae"	3
"Desulfobacterales"	"Desulfobacteraceae"	12
	"Desulfobulbaceae"	4
	"Desulfoarculaceae"	4
"Desulfuromonadales"	"Desulfuromonadaceae"	2
	"Geobacteraceae"	1
	"Pelobacteraceae"	2
"Synthrophobacterales"	"Synthrophobacteraceae"	4
	"Synthrophaceae"	2
"Bdellovibrionales"	"Bdellovibrionaceae"	3
Myxococcales	Myxococcaceae	2
	Archangiaceae	1
	Cystobacteraceae	3
	Polyangiaceae	3

[a]According to the second edition of *Bergey's Manual of Systematic Bacteriology* (Garrity and Holt, 2001). Quotation marks are used for names which have not yet been validated (as of mid 2002).

uptake, cooperative motility (swarming), and social development (Crespi, 2001). Intercellular communication plays an important role in these phenomena. The analysis of the genome sequence of *Myxococcus xanthus* (9.5 Mb; twice as large as the *E. coli* genome) should enhance the understanding of the molecular and evolutionary basis of the peculiar life style of these myxobacteria.

Bdellovibrio Group The bdellovibrios are small vibrioid prokaryotes living as predators on other Gram-negative bacteria. They are widespread in nature and have been isolated from soil, sewage, freshwater and marine environments. Wildtype bdellovibrios show a biphasic life cycle, alternating between an extracellular "attack-phase" flagellated form and an intracellular nonflagellated reproductive phase. After a bdellovibrio cell penetrates the wall of a Gram-negative prey cell (such as *Pseudomonas* or *Erwinia*), it performs a number of modifications on the outer membrane and the peptidoglycan of the invaded bacterium. The resulting spherical structure, consisting of the killed invaded cell and the developing bdellovibrio, is called a "bdelloplast." The bdellovibrio cell grows and multiplies in the periplasmic space of the prey cell and digests the periplasmic and cytoplasmic contents of the invaded cell (for more details, see Jurkevitch, 2001). The parasitic life style of *Bdellovibrio* is reflected in the size of its genome, which is only half of that of *E. coli*. Genomic heterogeneity has been observed among the bdellovibrios (Seidler et al., 1972; Baer et al., 2000): the genera *Bdellovibrio* and *Bacteriovorax* constitute separate lineages among the "Deltaproteobacteria." The 16S rDNA sequences of two other genera (*Micavibrio* and *Vampirovibrio*) have not been analyzed yet. The latter genus attacks cells of the green alga *Chlorella*.

Sulfate- and Sulfur-reducing Bacteria Sulfate- and sulfur-reducing bacteria occur in various major phylogenetic lineages, such as the Archaea (e.g., *Archaeglobus*), the phylum of the thermophilic Thermodesulfobacteria, the order Clostridiales (the spore-forming *Desulfotomaculum* and *Desulfosporosinus*), and the "Deltaproteobacteria" (such as the genera *Desulfovibrio*, *Desulfobacter* and *Desulfuromonas*). The proteobacterial sulfate- and sulfur-reducing bacteria are mostly mesophilic and constitute a physiological group of an otherwise phylogenetically heterogeneous assemblage of bacteria (Fig. 1). The number of genera and species has significantly increased during the last decade: in 2002, the group encompassed some 40 validly published genera and some 120 species, as well as a number of not yet culturable bacteria which emerge from molecular environmental studies (Akkermans et al., 1994). The proteobacterial sulfate- and sulfur-reducers are widespread in aquatic and humid terrestrial environments that become anoxic as a result of microbial decomposition. They are for the most part strictly anaerobic, performing the complete or partial oxidation of small organic molecules (e.g., lactate, pyruvate, acetate, and in a few cases, also alkanes and aromatic compounds) with oxidized S-compounds (such as sulfate or elemental sulfur) as terminal electron acceptors generating hydrogen sulfide. They play a crucial role in the sulfur cycle. Some sulfate reducers (e.g., *Desulfonema* and *Desulfosarcina*) can also grow autotrophically with hydrogen gas as electron donor and CO_2 as sole carbon source. Multiheme cytochromes (of the *c*3 type) play a key role in electron-transport reactions in the periplasm of sulfate- and sulfur-reducing bacteria. Strictly sulfur-reducing bacteria occur among the genera *Desulfuromonas*, *Desulfuromusa*, *Desulfurella* and *Hippea* (Liesack and Finster, 1994; Miroshnichenko et al., 1999).

Phylogenetically the sulfate- and sulfur-reducing proteobacterial taxa display a significant diversity; at present at least five major phylogenetic lineages can be discerned within the "Deltaproteobacteria" (Fig. 1). To make the picture perhaps even more complex, not all the taxa belonging to these phylogenetic clusters metabolize with sulfate or sulfur as terminal electron acceptor. These five major rRNA clusters are the *Desulfovibrio*, *Desulfuromonas*, *Desulfobacter*, *Synthrophobacter* and *Desulfurella* groups.

The *Desulfovibrio* rRNA group (encompassing the genera *Desulfovibrio*, *Bilophila* and *Lawsonia*) is phylogenetically related to the *Desulfohalobium* group, *Desulfomicrobium* and *Desulfonatronum*. Desulfovibrios occur in aquatic biotopes but are also common inhabitants of the intestines of mammals, including humans. Interestingly, *Lawsonia intracellularis* is an obligately intracellular bacterium occurring in the intestinal epithelial cells of pigs with proliferative enteropathy, a major disease affecting the economics of pig industries worldwide. *Lawsonia* does not reduce sulfate, but is clearly related to the *Desulfovibrio* group (McOrist et al., 1995). Similarly *Bilophila wadsworthia* is a common inhabitant of the human colon and has been associated with human appendicitis and was also reported as a common infective agent in neonatal pigs (McOrist et al., 2001). One species of *Desulfomicrobium* (*D. orale*) has been described as being involved in peridontal disease.

The *Desulfuromonas* group is more related to the *Pelobacter* group, which is mainly composed of fermenting bacteria, such as *Malonomonas*

rubra, growing by the decarboxylation of malonate to acetate. Some of the sulfate-reducing bacteria can also utilize Fe^{3+} as electron acceptor, which is typical for the various *Geobacter* species, also belonging to this rRNA cluster.

The *Desulfobacter* group is quite diverse as it contains 14 genera and is phylogenetically related to the *Desulfobulbus* and the *Desulfomonile* (or "*Desulfoarculus*") groups. The *Desulfobacter* group harbors also some psychrophilic sulfate-reducing bacteria such as *Desulfofrigus* and *Desulfofaba*, which were isolated from cold Arctic marine sediments (Knoblauch et al., 1999).

Sulfate reducers occur also among the *Syntrophobacter* group (Fig. 1), which is composed of the syntrophs classified in the genus *Syntrophobacter* and the sulfate-reducers belonging to *Desulforhabdus* and *Desulfovirga* (Tanaka et al., 2000) together with the thermophilic marine genera *Desulfacinum* and *Thermodesulforhabdus*. The latter two genera form part of a subsurface microbial community; the former was isolated from hydrothermal vent systems and the latter from hot North Sea oil field water. Other syntrophs of the "Deltaproteobacteria" such as the benzoate-degrading *Syntrophus* species and the propionate-degrading *Smithella* belong to the *Syntrophus* group (Fig. 1).

The genera *Desulfurella* and *Hippea* seem to occupy a separate position within the Proteobacteria; they are anaerobic, moderately thermophilic, sulfur-reducing bacteria, isolated from respectively terrestrial hot springs or submarine hot vents. Pending further studies, it is possible that in the future the *Desulfurella* group might be considered as a sixth (zeta) class among the Proteobacteria (Rainey et al., 1993).

The "Epsilonproteobacteria"

This is the smallest and a more recently recognized line of descent within the Proteobacteria (6 genera and some 50 species). Compared to the multiple changes in the classification of the four other proteobacterial classes, the classification of the ε-class remained quite stable, because it is a fairly new group whose taxonomy was right from its recognition based on comparisons of 16S rDNAs. Up to now, photosynthetic bacteria have not been reported and key representatives are the genera *Campylobacter* and *Helicobacter*, encompassing enteropathogens for man and/or animals. Most species of the "Epsilonproteobacteria" are microaerophilic, chemoorganotrophic nonsaccharolytic spiral-shaped or curved bacteria, typically motile with a corkscrew-like motion by means of polar flagella. They obtain their energy mainly from amino acids or tricarboxylic acid cycle intermediates. Menaquinones are the characteristic respiratory quinones (Table 3). In the 1980s, interest in campylobacters and related bacteria increased owing to the availability of adequate isolation and cultivation procedures. Some species require indeed fumarate, or formate plus fumarate, or hydrogen plus fumarate for growth in microaerobic conditions. Numerous new taxa were described, and the gastritis-causing *Campylobacter pylori* was transferred together with *C. mustelae* (isolated from the gastric mucosa of ferret) to the new genus *Helicobacter* by Goodwin et al. (1989). Some not yet culturable "Epsilonproteobacteria" have been reported as symbionts of shrimps and polychaetes. The complete genome sequences of *Campylobacter jejuni* (Parkhill et al., 2000) and *Helicobacter pylori* (Alm et al., 1999) have been determined. For recent reviews emphasizing taxonomic aspects of the major genera of the "Epsilonproteobacteria," see Vandamme (2000), On (2001), and Solnick and Vandamme (2001), and also Wassenaar and Newell (2001). Table 12 summarizes the names of taxa above the rank of genus within the "Epsilonproteobacteria" according to the new edition of *Bergey's Manual of Systematic Bacteriology* (Garrity, 2001).

The Campylobacteraceae *Campylobacter coli* and *C. jejuni* are the most frequently identified causative agents of acute infective diarrhea in humans. Campylobacters can enter the food chain via raw milk and raw meat (particularly poultry). Other *Campylobacter* and *Arcobacter* species are involved in abortion and infectious infertility in various animals (e.g., sheep and cows), whereas some *Campylobacter* species are also found in large numbers in the periodontal pockets of diseased gums of man. Although primarily known as human and animal associated bacteria which are of relevance in food microbiology, arcobacters were shown to be abundantly present in certain environmental niches including water reservoirs, sewage, oil field communities, and certain saline environments. Their role in the environment is not well documented, but some of these organisms were shown to be sulfide oxiders (with the production of sulfur) and it has been suggested that they play a role in the sulfur cycle by reoxidizing sulfide formed by

Table 12. The "Epsilonproteobacteria": orders, families and number of genera.[a]

Order	Family	Number of genera
"Campylobacterales"	Campylobacteraceae	4
	"Helicobacteraceae"	2

[a]According to the second edition of *Bergey's Manual of Systematic Bacteriology* (Garrity and Holt, 2001). Quotation marks are used for names which have not yet been validated (as of mid 2002).

microbial sulfate or sulfur reduction (Teske et al., 1996; Voordouw et al., 1996; Snaidr et al., 1997; Wirsen et al., 2002). The type species *A. nitrofigilis* occurs in the roots and the rhizosphere of salt marsh plants, where these bacteria fix nitrogen. The genus *Sulfurospirillum* harbors microaerophilic sulfur-reducing curved bacteria occurring in freshwater or marine surface sediments. Two recently described *Sulfurospirillum* species (*S. barnesii* and *S. arsenophilum*) can even use arsenate or selenate as electron acceptor (Stolz et al., 1999). The strictly anaerobic "*Dehalospirillum multivorans*" was isolated from activated sludge and can dechlorinate tetrachloroethene, a volatile man-made pollutant (Scholz-Muramatsu et al., 1995). This organism seems to be phylogenetically related to *Sulfurospirillum*.

The Helicobacter Group The helicobacters constitute together with *Wolinella* a distinct phylogenetic lineage within the "Epsilonproteobacteria" (Fig. 1). *Helicobacter pylori* has received considerable attention for its role in gastric and duodenal ulcers and in gastric cancer in humans. *Helicobacter* encompasses at present some 20 species and more will likely be discovered when the stomach of other animals will be screened for bacteria similar to *H. pylori*. The phylogenetic and taxonomic position of various so-called "gastrospirilla" occurring in the stomachs of humans and animals will only be resolved when significant advances in the culture methods for these difficult bacteria are made. The pig and cattle strains now constitute two provisional candidate species: respectively *Candidatus Helicobacter suis* and *Candidatus Helicobacter bovis* (De Groote et al., 1999a; De Groote et al., 1999b; see also Table 13). Similarly, polyphasic investigations are needed in the group of "Flexispira rappini," which is a provisional name for the spindle-shaped bacteria with periplasmic fibers and bipolar tufts of sheathed flagella, isolated from a variety of clinical and veterinary sources. The name "Flexispira rappini" refers to a characteristic morphotype shared by several distinct taxa and not to a distinct well-defined species (Dewhirst et al., 2001; Vandamme and On, 2001).

Wolinella succinogenes (formerly *Vibrio succinogenes*), a commensal of the bovine rumen, belongs also to the *Helicobacter* group. This organism can grow on hydrogen as electron donor and fumarate as electron acceptor.

Likely the full extent of the ecological diversity of the "Epsilonproteobacteria" still remains to be discovered: 16S rDNA gene sequence data of symbionts of shrimps (*Rimicaris exoculata*) and polychaete annelids (*Alvinella pompejana*) suggest that some "Epsilonproteobacteria" may occupy important niches in the habitats of deep-sea hydrothermal vents, where they contribute to overall carbon and sulfur cycling at moderate thermophilic temperatures (Campbell et al., 2001; Corre et al., 2001). Recently, such a thermophilic sulfur-reducing bacterium isolated from the deep-sea hydrothermal vent polychaete, *Alvinella pompejana*, was allocated to a new genus, *Nautilia* (Miroshnichenko et al., 2002). Another remarkable member of the "Epsilonproteobacteria" is *Thiovulum*, whose phylogenetic relationship was shown by partial rDNA analysis (Romaniuk et al., 1987). The sulfur-oxidizing *Thiovulum* possesses large ovoid cells with a diameter often reaching 25 µm. The cytoplasm contains orthorhombic sulfur inclusions, and the bacterium is considered as a chemolithotroph occurring in marine and freshwater environments, where sulfide-containing water and mud layers are in contact with overlaying oxygen-containing water. The environmental *Thiomicrospira denitrificans* belongs also to the "Epsilonproteobacteria," whereas the other six *Thiomicrospira* species, including the type species *T. pelophila*, are affiliated to the *Piscirickettsia* group of the "Gammaproteobacteria" (Brinkhoff et al., 1999).

Symbiotic, Parasitic and Not-Yet Cultured Proteobacteria, and the Alphaproteobacterial Origin of Mitochondria

The application of molecular biological methods to study the diversity of genes from environmental DNA without the need to isolate prokaryotic strains prior to molecular analyses has broadened immensely our insight into community structure and must be considered a quantum leap in microbial ecology (Hugenholz et al., 1998). Though problems are still associated with its ability to qualify and quantify individual partners of the community, the molecular approach has circumvented problems recognized with pure culture studies—the "great plate count anomaly," as phrased by Staley and Konopka (1985). Hundreds of habitats and selected environmental sites have been subjected to the direct analysis of community structure by ribosomal rDNA sequencing and, more recently, of community function by the analysis of genes involved in metabolism (sulfur, nitrogen, methanogenesis, etc.) as well as degradation, bioremediation or heavy metal resistance.

Symbiosis between prokaryotic and eukaryotic organisms seems to play a major role in evolution (Gray and Doolittle, 1982; Margulis, 1993; Margulis, 1996). A classical example is the symbiotic association between some diazotrophic

Table 13. Published *Candidatus* taxa among the Proteobacteria (as of mid 2002).

Name of *Candidatus* Reference	Comments/host	References
"Alphaproteobacteria"		
"*Candidatus* Liberibacter africanus"	Phloem-restricted bacterium associated with greening disease in citrus	Jagoueix et al., 1994 Murray and Stackebrandt, 1995
"*Candidatus* Liberibacter africanus subsp. capensis"	Phloem-restricted bacterium associated with leaf mottle symptoms in *Calodendrum capense* (Cape chestnut)	Garnier et al., 2000
"*Candidatus* Liberibacter asiaticus"	Phloem-restricted bacterium associated with greening disease in citrus	Jagoueix et al., 1994 Murray and Stackebrandt, 1995
"*Candidatus* Odyssella thessalonicensis"	Endosymbiont of *Acanthamoeba* spp. Phylogenetically related to *Caedibacter* and *Holospora*	Birtles et al., 2000
"*Candidatus* Xenohaliotis californiensis"	Endosymbiont causing withering syndrome of *Haliotis* spp. (abalone). Belongs to the Rickettsiaceae	Friedman et al., 2000
"Betaproteobacteria"		
"*Candidatus* Procabacter acanthamoebae"	Endosymbiont of *Acanthamoeba* spp.	Horn et al., 2002
"Gammaproteobacteria"		
"*Candidatus* Arsenophonus triatominarum"	Endosymbiont of *Triatoma infestans*. Closely related to *Arsenophonus nasoniae* (the triatomine bug)	Hypsa and Dale, 1997
"*Candidatus* Blochmannia floridanus"	Endosymbiont of *Camponotus floridanus* (carpenter ants)	Sauer et al., 2000
"*Candidatus* Blochmannia herculeanus"	Endosymbiont of *Camponotus herculeanus* (carpenter ants)	Sauer et al., 2000
"*Candidatus* Blochmannia rufipes"	Endosymbiont of *Camponotus rufipes* (carpenter ants)	Sauer et al., 2000
"*Candidatus* Phlomobacter fragariae"	Phloem-restricted bacterium associated with marginal chlorosis of strawberry	Zreik et al., 1998
"Epsilonproteobacteria"		
"*Candidatus* Helicobacter bovis"	Occurs in pyloric part of the abomasum of cattle	De Groote et al., 1999a
"*Candidatus* Helicobacter suis"	Occurs in stomach of pigs	De Groote et al., 1999b

"Alphaproteobacteria" (e.g., *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*) and leguminous plants, where the bacteria reduce atmospheric nitrogen in root or stem nodules. Some Proteobacteria live as obligate intracellular symbionts or parasites in animals. To study the long-term history of symbiotic associations, the phylogenetic trees of hosts and their symbionts have been compared. Few data only are available, the most convincing study being the one on the endosymbionts of aphids. The topology of the symbiont (*Buchnera aphidicola*) tree is completely concordant with host phylogeny, based upon morphology (Moran et al., 1993). The fossil and biogeographic time points for the host phylogeny have been used to calibrate the 16S rDNA of the closely related endosymbionts (>8% similarity). The value of 1% fixed substitutions per 25 to 50 milllion years (Moran et al., 1993) is similar to the value of 1% per 50 million years determined by Ochman and Wilson (1987) on the basis of a broader range of nonobligate symbiotic relationships (e.g., *Rhizobium*/legumes; *Photobacterium*/fish; and enterobacteria/mammals) and 1% per 60 million years suggested by Stackebrandt (1995) for the past 500 years.

The advantage to the host of the association has been unraveled in only a few cases (e.g., the removal of hydrogen produced from hydrogenosomes of ciliates, provision of nutritional carbon to the host bivalves by sulfur-oxidizing gill symbionts [Dando and Southward, 1986] or of essential amino acids to aphids by their endosymbionts [Baumann et al., 1995; Baumann et al., 1998]). Molecular tools have also been used to elucidate the transmission route of symbionts in tropical lunicid bivalves (Gros et al., 1998) and deep-sea bivalves (Krueger et al., 1996) by application of the polymerase chain reaction (PCR) techniques in ovaries, testis and gill tissue.

We can estimate that a considerable number of new proteobacterial taxa has not yet been detected or described, because suitable in vitro cultivation techniques do not yet exist for such endosymbiotic bacteria. However, DNA-based techniques allow the detection and visualization of such endosymbionts, and PCR amplification of their 16S rDNA by oligonucleotide probes (see Table 4), applied singly or in a combination of several probes, each representing a different taxonomic level, enables the determination of their phylogenetic position

(Manz et al., 1992; Amann et al., 1995; Amann and Ludwig, 2000).

An intracellular lifestyle has obvious advantages for a suitably adapted prokaryote, and the spread of intracellular bacteria seems to be guaranteed in hosts such as blood sucking insects. Hence, it is not surprising that intracellular bacteria have evolved in different phylogenetic groups. Classical examples among the Proteobacteria are *Coxiella*, *Rickettsiella* and the endosymbionts of aphids belonging to the genus *Buchnera* (all "Gammaproteobacteria") and various members of the Rickettsiaceae and allied groups (e.g., *Holospora* and *Caedibacter*), belonging to the "Alphaproteobacteria." Related to the Rickettsiaceae are the wolbachiae, which are symbionts of invertebrate hosts, affecting the reproduction of the host by induction of thelytokous parthenogenesis (i.e., male killing or feminization). The wolbachiae infect the reproductive cells of various insects and have thus the potential to be vertically transmitted by cytoplasmic inheritance. More details concerning intracellular prokaryotes can be found in the introductory chapter by Fredricks (Introduction to the Rickettsiales and other Intracellular Prokaryotes in this Volume) and some examples of symbiotic and not yet cultured Proteobacteria are shown in Table 14, together with their phylogenetic affiliation.

Most of these endosymbiotic bacteria cannot yet be cultivated and illustrate that only a very limited proportion of the bacterial species present in various habitats is actually known. Although a great part of the rDNA sequences of uncultured bacteria is available in public databases, no formal phenotypic description nor species names can be given for such organisms, and type strains cannot be made publicly available. Some of these uncultured bacteria have been partially described and provisionally allocated to the category of *Candidatus* (Murray and Schleifer, 1994; Murray and Stackebrandt, 1995). The proteobacterial *Candidatus* taxa together with some additional information are listed in Table 13 (see also the List of Bacterial Names with Standing in Nomenclature website).

The mitochondrion of eukaryotic cells can be considered as the ultimate prokaryotic endosymbiont. Like chloroplasts, mitochondria possess unique genomes that are distinct from the nuclear genomes of their associated eukaryotic cells, and mitochondrial ribosomes are clearly of the prokaryotic type and related to the "Alphaproteobacteria" (Lang et al., 1999). Among the many bacterial genomes that have now been sequenced, that of *Rickettsia prowazekii* (1.1 Mb) is most closely related to the mitochondrial genome (Andersson et al., 1998). *Rickettsia prowazekii* is the agent of epidemic

Table 14. Examples of symbiotic Proteobacteria and their phylogenetic affiliation.

Host species	Host trivial name	Phylogenetic affiliation of symbiont	Reference/EMBL accession number
Muscidifurax uiniraptor	House fly	α-class	Stouthamer et al., 1993
Bangasternus orientalis	Coleoptera, weevil	α-class	M85266
Rhinocyllus conicus	Coleoptera, weevil	α-class	M85267
Trichogramma deion	Parasite wasp of Lepidoptera	α-class	Stouthamer et al., 1993
Nasonia vitripennis	Parasitic wasp	α-class	Gherna et al., 1991
Crassostrea virginica	Eastern Oyster	α-class	Boettcher et al., 2000
Dysmicoccus neobrevipes	Homoptera, mealy bug	β-class	Munson et al., 1992
Acromyrmex octospinosus	Leave cutting ant	β-class	AF459796
Antonina crawii	Bamboo pseudococcoid, Homoptera	β, γ-class	Fukatsu et al., 2000
Solemya velum	Bivalve	γ-class	M90415
Heliothis virescens	Moth, tobacco budworm	γ-class	L22481
Bathymodiolus thermophilus	Bivalve	γ-class	Distel et al., 1988
Calyptogena elongata	Bivalve	γ-class	L25719
Calyptogena magnifica	Bivalve	γ-class	Distel et al., 1988
Vesicomya cordata	Bivalve	γ-class	L25713
Anodontia phillipiana	Bivalve	γ-class	L25711
Codakia orbicularis	Bivalve	γ-class	Distel et al., 1988
Thyasira flexuosa	Bivalve	γ-class	Distel and Wood, 1992
Lucina floridana	Bivalve	γ-class	L25707
Riftia pachyptila	Tube worm	γ-class	Distel et al., 1988
Anomalops katoptron	Deep sea anglerfish	γ-class	Z19081
Cryptosaras couesi	Deep sea anglerfish	γ-class	Haygood et al., 1992
Sitophilus zeamais	Coleoptera, maize weevil	γ-class	M85270
Acyrthosiphon sp.	Pea aphid	γ-class	Unterman et al., 1989
Camponotus floridanus	Giant ants	γ-class	Schröder et al., 1996
Glossina pallidipes	Tsetse fly	γ-class	Beard et al., 1993

louse-borne typhus in humans and multiplies in eukaryotic cells only. Most likely the genome types of mitochondria and rickettsiae have shared a common free-living alphaproteobacterial ancestor, which through genome reduction evolved via two descendent lineages (Gray, 1998; Gray et al., 1999). Studies on mitochondria of yeast reveal that the number of alphaproteobacterial descendents in the mitochondrial proteome is surprisingly small, and that a large number of novel mitochondrial genes likely originated from the eukaryotic nuclear genome complementing the remaining genes from the bacterial ancestor (Karlberg et al., 2000; Kurland and Andersson, 2000). Further genomic and proteomic analyses of other members of the "Alphaproteobacteria," such as *Bradyrhizobium* and *Rhodobacter*, may yield more information concerning the metabolic versatility of the proto-mitochondrion. Whether the origin of hydrogenosomes—the ATP-producing organelles of many anaerobic protists—is related to that of mitochondria remains to be investigated in more detail (Andersson and Kurland, 1999; Dyall et al., 2000).

Genome Analysis

The G+C content of genomic DNA of the Proteobacteria varies from 30 mol% (e.g., in *Campylobacter* and *Rickettsia*) to 70% (e.g., in *Alcaligenes*), indicating that almost the entire span of mol% G+C variation among living microorganisms is covered. Even within each of the proteobacterial subclasses, variation in G+C content of the genome is very large. Similarly the genome sizes of various Proteobacteria differ considerably. Commensals and free-living prokaryotic strains have a larger genome, ranging from 9.4 Mb (*Myxococcus xanthus*) to about 2.5 Mb (*Pasteurella multocida*), than that of obligate symbiotic and parasitic strains, e.g., *Buchnera aphidicola* (0.64 Mb) and *Rickettsia prowazekii* (1.1 Mb). Unexpectedly, the genome size of phylogenetically closely related species and even strains of the same species can differ significantly, as shown for three species of *Yersinia* (3.8–4.9 Mb) and various strains of *E. coli* (4.6–5.5 Mb) and *Burkholderia cepacia* (4.7–8.1 Mb; Lessie et al., 1996). Most Proteobacteria studied contain a single circular chromosome, but the presence of multiple chromosomes has been reported, particularly among the "Alphaproteobacteria": two different circular chromosomes in *Rhodobacter sphaeroides* (Suwanto and Kaplan, 1989) and *Brucella melitensis* (Michaux-Charachon et al., 1997); three circular chromosomes in *Rhizobium meliloti* (Sobral et al., 1991) and *Burkholderia cepacia* (Cheng and Lessie, 1994). Another unconventional organization has been detected in several strains of *Agrobacterium tumefaciens*, where the larger chromosome is circular (3.0 Mb), whereas the smaller (2.1 Mb) is linear (Jumas-Bilak et al., 1998). It should be noted that not all strains of *Brucella melitensis* or all species of the genus *Rhodobacter* contain multiple chromosomes, and the evolutionary significance of these peculiar genomic organizations remains to be unraveled (Jumas-Bilak et al., 1998).

A multitude of genes of various Proteobacteria have been sequenced; genetic and genomic aspects of *E. coli*, various *Pseudomonas* species, *Rhizobium* and many animal and human pathogenic Proteobacteria have been or are being studied in detail, and more importantly in recent years, considerable progress has been made in the sequencing of whole genomes of an increasing number of Proteobacteria. The first whole-genome sequence of a free-living microorganism was published in 1995 by Fleishmann et al. for the gammaproteobacterium *Haemophilus influenzae* (1.83 Mb). At present (mid 2002), the sequences have been completed for at least 9 α-, 3 β- (*Bordetella* and *Neisseria*), 17 γ- and 3 ε- (*Campylobacter* and *Helicobacter*) class members (sequenced genera are indicated by footnote "a" in Table 3; see also The Institute for Genomic Research website). Sequencing of a great number of proteobacterial genomes is in progress (see footnote "b" in Table 3 and The Institute for Genomic Research website, National Center for Biotechnological Information website, ([DOE Joint Genome Institute website] http://www.jgi.doe.gov/JGI_microbial/html and [GOLD Genome OnLine Database website] wit.integratedgenomics.com/GOLD/) and most of it pertains to bacteria of clinical or biotechnological importance. The sequencing projects will elucidate regulatory and structural functions of newly discovered genes and will also yield significant insights into the mechanisms of pathogenicity, bacterial photosynthesis, phylogeny and evolution.

Final Considerations

With the publication of the *Approved Lists of Bacterial Names* in 1980, the number of genera and species presently belonging in the Proteobacteria was approximately 130 and 500, respectively. Until 2002, the number of validly described genera and species increased to respectively about 460 and 1600, making the phylum Proteobacteria a heavily populated section

of the phylogenetic bacterial tree. Indeed, in terms of number of genera, the Proteobacteria encompass more than 40% of all prokaryotic genera. Recent insights into the community structure of as-yet uncultivated prokaryotes reveal that the vast majority of taxa have not yet been described. This is true not only for open environments, such as the open ocean (Giovannoni et al., 1990), soil (Liesack and Stackebrandt, 1992; Felske et al., 1999), deep subsurface (Chandler et al., 1997), waste treatment plants and bioreactors (Bond et al., 1995; Juretschko et al., 2002), and sediments of oceans and lakes (Bowman et al., 2000; Urakawa et al., 2000; Brambilla et al., 2001), but also for the microflora of eukaryotic hosts. Particularly the not-yet culturable Proteobacteria, the endosymbionts and those which are difficult to grow in axenic culture such as the helicobacters and allied taxa, are a goldmine for further studies on bacterial diversity. The most promising tools to explain the role of Proteobacteria in global cycling of gases and chemicals are: 1) for elucidating the phylogenetic structure of the community, DNA fragment electrophoresis, sequence analysis of universal and taxon-specific genes, and their identification in situ by FISH hybridization, and 2) for determining the physiological capacities of its members, identification of housekeeping genes by FISH techniques and by microautoradiography (Ouverney and Fuhrman, 1999; Lee et al., 1999). Horizontal transfer of genes (Klein et al., 2001) will shed light on the possible evolutionary history of the various large groups within Proteobacteria by tracing the origin of genes to the ancestors of recent species that are phylogenetically related to other prokaryotic phyla. The number of full genome sequences will continue to rise, providing microbiologists with an unparalleled wealth of information for scientific exploitation to the benefit of clinical, environmental, evolutionary and general microbiology. It is likely that the increasing number of genera and species as well as comparative studies on other semantic macromolecules will challenge the present five major subdivisions of the Proteobacteria: as the phylogenetic radiation of the proteobacterial lineages will become more complex, boundaries between the five major subgroups will become vague and the subgroups more ambiguous. On the other hand, information on orthologous genes other than ribosomal rRNA genes will provide systematicists with sets of molecules to be included in future studies on multi-locus sequence analysis. As recommended, this information will be used in the description of the taxon species (Stackebrandt et al., 2002) and possibly of higher taxa. Molecular studies and culture attempts will continue to go hand in hand. The scientific challenge in the near future will also include molecular determination of metagenomes from proteobacteria of selected sites as well as the cultivation of hitherto uncultured proteobacterial symbiotic and pathogenic organisms.

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SECTION 3.1

Alpha Subclass

Prokaryotes (2006) 5:41–64
DOI: 10.1007/0-387-30745-1_2

CHAPTER 3.1.1

The Phototrophic Alpha-Proteobacteria

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Introduction

The phototrophic purple α-Proteobacteria are purple nonsulfur bacteria able to perform anoxygenic photosynthesis. Owing to the presence of photosynthetic pigments, cell suspensions appear in various colors from beige, olive-green, peach-brown, brown, brown-red, red or pink and have characteristic absorption spectra. Photosynthetic pigments (bacteriochlorophyll *a* or *b* [esterified with phytol or geranylgeraniol] and various types of carotenoids) are located in the cytoplasmic membrane and internal membrane systems (vesicles, lamellae or membrane stacks).

Typically, phototrophic α-Proteobacteria grow under anoxic conditions in the light and their phototrophic growth, photosynthetic pigment synthesis and internal membrane formation are inhibited by oxygen but become derepressed at low oxygen tensions. Their metabolism is highly diverse and flexible. The preferred mode of growth is photoorganoheterotrophically, but many species also can grow photolithoautotrophically with molecular hydrogen, sulfide or thiosulfate as photosynthetic electron donor; some also can use ferrous iron. Growth factors are generally required, most commonly biotin, thiamine, niacin and *p*-aminobenzoic acid; growth of most species is enhanced by small amounts of yeast extract, and some have a complex nutrient requirement. Chemotrophic growth under microoxic to oxic conditions in the dark is common to most of these bacteria; some of them are very sensitive to minor levels of oxygen, while others grow equally well aerobically in the dark at the full atmospheric oxygen tension. Anaerobic dark growth by fermentation and oxidant-dependent growth also may occur.

Phylogeny

Phototrophic purple nonsulfur bacteria are a highly diverse and heterogeneous group of bacteria, both phenotypically and genetically. On the basis of 16S rDNA sequence similarities, phototrophic purple bacteria belong to the α-, β- and γ-Proteobacteria (Woese et al., 1984a; Woese et al., 1984b; Woese et al., 1985; Stackebrandt et al., 1988; Woese, 1987). While purple sulfur bacteria are γ-Proteobacteria, purple nonsulfur bacteria are found in the β- and α-Proteobacteria. The phototrophic purple β-Proteobacteria (including *Rhodocyclus* and relatives) are treated elsewhere in this volume (Imhoff). This chapter deals with the phototrophic purple α-Proteobacteria.

Three major phylogenetically distinct groups of phototrophic α-Proteobacteria are recognized (see Figs. 1–3). They are represented by *Rhodospirillum* and relatives (also called "α-1 Proteobacteria"; Fig. 1), by *Rhodopseudomonas* and relatives (also called "α-2 Proteobacteria"; Fig. 2) and by *Rhodobacter* and relatives (also called "α-3 Proteobacteria"; Fig. 3). While species of the α-3 group form a tight phylogenetic cluster, in the α-2 group *Rhodomicrobium* and *Rhodobium* species are at a greater distance to the other phototrophic species, and in the α-1 group, *Rhodovibrio*, *Rhodopila* and *Rhodothalassium* species form distinct lines separate from the cluster around *Rhodospirillum* and *Phaeospirillum* species. Close relatives of *Rhodovibrio* species and *Rhodothalassium salexigens* are not known and their assignment to the *Rhodospirillum* group is arbitrary. On the basis of 16S rDNA sequence similarities, chemotaxonomic characteristics and other properties, many representatives of the phototrophic α-Proteobacteria are very closely related to nonphototrophic, strictly chemotrophic bacteria. These similarities are taken as strong indication for the development of many nonphototrophic bacteria from phototrophic ancestors. A few examples of these relations are the following:

The acidophilic *Rhodopila globiformis* is closely related to other acidophilic bacteria including *Acidiphilium* species (Sievers et al., 1994).

Phaeospirillum species demonstrate a close relationship to *Magnetospirillum magnetotacticum* (Burgess et al., 1993).

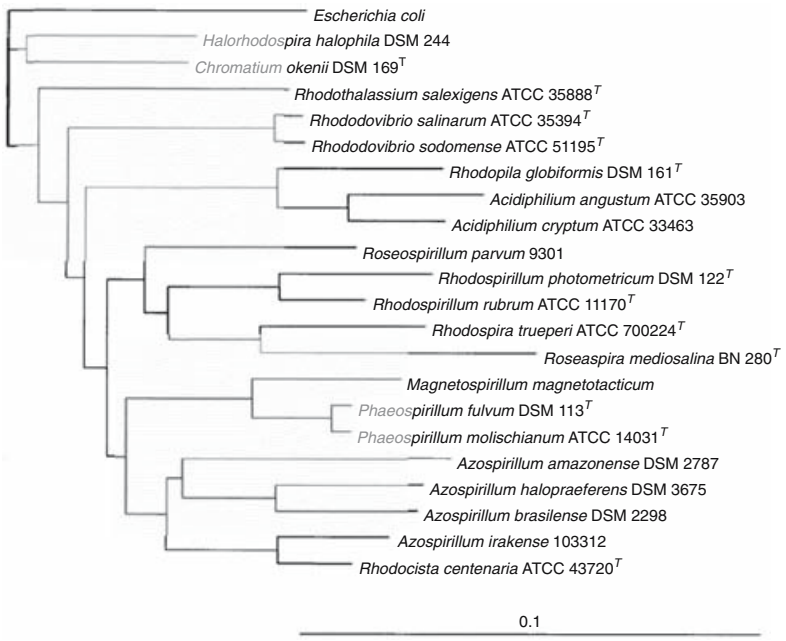

Fig. 1. Phylogenetic tree based on 16S rDNA sequences of the *Rhodospirillum*-group (α-1 Proteobacteria) in relation to other purple nonsulfur and purple sulfur bacteria and to representative close chemotrophic relatives.

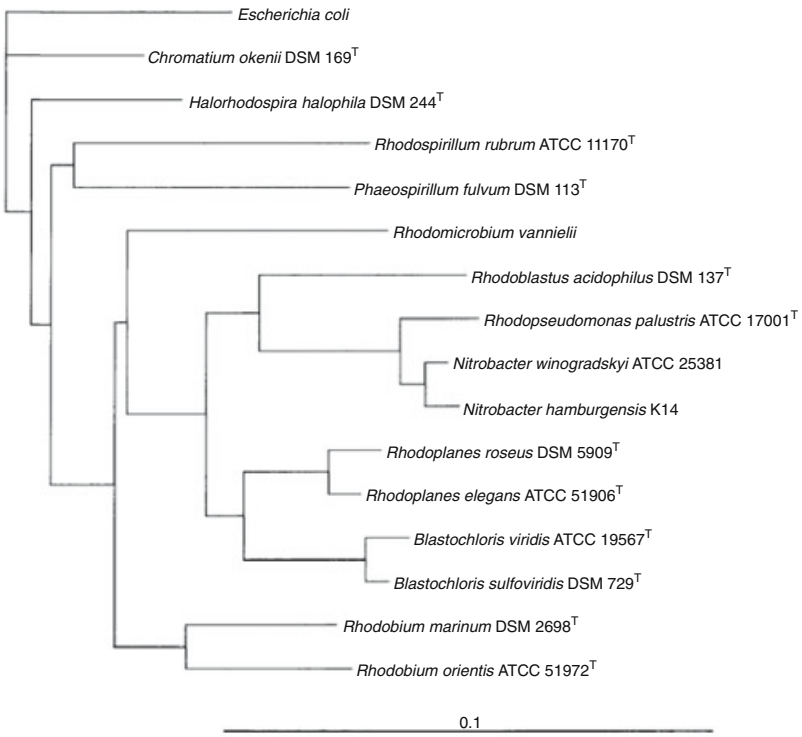

Fig. 2. Phylogenetic tree based on 16S rDNA sequences of the *Rhodopseudomonas*-group (α-2 Proteobacteria) in relation to other purple nonsulfur and purple sulfur bacteria and to representative close chemotrophic relatives.

Rhodocista centenaria has strong relations to *Azospirillum* species (Xia et al., 1994; Fani et al., 1995).

Within the α-2 Proteobacteria, *Rhodopseudomonas palustris* has strong relations to species of *Nitrobacter* (Seewaldt et al., 1982).

Rhodobacter and *Rhodovulum* species form a cluster closely associated to *Paracoccus denitrificans* (Imhoff, 1989a; Hiraishi and Ueda, 1994a).

Taxonomy

Since Molisch removed the purple sulfur bacteria from the Thiobacteria (Migula, 1900), pigmentation and ability to perform anoxygenic photosynthesis were considered of primary importance for assignment of bacteria to the Rhodobacteria (Molisch, 1907), later called "Rhodospirillales" (Pfennig and Trüper, 1971).

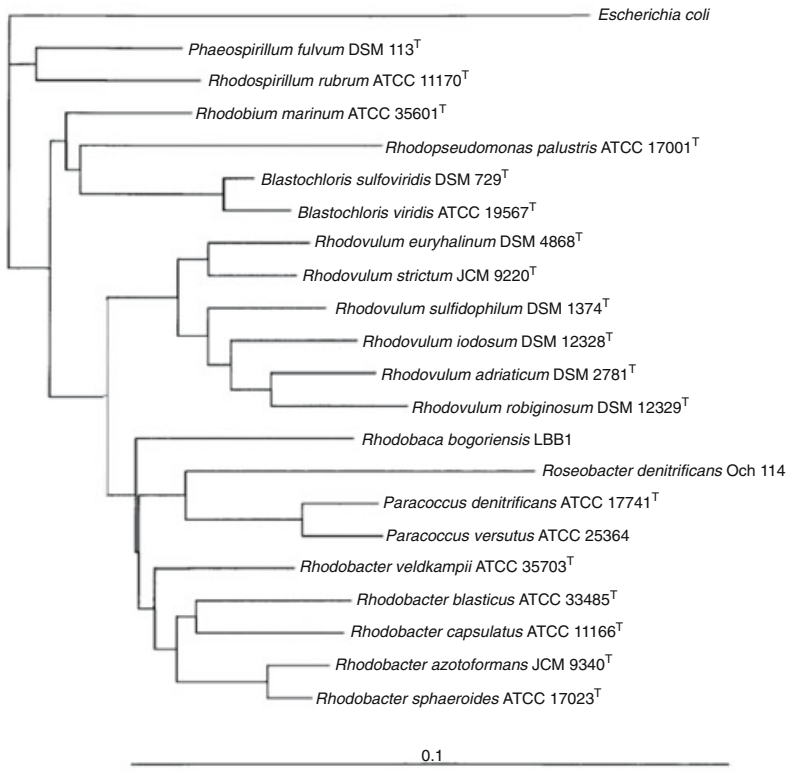

Fig. 3. Phylogenetic tree based on 16S rDNA sequences of the *Rhodobacter*-group (α-3 Proteobacteria) in relation to other purple nonsulfur and purple sulfur bacteria and to representative close chemotrophic relatives.

Because the Rhodospirillaceae (Pfennig and Trüper, 1971) do not represent a phylogenetically distinct group of bacteria, the use of the term "purple nonsulfur bacteria" (PNSB) was proposed for the α- and β-Proteobacteria that contain photosynthetic pigments and are able to perform anoxygenic photosynthesis under anoxic conditions (Imhoff et al., 1984b; Imhoff and Trüper, 1989b; Imhoff and Trüper, 1992b). Actual and historical aspects of the taxonomy of anoxygenic phototrophic purple bacteria have been discussed elsewhere (Imhoff, 1992a; Imhoff, 1995a; Imhoff, 1999; Imhoff, 2000).

Traditionally, purple nonsulfur bacteria have been classified into genera representing the rod-shaped *Rhodopseudomonas* species and the spiral-shaped *Rhodospirillum* species (Pfennig and Trüper, 1974b) and later into a third genus containing the half-circle- to circle-shaped *Rhodocyclus purpureus* (Pfennig, 1978). With the recognition of their genetic relationships and chemotaxonomic diversity, purple nonsulfur bacteria of the α- and β-Proteobacteria were taxonomically separated (Imhoff et al., 1984b; Imhoff and Trüper, 1989b). Later, bacteria within these groups were rearranged according to phylogeny, chemotaxonomic characteristics and ecophysiological properties. Despite the fact that many of the phototrophic purple nonsulfur bacteria are closely related to strictly chemotrophic relatives, the genus definitions of genera of the anoxygenic phototrophic bacteria still include phototrophic capability and content of photosynthetic pigments. At higher taxonomic ranks, phototrophic bacteria are treated together with nonphototrophic relatives.

Alpha-1 Proteobacteria

Most of the phototrophic bacteria that belong to the α-1 Proteobacteria (also known as the *Rhodospirillum* group) have been previously known as *Rhodospirillum* species and are of spiral shape. At present, the only nonspiral representative is *Rhodopila globiformis*. Genera included in this group are *Rhodospirillum*, *Phaeospirillum*, *Rhodospira*, *Roseospira*, *Rhodocista*, *Roseospirillum* and also *Rhodopila*, *Rhodothalassium* and *Rhodovibrio*.

In addition, 16S rDNA sequence data of purple nonsulfur bacteria implied that the spiral-shaped phototrophic α-Proteobacteria are phylogenetically quite distantly related to each other and do not warrant classification in one and the same genus (Kawasaki et al., 1993a; Imhoff et al., 1998). These bacteria also demonstrate great phenotypic diversity. Therefore, a reclassification of the spiral-shaped phototrophic α-Proteobacteria was proposed, based on distinct phenotypic properties and 16S rDNA sequence similarities. *Rhodospirillum centenum* was transferred to a new genus as *Rhodocista*

centenaria (Kawasaki et al., 1992). Other Rhodospirillum species were transferred to the new genera *Phaeospirillum*, *Rhodovibrio*, *Roseospira* and *Rhodothalassium* (Imhoff et al., 1998). Only *R. rubrum* and *R. photometricum* were maintained as species of the genus *Rhodospirillum*. In addition, new species were described of this group: *Rhodospira trueperi* was assigned to a new genus on the basis of significant phenotypic and genotypic differences from *Rhodospirillum rubrum* and other known PNSB (Pfennig et al., 1997); for similar reasons, the new bacterium *Roseospirillum parvum* was assigned to a new genus (Glaeser and Overmann, 1999).

Alpha-2 Proteobacteria

Most characteristic of phototrophic bacteria of the α-2 (*Rhodopseudomonas*) group is the budding mode of growth and cell division and the presence of lamellar internal membranes lying parallel to the cytoplasmic membrane. Most of these phototrophic bacteria have been previously known as *Rhodopseudomonas* species. Genera of this group now include *Rhodopseudomonas*, *Rhodoplanes*, *Rhodoblastus*, *Blastochloris*, *Rhodomicrobium* and *Rhodobium*.

After the removal of purple nonsulfur bacteria that contained vesicular internal photosynthetic membranes and those that were β-Proteobacteria from the genus *Rhodopseudomonas*, only those species remained within this genus that had lamellar internal membrane structures and grew and reproduced by budding (Imhoff et al., 1984b). The bacteria removed from *Rhodopseudomonas* are now recognized as species of *Rhodopila*, *Rhodobacter*, *Rhodovulum* and *Rubrivivax*. Thereafter, what remained of the genus *Rhodopseudomonas* (together with *Rhodomicrobium vannielii*) still represented a heterogeneous assemblage of species (Imhoff et al., 1984b) now recognized as genera of the α-2 Proteobacteria. Primarily due to the availability of sequence data of the 16S rDNA (Kawasaki et al., 1993a) and in part supported by the isolation and description of new species and additional data, the following proposals have been made. *Rhodopseudomonas marina* was transferred to the new genus *Rhodobium* as *Rhodobium marinum* together with the new species *Rhodobium orientis* (defined as the type species of this genus; Hiraishi et al., 1995b). *Rhodopseudomonas rosea* was transferred to the new genus *Rhodoplanes* and designated as the type species of this genus, *R. roseus* (Hiraishi and Ueda, 1994b). At the same time, *Rhodoplanes elegans* was described as a new species of this genus. *Rhodopseudomonas viridis* and *Rhodopseudomonas sulfoviridis* were assigned to the new genus *Blastochloris* as *B. viridis* and *B. sulfoviridis* (Hiraishi, 1997). Quite recently, *Rhodopseudomonas acidophila* was transferred to a new genus as *Rhodoblastus acidophilus* (Imhoff, 2001).

Rhodopseudomonas blastica was removed from this genus and transferred to *Rhodobacter blasticus* (Kawasaki et al., 1993b). Its 16S rDNA sequence is most similar to and clusters with those of the *Rhodobacter* species. *Rhodopseudomonas rutila* (Akiba et al., 1983) was considered as a later subjective synonym of *Rhodopseudomonas palustris* (Hiraishi et al., 1992). In addition to *Rhodopseudomonas palustris*, *Rhodopseudomonas julia* (Kompantseva, 1989) and *Rhodopseudomonas cryptolactis* (Stadtwald-Demchick et al., 1990) have been affiliated to this genus, though both species so far have not been validated and no 16S rDNA sequence of them is available.

Alpha-3 Proteobacteria

A characteristic feature of the phototrophic α-3 Proteobacteria (*Rhodobacter* group) is the presence of carotenoids of the spheroidene series and their extraordinary metabolic versatility and flexibility. These bacteria have been previously known as *Rhodopseudomonas* species and belong to the genera *Rhodobacter* and *Rhodovulum* (Pfennig and Trüper, 1974b; Imhoff et al., 1984b; Hiraishi and Ueda, 1994a). The former are freshwater bacteria and the latter true marine bacteria. Species of both genera have distinct 16S rDNA sequences (Hiraishi and Ueda, 1994a; Hiraishi and Ueda, 1995a; Hiraishi et al., 1996). Two new species, *Rhodovulum iodosum* and *Rhodovulum robiginosum*, have been described that use ferrous iron as photosynthetic electron donor (Straub et al., 1999). *Rhodobaca borogenensis*, a new isolate from an alkaline soda lake with low salt concentrations, has adapted in its salt response to this habitat (Milford et al., 2000).

Habitats

Ecological niches of phototrophic α-Proteobacteria are those anoxic parts of waters and sediments that receive light of sufficient quantity and quality to allow phototrophic development. Representatives of the purple nonsulfur bacteria are widely distributed in nature and are found not only in all kinds of stagnant water bodies, including lakes, waste water ponds, coastal lagoons, and in other aquatic habitats, but also in sediments, moist soils, and paddy fields. They live in aquatic habitats with significant amounts of soluble organic matter and low oxygen tension, but

rarely form colored blooms, like those of purple sulfur bacteria. However, often they are found accompanying the purple sulfur bacteria in stratified environments.

They have been found not only in freshwater, marine and hypersaline environments, and most frequently in habitats of moderate temperatures, but also in thermal springs and in cold polar habitats. Most purple nonsulfur bacteria have been isolated from all kinds of freshwater habitats where they also are most abundant. The greatest variety of species and the largest numbers of cells have been found in mud and water of eutrophic ponds, ditches and lakes. In the flat shore area of eutrophic lakes, 10^3 to more than 10^8 cells/ml of purple nonsulfur bacteria have been found in mud and water samples (Kaiser, 1966; Biebl and Drews, 1969). In pelagic water, the numbers usually are much lower (Biebl, 1973; Swoager and Lindstrom, 1971).

Quite a number of purple nonsulfur bacteria occur in marine and hypersaline environments. They are usually found in water bodies and sediments of intertidal flats, salt marshes, and polluted harbor basins, but not in the open sea. While freshwater isolates have a very low tolerance to sulfide, the sulfide tolerance of most marine species is much higher, and they even use sulfide and thiosulfate as photosynthetic electron donors. This is certainly an adaptation to this environment, which characteristically has a high activity of sulfate reduction and where consequently anoxic conditions are coincident with the presence of hydrogen sulfide. Although some isolates (strains of *Rhodopseudomonas palustris* and *Rhodomicrobium vannielii*) from marine habitats are not typical marine bacteria and do not require salt for optimum growth, most of the purple nonsulfur bacteria found in marine habitats are typical marine bacteria and are not found in freshwater habitats (Imhoff, 1988a). The marine forms include species of the genera *Rhodovulum* and *Rhodobium*, as for example *Rhodovulum sulfidophilum* (Hansen and Veldkamp, 1973), *Rhodovulum adriaticum* (Neutzling et al., 1984), *Rhodovulum euryhalinum* (Kompantseva, 1985), *Rhodobium marinum* (Imhoff, 1983a) and *Rhodobium orientis* (Hiraishi et al., 1995b). *Rhodospira trueperi* and *Roseospirillum parvum* are from a marine salt marsh and also represent typical marine bacteria. In addition, *Roseospira mediosalina* was isolated from a hot spring with low salt concentrations (2% salts) and is growing optimally between 5 and 7% NaCl (Kompantseva and Gorlenko, 1984).

Other halophilic species are well adapted to hypersaline environments. *Rhodothalassium salexigens* and *Rhodovibrio salinarum* are common to evaporated seawater pools and marine salterns and sometimes form colored layers in salt deposits or sediments (Drews, 1981; Nissen and Dundas, 1984; Rodriguez-Valera et al., 1985), whereas *Rhodovibrio sodomensis* is from Dead Sea sediments (Mack et al., 1993).

Some purple nonsulfur bacteria occur in acidic, boggy waters and soils. Most frequently, *Rhodoblastus acidophilus*, which grows optimally at pH 5.5–5.8, is found in such environments, often accompanied by *Rhodopseudomonas palustris* (Pfennig, 1969). *Rhodopseudomonas palustris* is very common and was isolated from all kinds of aquatic habitats (lakes, sewage and brackish waters), even from wet decaying leaves and from soils (Biebl and Pfennig, 1981; Imhoff and Trüper, 1992b). A preference for low pH values (pH 4.8-5.0) also is found in *Rhodopila globiformis* (Pfennig, 1974a).

Purple nonsulfur bacteria are found regularly in all stages of conventional sewage plants. Their numbers may increase dramatically from raw sewage to the activated sludge stage (from 2,000 to 100,000 cells/ml; Siefert et al., 1978). When anaerobic sewage is incubated in the light, it often spontaneously becomes red-brown in color owing to the presence of phototrophic bacteria. This suggests that it should be possible to easily direct the processes in the activated sludge towards photoassimilation of organic substrates by phototrophic bacteria. The first sewage treatment plant based on this principle was established in Japan (Kobayashi et al., 1971; see below).

Isolation

Two different strategies may be applied to isolate purple nonsulfur bacteria from their natural environment. Depending on the aims of the studies either the one or the other has clear preferences. According to classical enrichment techniques, phototrophic bacteria may be selectively enriched in suitable media under anoxic conditions and in the light; after visible growth, cells are separated in agar dilution series or on agar plates and isolated in pure culture. Alternatively pure cultures may be obtained by directly inoculating agar media from natural samples without prior enrichment.

The first strategy is the method of choice whenever the isolation of bacteria with particular physiological features is attempted. With a proper environmental sample and the right medium, bacteria with the desired properties may be selected and isolated. The second strategy has to be used whenever the natural diversity is to be analyzed and information on the natural abundance and distribution of the species in a sample is required. While specifically adapted

media are essential for the success of the first strategy, in the second one nonselective media are used with substrates (such as acetate, succinate or malate) that enable the development of most purple nonsulfur bacteria (Biebl and Pfennig, 1981; Imhoff and Trüper, 1992b). Even if the medium used can support only poor growth of a specific strain, after separation in or on agar and when the incubation time is adapted to the slow growth rates, it will grow out to a small colony and can be picked up for further transfers.

Selective Enrichment

Selective enrichment techniques for phototrophic bacteria were first used by Sergei Winogradsky (Winogradsky, 1888), and his technique is known as the "Winogradsky column." Variations of this column technique by other authors are discussed by Pfennig (1965) and Van Niel (1971). Van Niel (1944) elaborated the physiological basis for the enrichment of purple nonsulfur bacteria and was the first to develop a defined medium which could be used for their enrichment and cultivation.

For selective enrichment of purple nonsulfur bacteria, media have been used with lowered sulfate concentration to avoid production of sulfide by sulfate-reducing bacteria (Van Niel, 1944; Van Niel, 1971). An anaerobic enrichment culture using a common organic substrate and placed in the light usually will give rise to the development of purple nonsulfur bacteria. In many cases, in particular with marine samples, small Chromatiaceae—owing to their organotrophic capacity—compete with purple nonsulfur bacteria even if the sulfate concentration in the enrichment media is strongly reduced. The choice of the carbon source is not critical for the success of an enrichment culture because fermentative processes usually result in the formation of acetate or other acids (propionate, butyrate and lactate), which are good substrates for the majority of the purple nonsulfur bacteria. Some species of the purple nonsulfur bacteria, however, such as *Rhodovulum sulfidophilum* (Hansen and Veldkamp, 1973), *Rhodovulum adriacticum* (Neutzling et al., 1984) and *Blastochloris sulfoviridis* (Keppen and Gorlenko, 1975), tolerate and/or even require sulfide as a reduced sulfur source and/or photosynthetic electron donor.

Besides the mineral salts composition and the concentration of nutrients of the media, also the addition of vitamins, the pH, temperature, light intensity and light regime are of general importance for the selectivity of enrichment cultures. If samples from marine and hypersaline environments are investigated, the salinity and the mineral composition of the medium are of special importance.

A few examples of selective parameters for the enrichment of purple nonsulfur bacteria are the following:

Selective carbon sources for *Rhodopseudomonas palustris* are benzoate and formate (Qadri and Hoare, 1968). Because this species is very common in nature, many enrichments for purple nonsulfur bacteria will end up with the development of this species even without particularly selective conditions.

Enrichments under photoautotrophic conditions with hydrogen as electron donor favor growth of *Rhodobacter capsulatus*, which grows faster under these conditions than other phototrophic bacteria do (Klemme, 1968). Also this species often becomes predominant in unselective media.

Higher fatty acids like pelargonate and caproate (not more than 0.04% at pH 7.5) provide selective growth conditions for *Phaeospirillum fulvum* and *Phaeospirillum molischianum* (Pfennig, 1967; Van Niel, 1944).

For enrichment of *Rhodospirillum photometricum*, the anaerobic infusion method of Molisch (Giesberger, 1947) is still the best method (Biebl and Pfennig, 1981). Hay or other dried plant materials are suitable sources which may be used in large test tubes or cylinders under continuous illumination. *Rhodospirillum photometricum* also can be readily enriched from activated sludge incubated anaerobically in the light (1,000 lux, 30°C; Siefert et al., 1978).

The use of amino acids as carbon substrates favors the development of the *Rhodospirillum* species, in particular of *Rhodospirillum rubrum* (Biebl and Pfennig, 1981).

A succinate-mineral salts medium without growth factors and with an initial pH of 5.5 is highly selective for both *Rhodoblastus acidophilus* and *Rhodomicrobium vannielii* (Pfennig, 1969). If yeast extract or the required vitamins are present *Rhodopseudomonas palustris* may also develop under acidic conditions.

Enrichments in methanol bicarbonate medium select for *Rhodoblastus acidophilus* (and *Rubrivivax gelatinosus*).

Typical marine bacteria will not or only poorly grow in media without NaCl, though most freshwater species are inhibited by NaCl concentrations of sea water. Therefore, the addition of 3% NaCl is a selective factor for marine purple nonsulfur bacteria.

Salt concentrations of more than 10% are highly selective for moderately halophilic species like *Rhodothalassium salexigens*, *Rhodovibrio salinarum* and *Rhodovibrio sodomensis* that will not grow in media for freshwater or marine phototrophic bacteria.

Isolation Procedures

Direct Isolation. Methods of direct isolation of the phototrophic bacteria from a natural sample use agar dilution series or inoculation of agar plates to separate the cells prior to incubation. For isolation with solid media, water samples are most appropriate. A sample of mud, sludge, or soil may be used as a homogeneous suspension in medium or filter-sterilized water from the habitat. Samples containing less than 10 cells/ml need to be concentrated by centrifugation (agar dilution series) or filtration (agar plates). All methods for direct isolation are suitable for the determination of living cell counts, when known amounts of the sample are used in appropriate dilutions.

Preparation of Agar Dilution Series. Agar dilution series are prepared either with enrichment cultures or with promising natural samples by direct inoculation with an environmental sample. Selective media are not required and nonselective ones are preferred for a direct isolation without prior enrichment procedure.

In a modification of the method of Pfennig (Pfennig, 1965; Trüper, 1970), purified agar is dissolved (1.8%) and distributed in amounts of 3 ml into cotton-plugged test tubes. The agar is sterilized by autoclaving. The liquid agar is kept at 50°C in a water bath until use. A suitable medium is placed in the same water bath, and 6 ml of the prewarmed medium is added to each test tube. Medium and agar are mixed thoroughly by turning the tubes upside down and back and kept at 50°C. Eight tubes are sufficient for each dilution series. The first tube is inoculated with a natural sample or enrichment culture and mixed carefully; approximately 0.5 to 1.0 ml is transferred to a second tube, mixed carefully, and the procedure continued up to the last tube. The tubes are immediately placed into a cold water bath. After the agar has hardened, they are sealed with a paraffin mixture (3 parts paraffin oil and 1 part paraffin) and kept in the dark for several hours before they are incubated in the light (ca. 500–2,000 lux). After cells have grown to visible colonies, the paraffin layer is removed by melting, and well separated colonies are picked with a Pasteur pipette (the tip drawn out to a thin capillary) and transferred to a second dilution series. In general, three to four such dilution series are necessary to obtain pure cultures. When pure cultures have been obtained, single colonies are inoculated into liquid medium.

Cultivation on Agar Plates. Purple nonsulfur bacteria have quite often been successfully isolated on agar plates. When high numbers of phototrophic bacteria are present in the sample, streaking by conventional methods is appropriate. Samples containing low numbers of phototrophic bacteria can be easily concentrated on membrane filters (e.g., cellulose acetate or cellulose nitrate with 0.4 μm pore size; Biebl, 1973; Biebl and Drews, 1969; Swoager and Lindstrom, 1971; Westmacott and Primrose, 1975). The filter assembly is used after autoclaving, and the filters are transferred onto the agar surface with sterile tweezers. Samples with high numbers of purple nonsulfur bacteria (more than 100 cells/ml) may be diluted prior to filtration or distributed on the agar with a Drigalsky spatula (approximately 0.1 ml of a sample containing 100–300 cells/ml; Biebl and Drews, 1969).

Incubation of the plates is recommended in an anaerobic jar in which the air is replaced by an oxygen-free mixture of nitrogen with 5% carbon dioxide and 3% hydrogen. Remaining traces of oxygen are removed by reaction with hydrogen over a palladium catalyst. Alternatively the GasPak system (Becton, Dickinson and Co.) or comparable systems may be used. A more detailed description of these methods is given by Biebl and Pfennig (1981). Normally, an incubation time of five or more days is required before intensely colored colonies of purple nonsulfur bacteria become visible. Occasionally, unicellular green algae develop and form flat spots of grass-green color. Sometimes purple sulfur bacteria, in particular *Thiocapsa roseopersicina* and *Allochromatium vinosum*, also develop.

Media

A large number of media have been used for the enrichment and cultivation of purple nonsulfur bacteria (Biebl and Pfennig, 1981; Drews, 1965; Haskins and Kihara, 1967; Imhoff, 1982b; Imhoff and Trüper, 1976; Kaiser, 1966; Mack et al., 1993; Pfennig, 1969; Pfennig, 1978; Pratt and Gorham, 1970; Swoager and Lindstrom, 1971; Van Niel, 1944).

For the isolation and cultivation of purple nonsulfur bacteria from freshwater and marine sources, the AT medium (Imhoff and Trüper, 1976; Imhoff, 1982b; Imhoff, 1988c) with numerous slight modifications has been successfully used as a basic medium for more than 20 years in the author's laboratory. It can be used for cultivation of the great majority of the purple nonsulfur bacteria. It is also well suited for enrichment cultures of freshwater and marine purple nonsulfur bacteria. For the purpose of selective enrichment cultures, sulfate is omitted, a sulfate-free trace element solution (SLA) is used and selective carbon and nitrogen substrates are added. With nonselective carbon sources such as acetate, pyruvate, malate, succinate or fumarate, this medium is well suited for the isolation and enumeration of purple nonsul-

fur bacteria in agar dilution series or on agar plates. A vitamin solution (VA) is used that adds sufficient amounts of all vitamins required by known purple nonsulfur bacteria (Imhoff, 1988c; see below).

AT Medium

Dissolve in and dilute to 1 liter with distilled water:

KH_2PO_4	1.0 g
$MgCl_2 \cdot 6H_2O$	0.5 g
$CaCl_2 \cdot 2H_2O$	0.1 g
Na_2SO_4	0.7 g
NH_4Cl	1.0 g
$NaHCO_3$	3.0 g
NaCl	1.0 g
Na-acetate (or other carbon source)	1.0 g

Add: 1 ml of trace element solution SLA (see below)
1 ml of vitamin solution VA (see below)
10 ml of 5% sodium ascorbate
Adjust pH to 6.9.

The medium is filter-sterilized, collected in an autoclaved 2-liter Erlenmeyer flask with an outlet at the bottom and distributed from this vessel into sterile screw-cap bottles of desired volume (usually 50-ml and 1-liter bottles) under sterile conditions. These bottles are filled completely (not more than a pea-sized air bubble should be left) and can be stored for several months. To achieve anoxic conditions and to remove the oxygen from the medium, 0.05% sodium ascorbate is added. To avoid oxidation of the ascorbate prior to distribution into the bottles, it is added to the medium immediately before sterile filtration.

Yeast extract stimulates growth of most of the known purple nonsulfur bacteria. It is used as a source of growth factors and may be added at a concentration of 0.05%.

For *Phaeospirillum* and *Rhodospirillum* species addition of 0.01% Fe-citrate is growth stimulating. It may also be routinely added to the medium, except when sulfide is present.

For the cultivation of *Rhodoblastus acidophilus* and *Rhodomicrobium vannielli*, the pH is adjusted to 5.5.

For marine species, NaCl is added. A saline modification of the medium, the SAT medium, contains 3% NaCl.

For iron-oxidizing purple nonsulfur bacteria, 10 mM ferrous iron is added to completely anoxic media buffered with 20 mM bicarbonate at pH 7.0 (Ehrenreich and Widdel, 1994). An anoxic stock solution of $FeSO_4$ is autoclaved, maintained under nitrogen, and used to supplement the media. To increase the solubility of iron, chelators such as citrate or nitrilotriacetic acid may be added.

Some purple nonsulfur bacteria can tolerate sulfide to various degrees and/or use it as a photosynthetic electron donor. For these species, sodium sulfide is added at low concentrations of 0.4–1.0 mM (up to 2 mM for more tolerant strains). Some species even are dependent on the presence of reduced sulfur sources because they lack the ability to use sulfate as an assimilatory sulfur source. For these bacteria also low concentrations of sulfide may be used, or alternative sources of reduced sulfur such as thiosulfate, cysteine or methionine may be applied.

Vitamin Solution VA

(Modified after Imhoff and Trüper, 1977)

Biotin	10 mg
Niacin	35 mg
Thiamine dichloride	30 mg
p-Aminobenzoic acid	20 mg
Pyridoxolium hydrochloride	10 mg
Ca-panthothenate	10 mg
Vitamin B_{12}	5 mg

This vitamin solution meets the vitamin requirements of all purple nonsulfur bacteria. More specifically, for the culture of individual strains or species, the vitamins may be added individually or in desired combinations at the indicated concentration. Dissolve in 100 ml of distilled water, sterilize by filtration, keep refrigerated, and add 1 ml per liter of medium.

Trace Element Solution SLA

(Modified after Imhoff and Trüper, 1977)

$FeCl_2 \cdot 4H_2O$	1,800 mg
$CoCl_2 \cdot 6H_2O$	250 mg
$NiCl_2 \cdot 6H_2O$	10 mg
$CuCl_2 \cdot 2H_2O$	10 mg
$MnCl_2 \cdot 4H_2O$	70 mg
$ZnCl_2$	100 mg
H_3BO_3	500 mg
$Na_2MoO_4 \cdot 2H_2O$	30 mg

These salts are dissolved separately in a total of 900 ml of double-distilled water; the solutions are mixed, the pH is adjusted to about 2–3 with 1 N HCl, and the final volume is brought to 1 liter.

Medium for *Rhodopila globiformis*

(Modified after Pfennig, 1974a)

Mannitol	1.5 g
Gluconate	1.5 g
KH_2PO_4	0.4 g
NaCl	0.4 g
$CaCl_2 \cdot 2H_2O$	0.05 g
$MgCl_2 \cdot 6H_2O$	0.4 g
NH_4Cl	0.4 g
Na-thiosulfate	0.2 g

Dissolve the above in and dilute up to 1 liter with distilled water.

Add separately: 1 ml of SLA (or equivalent trace element solution)
1 ml of VA
5 ml of 0.1% Fe-citrate solution
Adjust pH to 4.9.

Complex Medium

(Modified after Nissen and Dundas, 1984)

$MgCl_2 \cdot 6H_2O$	3.5 g
KH_2PO_4	0.3 g
NaCl (depending on desired salinity)	100 g
Yeast extract (Difco)	1.5 g
Proteose peptone (Difco)	1.5 g
Na-malate	10 mM
Trace element solution SLA	1 ml

Dissolve the above in and dilute up to 1 liter of distilled water. Then adjust pH to 7.0. All strains of halophilic purple nonsulfur bacteria including our own isolates grow well in this medium. This and the complex medium described by Drews (1981) are suitable for growth of *Rhodothalassium salexigens* and *Rhodovibrio salinarum*.

Synthetic Medium for Halophilic (Purple Nonsulfur) Phototrophic α-Proteobacteria

(Modified from Imhoff, 1988c)

$NaHCO_3$	3.9 g
KH_2PO_4	0.5 g
KCl	1.0 g
$CaCl_2 \cdot 2H_2O$	0.05 g
$MgCl_2 \cdot 6H_2O$	3.5 g
Na_2SO_4	1.0 g
NaCl (depending on desired salinity)	40–150 g
Proline	5.0 mM
Acetate	2.0 mM
Pyruvate	2.0 mM
K_3 citrate	10.0 mM
Glycine betaine	10.0 mM
Na-glutamate	5.0 mM
Vitamin solution VA (see above)	1 ml
Trace element solution SLA (see above)	1 ml

Dissolve the above in and dilute up to 1 liter of distilled water. Adjust pH to 7.0. Sterilize by filtration.

For special biochemical and physiological studies, synthetic media (such as this one) are required. The NaCl concentration is varied according to the desired salinity.

Identification

Identification of new isolates can be obtained only by detailed studies of physiological and morphological properties together with chemotaxonomic information and genetic sequence data. To distinguish closely related strains and species of the same genus, often DNA-DNA hybridization studies are required.

Because of the great diversity of the phototrophic α-Proteobacteria, a tentative assignment to one of the known genera often is much easier. Owing to the different physicochemical requirements of the species (e.g., salinity, pH and possibly temperature) and their physiological potential, the choice of the medium and the culture conditions for enrichment and isolation restrict the number of possible species that will be able to develop. Important additional information can be obtained from the color, size and consistency of the colonies on agar plates, the color of cell suspensions, and microscopic examinations. The shape of the cells, cell width and length, motility, mode of division, formation of cell aggregates, and presence of slime capsules are relevant properties that can be recognized in the light microscope. The cell morphology of a few representative species using light microscopy is shown in Fig. 4. Ultrathin sections under the electron microscope reveal the fine structure of the cells, in particular the type of the internal membrane system.

In many species, the color of cell suspensions is indicative of the major type of carotenoids present (Schmidt, 1978). Spirilloxanthin as the major component gives a pink or red color, increasing amounts of additional rhodopin turn the color to brown-red, rhodopin without significant amounts of spirilloxanthin results in brown, okenone results in purple-red, and rhodopinal results in purple-violet. Carotenoids of the spheroidene series give colors from yellowish brown to brownish red (depending on the content of oxygenated derivatives formed in the presence of oxygen) and greenish to beige-brown under strongly reducing conditions. Carotenoidless mutants of *Rhodospirillum rubrum* and *Rhodobium marinum* are blue in color.

In addition to the color of cell suspensions, absorption spectra yield preliminary information on the predominant bacteriochlorophylls and on the kind of bacteriochlorophyll-protein complexes. The carotenoids absorb at 480–550 nm. Absorption bands of bacteriochlorophyll a in vivo are at 380, 590–600, and 800–900 nm. Owing to the formation of different light-harvesting complexes, absorption spectra show a remarkable variation in the long wavelength range from 800–900 nm. Absorption spectra of whole cells are measured with cell suspensions washed twice in medium or appropriate salt solutions and then suspended in 60% sucrose solution (Biebl and Drews, 1969). Better results are often achieved by using isolated internal membranes suspended in buffer. For this purpose, it is sufficient to break the cells by ultrasonication or with a French press and to separate whole cells and large cell fragments from the internal membranes by centrifugation at 15,000 g.

For the detailed description of a new bacterium, careful physiological studies are required, including the utilization of substrates, relations to oxygen, the ability to grow in darkness, respiratory and fermentative growth, vitamin requirement, and ranges and optima of salt concentration, pH and temperature.

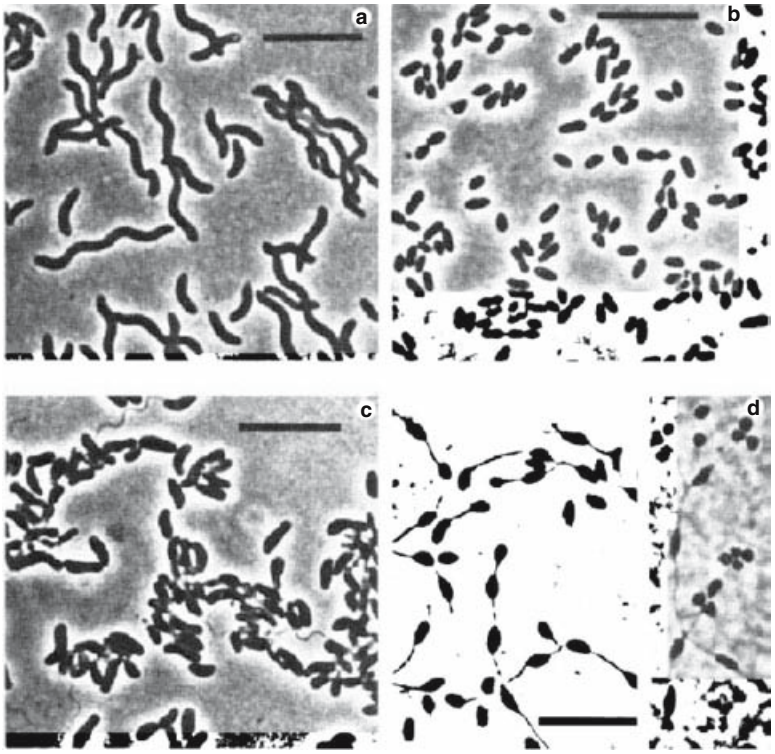

Fig. 4. Morphology of *Rhodospirillum rubrum* (a); *Rhodobacter sphaeroides* (b); *Rhodopseudomonas acidophilia* (c); *Rhodomicrobium vannielii* (d), left: Vegetative cells with polyhedral exospores; phrase contrast micrographs. (Bar = 10 μm.)

In addition to the phenotypic characterization, information on the genetic relatedness of a new isolate has to be obtained. To achieve a phylogenetic classification, 16S rDNA sequences are used and when the distinction of closely related strains and species is required (DeBont et al., 1981), DNA-DNA hybridization studies are performed. For the description of a new species, also the determination of the G+C content of the DNA is recommended.

Also chemotaxonomic properties have been found to be quite helpful to identify and classify new isolates of phototrophic bacteria (Hiraishi et al., 1984; Imhoff, 1984a; Imhoff, 1988b; Imhoff et al., 1982c; Imhoff et al., 1984b; 1998; Imhoff and Bias-Imhoff, 1995b; Thiemann and Imhoff, 1996; Imhoff and Süling, 1996; Pfennig et al., 1997). In particular the ring structure and the isoprenoid chain length of respiratory quinones and the fatty acid composition of the cell membranes are quite useful in identification (Hiraishi et al., 1984; Imhoff, 1984a). The structure and composition of polar lipids and lipopolysaccharides also are diagnostic properties of high value (Imhoff and Bias-Imhoff, 1995b; Weckesser et al., 1995). The need for sophisticated analytical methods, however, makes these properties less accessible for diagnosis.

Characteristic properties of presently known species of the phototrophic purple α-Proteobacteria that are of diagnostic value are shown in Tables 1–3. An arbitrary selection of outstanding properties of a few selected species follows:

Cultures of *Blastochloris viridis* and *Blastochloris sulfoviridis* are olive-green in color and contain bacteriochlorophyll β with an absorption maximum at 1020–1030 nm.

Cultures of *Rhodospira trueperi* are peach colored and reveal unusually low long-wavelength absorption maxima of bacteriochlorophyll b at 986 nm.

The in vivo absorption spectra of *Rhodospirillum rubrum* and *Rhodobium marinum* are characterized by an unusually low absorption maximum at approximately 803 nm and a single dominant peak without a shoulder (should be checked in derivative spectra) at 870–885 nm. This is taken as indication of the lack of peripheral light-harvesting complexes. Additional species with such spectra are *Rhodovibrio sodomensis*, *Rhodobaca bogoriensis* and *Rhodocista centenaria*.

For *Rhodomicrobium vannielii*, the peritrichous flagellation of swarmer cells, the formation of cell aggregates connected by thin filamentous tubes and the formation of exospore-like cysts of moderate heat resistance are typical.

Swarming motility on agar surfaces is characteristic for *Rhodocista centenaria*, which also forms desiccation- and heat-resistant cysts (Favinger et al., 1989).

A definitive growth requirement for salt is found in the marine species of *Rhodovulum* and *Rhodobium* and also in *Rhodospira trueperi*, *Roseospira mediosalina* and *Roseospirillum parvum*.

Table 1. Characteristic properties of the phototrophic *Rhodospirillum* and related phototrophic purple nonsulfur bacteria.

	Rhodospirillum		*Phaeospirillum*		*Rhodocista*	*Rhodovibrio*		*Rhodospira*		*Roseospira*		*Rhodopila*
Genus Species	*Rsp.* *rubrum*	*Rsp.* *photometricum*	*Phs.* *fulvum*	*Phs* *molischianum*	*Rci.* *centenaria*	*Rhv.* *salinarum*	*Rhv.* *sodomensis*	*Rsa.* *trueperi*	*Roseospirillum* *Reo. parvum*	*Ros.* *mediosalina*	*Rhodothalsassium* *Rts. salexigens*	*Rpi.* *globiformis*
Characteristic												
Cell diameter (μm)	0.8–1.0	1.1–1.5	0.5–0.7	0.7–1.0	1.0–2.0	0.8–0.9	0.6–0.7	0.6–0.8	0.4–0.6	0.8–1.0	0.6–0.7	1.6–1.8
ICM	Vesicles	Stacks	Stacks	Stacks	Lamellae	Vesicles	Vesicles	Vesicles	Lamellae	Vesicles	Lamellae	Vesicles
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Color	Red	Brown	Brown	Brown	Pink	Red	Pink	Beige	Pink	Pink	Red	Purple-red
Bacteriochlorophyll	*a*	*a*	*a*	*a*	*a*	*a*	*a*	*b*	*a*	*a*	*a*	*a*
Vitamins required	b	n	paba	aa	b, B_{12}	c, n	c, n	b, t, pan	o	t, paba, n	glu	b, paba
Aerobic growth	+	–	–	–	+	+	+	–	+	(+)	+	(+)
Oxidation of sulfide	+	–	–	–	o	–	o	+	+	+	–	–
Salt requirement	None	None	None	None	None	4–6% (3–24)	10–14% (8–18)	2% (0.5–5)	1–2	4–7% (0.5–15)	6–8% (5–20)	None
Optimal temperature	30–35	25–30	25–30	30	40–45	42	35–40	25–30	30	30–35	40	30–35
Optimal pH	6.8–7.0	6.5–7.5	7.3	7.3	6.8	7.5–8.0	7	7.0–7.5	7.9	7	6.6–7.4	4.8–5.0
Habitat	Fresh water	Fresh water	Fresh water	Fresh water	Fresh water warm springs	Saltern	Salt lakes	Marine sediments	Marine sediments	Salty springs	Saltern	Fresh water acidic springs
DNA base ratio[a]	63.8–65.8	64.8–65.8	64.3–65.3	60.5–64.8	69.9	67.4	66.2–66.6	65.7	71.2	66.6	64.0	66.3
Cytochrome *c* size	Large	Large	Small	Small	o	None	None	o	o	o	o	Small
Major quinones	Q-10, RQ-10	Q-8, RQ-8	Q-9, MK-9	Q-9, MK-9	Q-9	Q-10, MK-10	o	Q-7, MK-7	o	o	Q-10, MK-10	Q-9/10, MK-9/10 RQ-9/10

Symbols: +, positive in most strains; –, negative in most strains; +/–, variable in different strains; o, not determined; (+), weak growth or microaerobic growth only.
Abbreviations: b, biotin; t, thiamine; n, niacin; paba, *p*-aminobenzoic acid; pan, pantothenate; glu, glutathione; ICM, internal cell membrane; Q-10, ubiquinone 10; MK-10, menaquinones 10; and RQ-10, rhodoquinone 10.
[a]Mol%.

Table 2. Characteristic properties of *Rhodopseudomonas* and related phototrophic purple nonsulfur bacteria.

	Rhodopseudomonas			*Rhodoblastus*	*Rhodoplanes*		*Blastochloris*			*Rhodobium*	
Genus Species	*Rps. palustris*	*Rps. julia*	*Rps. cryptolactis*	*Rbl. acidophilus*	*Rpl. roseus*	*Rpl. elegans*	*Blc. viridis*	*Blc. sulfoviridis*	*Rhodomicrobium Rmi. vannielii*	*Rbi. orientis*	*Rbi. marinum*
Characteristic											
Cell diameter (μm)	0.6–0.9	1.0–1.5	1	1.0–1.3	1.0	0.8–1.0	0.6–0.9	0.5–0.9	1.0–1.2	0.7–0.9	0.7–0.9
Type of budding	Tube	Sessile	Sessile	Sessile	Sessile	Tube	Tube	Sessile	Tube	Sessile	Sessile
Rosette formation	+	+	+	–	–	+	+	–	Large cell aggregates	–/+	–
Internal membrane system	Lamellae	Lamellae	Lamellae	Lamellae	Lamellae	Lamellae	Lamellae	Lamellae	Lamellae	Lamellae	Lamellae
Motility	+	+	+	+	+	+	+	+	+	+	+
Color of cultures	Brown-red to red	Pink	o	Red to orange-red	Pink	Pink	Green to olive-green	Olive-green	Orange-brown to red	Pink to red	Pink to red
Bacteriochlorophyll	*a*	*a*	*a*	*a*	*a*	*a*	*b*	*b*	*a*	*a*	*a*
Salt requirement	None	o	None	None	None	None	None	None	None	4–5%	1–5%
Optimum pH	6.9	6.0	6.8–7.2	5.5–6.0	7.0–7.5	7.0	6.5–7.0	7.0	6.0	7.0–7.5	6.9–7.1
Optimum temperature (°C)	30–37	25–35	40	25–30	30	30–35	25–30	28–30	30	30–35	25–30
Aerobic dark growth	+	+	+	+	+	+	(+)	(+)	+	+	(+)
Denitrification	+/–	–	o	–	+	+	–	–	o	+	–
Photoautotrophic growth with	H_2, TS, Sulfide	Sulfide, S^o	–	H_2	TS	TS	–	TS, Sulfide	H_2, Sulfide	TS	Sulfide
Vitamins required	Paba (biotin)	None	B_{12}, niacin, paba	None	Niacin	Thiamine, paba	Biotin, paba	Biotin, paba pyridoxine	None	Biotin, paba	o
Utilization of:											
Benzoate	+	–	–	–	–	–	–	–	–	–	–
Citrate	+/–	–	o	+	+	+	+/–	–	–	–	+/–
Formate	–	+	o	+	–	–	–	–	+/–	–	+
Tartrate	–	–	o	–	+	+	–	–	–	–	–
Glucose	–	+	–	–	–	–	(+)	+	–	+	+
Thiosulfate	+	o	o	–	+	+	–	+		+	–
Sulfide	+	+	o	–	–	–	–	+	+	–	(+)
Cytochrome c_2 size	Large	o	o	Small	o	o	Small	o	Small	o	o
Major quinones	Q-10	o	o	Q-10, MK-10, RQ-10	Q-10, RQ-10	Q-10, RQ-10	Q-9, MK-9	Q-8/10, MK-7/8	Q-10, RQ-10	Q-10, MK-10	Q-10, MK-10
Mol% G+C of the DNA	64.8–66.3 (Bd)	63.5	68.8	62.2–66.8 (Bd)	66.8 (HPLC)	69.6–69.7 (HPLC)	66.3–71.4 (Bd)	67.8–68.4 (CA)	61.8–63.8 (Bd)	65.2–65.7 (HPLC)	62.4–64.1 (HPLC)

Symbols: +, positive in most strains; –, negative in most strains; +/–, variable in different strains; o, not determined; and (+), weak growth or microaerobic growth only.
Abbreviations: paba, *p*-aminobenzoic acid; (biotin), biotin is required by some strains; Q-10, ubiquinone 10; MK-10, menaquinones 10; RQ-10, rhodoquinone 10; Bd, buoyant density; HPLC, high-pressure liquid chromatography; and TS, thiosulfate.

Table 3. Characteristic properties of *Rhodobacter* and related phototrophic purple nonsulfur bacteria.

	Rhodobacter					*Rhodobaca*	*Rhodovulum*					
Genus Species	*Rba. azotoformans*	*Rba. blasticus*	*Rba. capsulatus*	*Rba. sphaeroides*	*Rba. veldkampii*	*Rhb. bogoriensis*	*Rhv. strictum*	*Rhv. sulfidophilum*	*Rhv. adriaticum*	*Rhv. euryhalinum*	*Rhv. iodosum*	*Rhv. robiginosum*
Characteristic												
Cell diameter (μm)	0.6–1.0	0.6–0.8	0.5–1.2	2.0–2.5	0.6–0.8	0.8–1.0	0.6–1.0	0.6–0.9	0.5–0.8	0.7–1.0	0.5–0.8	0.5–0.8
Motility	+	–	+	+	–	+	+	+	–	+	–	–
Internal membrane system	Vesicle	Lamellae	Vesicle	Vesicle	Vesicle	Vesicle	Vesicle	Vesicle	Vesicle	Vesicle	Vesicle	Vesicle
Cell division	Binary fission	Budding	Binary fission	Binary fission	Binary fission	Binary fission	Binary fission	Binary fission	Binary fission	Binary fission	Binary fission	Binary fission
NaCl required (%)	–[a]	–	–[a]	–[a]	–	1–2	0.8–1.0	1–6	2.5–7.5	0.5–12	2.5–5	2.5–5
Sulfate assimilated	+	+	+	+	–	o	+	+	–	–	–	–
Oxidation products of sulfide	S^o	–	S^o	S^o	S^o/sulfate	o	Sulfate	Sulfate	S^o/sulfate	S^o/sulfate		
Aerobic dark growth	+	+	+	+	+	+	+	+	–	–	+	+
Vitamins required	b, n, t	b, n, t, B_{12}	t, (b, n)	b, t, n	b, paba, t	b, t, [B_{12}]	b, paba, t	b, n, paba, t	b, t	b, n, paba, t	b, n	b, n, B_{12}
Utilization of:												
Formate	+	–	+	–	–	–	+	+	+	+/–	–	–
Citrate	o	+	+/–	+	–	–	+	–	–	–	–	–
Tartrate	–	–	–	+	–	o	(+)	–	–	–	o	o
Mannitol	+	+	+/–	+	–	+	–	+/–	–	+/–	+	+
Glycerol	+	+	–	+	–	o	–	+	+	+	–	+
Ethanol	o	–	–	+	–	–	–	+/–	+	+/–	–	–
Hydrogen	o	+	+	+	–	o	–	+	–	+	+	+
Thiosulfate	–	–	–	–	+	o	+	+	+	+	+	+
Ferrous iron	o	o	o	o	o	o	o	–	–	–	+	+
Mol% G+C of DNA	69.5–70.2 (HPLC)	65.3 (Bd)	65.5–66.8 (Bd)	68.4–69.9 (Bd)	64.4–67.5 (Tm)	58–59	67.3–67.7 (HPLC)	66.3–66.6 (HPLC)	64.9–66.7 (Tm)	62.1–68.6 (Tm)	66 (HPLC)	69 (HPLC)

Symbols: +, positive in most strains; –, negative in most strains; +/–, variable in different strains; o, not determined; and (+), weak growth or microaerobic growth only.
Abbreviations: b, biotin; t, thiamine; n, niacin; paba, *p*-aminobenzoic acid; (biotin), biotin is required by some strains; [B_{12}], vitamin B_{12} growth stimulating but not absolutely required; Bd, buoyant density; Tm, thermal denaturation; and TS, thiosulfate.
[a]Optimal growth in the absence of NaCl, but able to grow at 3% NaCl.

The tolerance of salt concentrations of more than 10% and growth under these conditions are characteristic for the halophilic species *Rhodothalassium salexigens*, *Rhodovibrio salinarum* and *Rhodovibrio sodomensis*.

Preservation

For short-term preservation, well-grown cultures may be kept in closed, air-tight screw-cap bottles at 6–10°C in a refrigerator (or cold room) or at room temperature for several months, even years. Maintenance transfer of liquid cultures should occur at intervals of 2–6 months. In particular *Rhodopseudomonas palustris* and some *Rhodobacter* species have been shown to maintain viable cells over very long time periods. Stock cultures of the brown-colored *Rhodospirillum* and *Phaeospirillum* species and of other oxygen-sensitive species should be grown in the presence of 0.05% sodium ascorbate and transfers should be made every 1–2 months. Also, stock cultures of *Blastochloris sulfoviridis* and *Rhodopila globiformis* should be transferred more frequently. Stock cultures are incubated anaerobically in screw-cap 50-ml bottles in the described media at 25–30°C and 2,000 lux and then taken from the light during late exponential growth phase. Stock cultures also may be maintained for prolonged times in agar stab cultures or in appropriate dilutions of agar dilution series, sealed with paraffin and kept in the dark.

For long-term storage, preservation in liquid nitrogen is recommended. Well grown cultures are supplemented with 50% dimethyl sulfoxide to give a final concentration of 5%, thoroughly mixed, dispensed in plastic ampoules, sealed and frozen in liquid nitrogen. Preservation and storage in liquid nitrogen are possible with all strains tested with either dimethylsulfoxide (5%) or glycerol (10%) as a protecting agent.

Several purple nonsulfur bacteria were succsessfully preserved by freeze-drying (Biebl and Malik, 1976). The best protecting agent was skim milk (20%) supplemented with 10% sucrose, but most strains also survived in 10% sucrose alone. The brown-colored *Phaeospirillum* and *Rhodospirillum* species could not be lyophilized successfully (Biebl and Malik, 1976).

Physiology

A comprehensive treatment of the various aspects of the physiology of purple nonsulfur bacteria including structure, function and genetics of the photosynthetic apparatus is found in various chapters of *The Photosynthetic Bacteria* (Clayton and Sistrom, 1978) and *Anoxygenic Phototrophic Bacteria* (Blankenship et al., 1995). A short overview on physiology and photosynthesis is given by Drews and Imhoff (1991). In the following, the basic principles and a few specific examples of metabolic properties of the phototrophic α-Proteobacteria are presented.

Photosynthesis

Purple nonsulfur bacteria are anoxygenic phototrophic bacteria, growing phototrophically under anoxic conditions in the light without producing oxygen. All species can grow photoorganoheterotrophically using organic substrates as photosynthetic electron donors and carbon sources. Many representatives also grow under photolithoautotrophic conditions with reduced sulfur compounds, with hydrogen or ferrous iron ions as electron donor and CO_2 as sole carbon source.

Anoxygenic photosynthesis depends on the presence of a complex membrane-bound photosynthetic apparatus, which includes reaction center and light harvesting (antenna) pigment-protein complexes. The proteins of reaction center and antenna noncovalently bind bacteriochlorophyll, carotenoids and other cofactors in stoichiometric ratios. Most purple nonsulfur bacteria have two antenna complexes. The complexes of the reaction center are surrounded by core antenna (a B870 or B890 antenna complex with bacteriochlorophyll a and a B1020 complex with bacteriochlorophyll *b*) and additional peripheral antenna (B800-850 and B800-820 complexes with bacteriochlorophyll *a*). Species such as *Rhodospirillum rubrum* and *Rhodobium marinum* lack peripheral antenna complexes and can be recognized by their minor absorption maxima at 803 nm. Most purple nonsulfur bacteria have one type of peripheral antenna complex (B800-850). The synthesis of multiple forms of peripheral antenna polypeptides with varying proportions under different growth conditions (as found in *Rhodoblastus acidophilus* and *Rhodopseudomonas palustris*) and the formation of different peripheral antenna complexes result in a complex pattern of absorption maxima that is only resolved in derivative spectra (Brunisholz and Zuber, 1992; Zuber and Cogdell, 1995).

The principal function of the photosynthetic apparatus is the light-mediated excitation of a bacteriochlorophyll molecule in the reaction center followed by charge separation and resulting in electron transfer through the membrane. Most purple nonsulfur bacteria have an internal membrane system in which the photosynthetic apparatus is integrated. These internal membranes form vesicles, tubules or lamellar structures and are interconnected to the cytoplasmic

membrane. They can be isolated by cell rupture and fractionated centrifugation. Quite characteristically, the production of photosynthetic pigments, pigment-protein complexes and of the photosynthetic membrane structures is suppressed by oxygen. A notable exception is *Rhodocista centenaria* (Yildiz et al., 1991).

(Note: We distinguish between the anoxygenic phototrophic purple nonsulfur bacteria [which use photosynthesis as primary energy source and are well adapted to the anoxic way of life] and the aerobic bacteriochlorophyll-containing bacteria [which primarily gain energy by aerobic respiration but are unable to grow by anoxygenic photosynthesis under anoxic conditions]. The latter are treated elsewhere.)

Respiration

Chemoorganoheterotrophic growth in the presence of oxygen is common among purple nonsulfur bacteria and most of the known species are facultatively chemotrophic. While some species are very sensitive to oxygen, others grow equally well under oxic conditions in the dark at the full oxygen tension of air. Also chemolithoautotrophic growth with hydrogen or reduced sulfur compounds as electron donors and oxygen as electron acceptor has been demonstrated (Madigan and Gest, 1979; Siefert and Pfennig, 1979). Under anoxic dark conditions, growth of several species is also supported by respiratory electron transport in the presence of nitrate, nitrite, and nitrous oxide. Denitrification is induced by nitrate, either in the dark or in the light, but is suppressed by oxygen. A single strain of *Rhodobacter sphaeroides*, described as a subspecies, *Rhodobacter sphaeroides* f. sp. *denitrificans* (Satoh et al., 1976), but later recognized as a regular strain of *Rhodobacter sphaeroides* (De Bont et al., 1981; Imhoff, 1989a), was the first phototrophic purple bacterium known to use nitrate as an electron acceptor under anoxic dark conditions. Later, nitrate reduction was found in additional strains of *Rhodobacter sphaeroides* (Michalski and Nicholas, 1988), in strains of *Rhodopseudomonas palustris* (Klemme et al., 1980), and in *Rhodobacter capsulatus* (McEwan et al., 1984). Some of these strains could not grow with nitrate as sole nitrogen source, but dinitrogen produced during denitrification might be assimilated under these growth conditions (Dunstan et al., 1982). Also, *Rhodobacter azotoformans*, *Rhodobium orientis* and *Rhodoplanes roseus* and *Rhodoplanes elegans* are able to denitrify (Hiraishi and Ueda, 1994b; Hiraishi et al., 1995b; Hiraishi et al., 1996).

Anaerobic growth on sugars with dimethylsulfoxide (DMSO) or trimethylamine-*N*-oxide (TMAO) as an additional oxidant has been observed first in *Rhodobacter capsulatus* (Yen and Marrs, 1977) and later also in other species. Energy generation during growth with fructose and TMAO was proposed to be due to anaerobic respiration (Schultz and Weaver, 1982), and the generation of a membrane potential under these conditions has been demonstrated (McEwan et al., 1983). Later it was questioned that a true electron transport chain is involved in DMSO and TMAO reduction. The physiological role of these external oxidants was discussed in detail (Ferguson et al., 1987; Zannoni, 1995).

Fermentation

In the absence of external electron acceptors, a number of purple nonsulfur bacteria can use fermentative processes for energy generation (Uffen, 1978). During fermentative growth, substrates or storage products are oxidized incompletely, and reduced organic compounds as well as CO_2 and H_2 are produced. *Rhodospirillum rubrum* forms succinate, acetate, propionate, formate, CO_2 and H_2 during fermentation of fructose (Schön and Biedermann, 1973), whereas acetate, formate and equimolar amounts of CO_2 and H_2 are produced from pyruvate (Uffen, 1973). *Rhodobacter capsulatus* produces succinate, lactate, acetate, CO_2 and H_2 under identical conditions (Schultz and Weaver, 1982).

Carbon Metabolism

Organic carbon sources have quite different functions under phototrophic, respiratory and fermentative metabolism. Under phototrophic growth conditions, they serve primarily as a source of cellular carbon, but in addition may function as an electron source for photosynthetic electron transport; in the presence of inorganic electron donors, they may be exclusively photoassimilated. During respiratory growth, the major part of the carbon sources is completely oxidized and only a minor fraction is assimilated into cell substance. Enzymatic reactions of the citric acid cycle involved in substrate oxidation would be expected at elevated levels under these conditions and indeed have been found to be increased during respiratory growth of *Rhodobacter capsulatus* (Beatty and Gest, 1981). During fermentation, substrates or storage products are oxidized incompletely on a large scale, and reduced organic compounds and hydrogen are excreted to achieve a redox balance of the cells.

Most of the purple nonsulfur bacteria can use a variety of different organic carbon sources. Intermediates of the tricarboxylic acid cycle in addition to acetate and pyruvate are generally used. A number of purple nonsulfur bacteria use straight-chain saturated fatty acids with 5–18 car-

bon atoms (Janssen and Harfoot, 1987). Also organic acids, amino acids, alcohols and carbohydrates support growth of many of these bacteria. Carbon substrates such as citrate and aromatic compounds are used by a few species only. Citrate for instance is used by *Rubrivivax gelatinosus* (a β-Proteobacterium), *Rhodobacter sphaeroides*, *Rhodobacter blasticus*, *Rhodovulum strictum*, *Rhodothalassium salexigens*, *Rhodoblastus acidophilus*, *Rhodoplanes roseus*, *Rhodoplanes elegans* and by some strains of *Blastochloris viridis*, *Rhodobium marinum* and *Rhodopseudomonas palustris*. Some of these bacteria grow weakly with citrate.

A small number of species (*Rhodopseudomonas palustris*, *Rubrivivax gelatinosus*, *Phaeospirillum fulvum*, *Rhodocyclus purpureus*, *Rhodoblastus acidophilus* and *Rhodomicrobium vannielii*) can grow at the expense of aromatic organic compounds, such as benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 1,3,5-trihydroxybenzene and other hydroxylated, methoxylated aromatic acids and aldehydes (Dutton and Evans, 1978). The ability to use aromatic compounds is best developed in *Rhodopseudomonas palustris*. This species also metabolizes phenolic acids, phenylalkane carboxylates, 4-hydroxy-cinnamate and related compounds (Harwood and Gibson, 1988; Gibson and Harwood, 1995). Most of these compounds support both phototrophic (anoxic conditions in the light) and chemotrophic (oxic conditions in the dark) growth; some are degraded only under the one or the other condition. During phototrophic growth, a reductive pathway for cleavage of the aromatic ring structure is used (Dutton and Evans, 1969; Gibson and Harwood, 1995).

Acetate is assimilated by almost all purple nonsulfur bacteria. The metabolic pathways of acetate assimilation, however, are quite different among the species. In many phototrophic purple bacteria, the primary reaction of acetate metabolism is the ATP-dependent formation of acetyl CoA, which is the substrate for further reactions. The two key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, are present in most of these bacteria and acetate is assimilated via this pathway. There is, however, some variance in the literature regarding the presence of isocitrate lyase, in particular in *Rhodospirillum rubrum* and *Rhodobacter sphaeroides* (Tabita, 1995). Certainly, alternative pathways of acetate assimilation are possible. For *Rhodospirillum rubrum*, the conversion of acetate to oxalacetate by two carboxylation reactions from acetate to pyruvate and further to oxalacetate has been postulated (Buchanan et al., 1967). In *Rubrivivax gelatinosus* (a β-Proteobacterium), photoassimilation of acetate is possible via the serine-hydroxypyruvate pathway (Albers and Gottschalk, 1976).

For purple nonsulfur bacteria, CO_2 is an important carbon source. Under autotrophic growth conditions with CO_2 as sole carbon source, the Calvin cycle with ribulose bisphosphate carboxylase (RubisCO) as key enzyme is employed (Tabita, 1995). This enzyme is well studied and constitutes a major fraction of the cellular protein in bacteria that grow with CO_2 as sole carbon source and use the Calvin cycle. Also, CO_2 is required under heterotrophic growth conditions during assimilation of several reduced organic substrates. A number of carboxylating enzyme activities are responsible for this "heterotrophic CO_2 fixation" (see Kondratieva, 1979; Tabita, 1995). For instance, assimilation of propionate is connected with a carboxylation to succinate. Also, long-chain fatty acids and other highly reduced substrates such as methanol require CO_2 to elevate the oxidation-reduction level of these substrates to that of the cell material.

One-carbon compounds, such as methanol and formate, also are used by strains of a limited number of species. *Rhodomicrobium vannielii*, *Rhodobium marinum*, *Rhodoblastus acidophilus*, *Rhodospirillum rubrum*, *Rubrivivax gelatinosus*, *Rhodocyclus tenuis* and several *Rhodobacter* and *Rhodovulum* species use formate as carbon source. Reasonable growth rates with methanol were found only in strains of *Rhodoblastus acidophilus* (Douthit and Pfennig, 1976). Apparently, the RubisCO pathway is involved in carbon assimilation of *Rhodoblastus acidophilus* also during growth on methanol and formate; both substrates are used as electron donors and are oxidized to CO_2, which in turn is assimilated (Quale and Pfennig, 1975; Sahm et al., 1976). *Rubrivivax gelatinosus* is able to grow anaerobically in the dark with CO as sole source as carbon and energy. Under these conditions, CO is transformed into CO_2 and H_2, and RubisCO could be involved in assimilation of the latter (Uffen, 1983). In *Rhodospirillum rubrum*, CO is used under anoxic conditions during phototrophic growth and induces an oxygen-sensitive CO dehydrogenase (Bonam et al., 1989).

Sulfur Metabolism

The role of reduced sulfur compounds as photosynthetic electron donors for purple nonsulfur bacteria was realized together with the recognition of their importance in the marine environment. Purple nonsulfur bacteria vary greatly in their sulfur metabolism.

Most purple nonsulfur bacteria, in particular freshwater species, are inhibited by sulfide even

at low concentrations. Some species, however, are quite tolerant to this toxic compound and use it as a photosynthetic electron donor (Hansen and Imhoff, 1985; Hansen and van Gemerden, 1972; Imhoff, 1982a; Imhoff, 1983a; Neutzling et al., 1984). The tolerance of *Rhodovulum sulfidophilum* is high and comparable to that of *Allochromatium vinosum*. *Rhodomicrobium vannielii* tolerates concentrations of 2–3 mM, whereas growth of *Rhodobacter capsulatus* is completely inhibited at 2 mM. At low concentrations of sulfide (0.4–0.8 mM), however, growth of this species is quite rapid. Growth of *Rhodopseudomonas palustris*, as of most of the purple nonsulfur bacteria, is inhibited at concentrations as low as 0.5 mM sulfide (Hansen and van Gemerden, 1972). In general, small amounts of yeast extract in the media increase the tolerance towards sulfide.

In addition to sulfide, some purple nonsulfur bacteria can use thiosulfate as an electron donor. Extracellular elemental sulfur is the final oxidation product during sulfide oxidation of a number of purple nonsulfur bacteria, such as *Rhodospirillum rubrum*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides* (Hansen and Veldkamp, 1973) and *Roseospira mediosalina* (Kompantseva and Gorlenko, 1984). Elemental sulfur occurs outside the cells. Only with *Rhodopseudomonas julia* elemental sulfur was microscopically observed outside as well as inside the cells (Kompantseva, 1989). Oxidation of elemental sulfur is found in a limited number of species, as in *Rhodobacter veldkampii*, *Rhodovulum euryhalinum*, *Rhodovulum adriaticum* and *Rhodopseudomonas julia*. In these species, elemental sulfur is an intermediate product during oxidation to sulfate (Hansen, 1974; Hansen and Imhoff, 1985; Neutzling et al., 1984; Kompantseva, 1985; Kompantseva, 1989). Sulfate is formed from sulfide without formation of elemental sulfur in *Rhodovulum sulfidophilum*, *Rhodovulum strictum*, *Rhodopseudomonas palustris* and *Blastochloris sulfoviridis* (Hansen, 1974; Hiraishi and Ueda, 1995a; Neutzling et al., 1985). Tetrathionate is the only oxidation product of *Rhodomicrobium vannielii* grown in a chemostat, but in batch culture, sulfide reacts with the tetrathionate formed, so that thiosulfate is the major product (together with minor amounts of elemental sulfur) accumulated under these conditions; sulfate is not formed under either condition (Hansen, 1974).

With a few notable exceptions, the purple nonsulfur bacteria can use sulfate as an assimilatory sulfur source. Apparently two different pathways occur in these bacteria, involving either adenosine 5′-phosphosulfate (APS) or 3′-phosphoadenosine 5′-phosphosulfate (PAPS; Imhoff, 1982b). In the presence of reduced sulfur compounds, these are preferentially assimilated and the energetically expensive assimilation of sulfate is repressed (Imhoff et al., 1983b). Common alternative assimilatory sulfur sources include sulfite, thiosulfate, cysteine, glutathione, methionine and sulfide. *Rhodovulum adriaticum*, *Rhodovulum iodosum*, *Rhodovulum robiginosum*, *Rhodovulum euryhalinum*, *Blastochloris sulfoviridis*, *Rhodopseudomonas julia*, *Rhodobacter veldkampii* and *Roseospirillum parvum* require reduced sulfur sources and are unable to assimilate sulfate (Keppen and Gorlenko, 1975; Kompantseva, 1985; Kompantseva, 1989; Neutzling et al., 1984; Hansen and Imhoff, 1985; Pfennig, 1974a; Straub et al., 1999; Glaeser and Overmann, 1999). Growth of *Rhodopila globiformis* is inhibited by high concentrations of sulfate although at low concentration this can serve as an assimilatory sulfur source (Imhoff et al., 1981). Also *Rhodobium marinum* grows poorly with sulfate as an assimilatory sulfur source, but much better in the presence of low concentrations of reduced sulfur sources.

Nitrogen Metabolism

Ammonia, dinitrogen, and several organic nitrogen compounds (e.g., glutamate, aspartate or yeast extract) are the most appropriate nitrogen sources of most purple nonsulfur bacteria. Nitrate is assimilated only by a few species (strains of *Rhodospirillum rubrum*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus* and *Rhodopseudomonas palustris*) and growth yields with nitrate are considerably lower than with other nitrogen sources (Göbel, 1978; Imhoff, 1982a). Nitrate assimilation is inducible by nitrate but repressed by ammonia and glutamate.

Assimilation of ammonia is strongly regulated and two different major pathways occur. The primary pathway in purple nonsulfur bacteria is via glutamine synthetase and glutamate synthase, which is active if cellular concentrations of ammonia are low and if growth is occurring at low concentrations of ammonia or on dinitrogen and nitrate (Drews and Imhoff, 1991). The second pathway present in most purple nonsulfur bacteria is via glutamate dehydrogenase, which is active when ammonia concentrations are high.

The ability to fix dinitrogen is a common property of most phototrophic purple bacteria (Madigan, 1995). Dinitrogen fixation occurs under phototrophic and chemotrophic growth conditions, and the activity and expression of nitrogenase underlie a complex regulatory cascade (Drews and Imhoff, 1991; Haselkorn, 1986; Ludden and Roberts, 1995; Madigan, 1995). It has been most intensively studied in *Rhodospirillum rubrum*, where it was first discovered by Kamen and Gest (1949), and in *Rhodobacter capsulatus*.

Nitrogenase activity is repressed and inactivated by oxygen as in other dinitrogen-fixing bacteria. Its synthesis is derepressed at low concentrations of ammonia, i.e., under conditions of nitrogen limitation.

Hydrogen Metabolism

A great number of phototrophic purple bacteria can photoproduce hydrogen. With dinitrogen, glutamate or aspartate as nitrogen source, a number of carbon substrates (lactate, acetate, butyrate, malate and others) may be completely transformed to CO_2 and H_2, and these in turn may serve as substrates for photoautotrophic growth. After consumption of the organic substrate, cell suspensions of *Rhodobacter capsulatus* that produced, e.g., H_2 from lactate, rapidly started to consume the produced H_2 (Kelley et al., 1977). Similar observations were obtained with *Rhodospirillum rubrum* (Schick, 1971). Photoevolution of H_2 is catalyzed by nitrogenase, which is inhibited by ammonia, high concentrations of yeast extract and amino acids degraded to produce ammonia. The reaction is not reversible, insensitive to CO (a common inhibitor of hydrogenases) and independent of the partial pressure of H_2.

Hydrogen evolution in purple nonsulfur bacteria also occurs during fermentative growth under anoxic dark conditions. This hydrogen evolution is catalyzed by a reversible hydrogenase or by formate hydrogenlyase and underlies similar regulatory rules as in other fermenting bacteria, i.e., it is strongly inhibited by CO (Drews and Imhoff, 1991; Sasikala et al., 1993).

Hydrogen is not only produced, but also serves as an excellent photosynthetic electron donor for many purple nonsulfur bacteria and enables these bacteria to grow photolithoautotrophically. Hydrogen uptake is catalyzed by a reversible, membrane-bound hydrogenase, which is induced by hydrogen and independent of the nitrogen source. The membrane-bound hydrogenase is not inhibited by ammonia, but strongly inhibited by CO. During growth conditions of dinitrogen fixation, this uptake hydrogenase recycles the hydrogen produced by nitrogenase, and mutants lacking this hydrogenase demonstrate an increased hydrogen production during nitrogen fixation (Drews and Imhoff, 1991).

Applications

Most prominent examples of the application of phototrophic purple nonsulfur bacteria are their use in sewage treatment processes and for production of biomass, biopolymers and molecular hydrogen. They may be used as a source for cell-free systems performing photosynthesis and ATP formation and for the production of vitamins and other organic molecules.

Sewage contains a complex mixture of small organic molecules that are good substrates for purple nonsulfur bacteria. Phototrophic bacteria are regularly found in conventional sewage treatment plants (Holm and Vennes, 1971; Siefert et al., 1978). Facultative chemotrophic purple phototrophic bacteria compete best under such conditions. Because light-driven energy generation enables them to use compounds produced during anaerobic degradation processes, these bacteria are good candidates for application to the final stages of sewage treatment. A highly advanced system using phototrophic bacteria in the purification of municipal and industrial waste water is that developed by Kobayashi (Kobayashi et al., 1971; Kobayashi and Tschan, 1973; Kobayashi, 1977; Kobayashi and Kobayashi, 1995). This process uses the natural sequence of aerobic and anaerobic degradation, followed by culture of anoxygenic phototrophic bacteria and of green algae in separated reaction tanks (bioreactors). Phototrophic bacteria used in Kobayashi's system include *Rhodopseudomonas palustris*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides* and *Rubrivivax gelatinosus*. The use of phototrophic bacteria for sewage treatment was recently summarized by Kobayashi and Kobayashi (1995).

A number of different sources and processes have been used to produce bacterial biomass of phototrophic bacteria. Animal wastes (Ensign, 1977; Sasaki et al., 1990), soybean wastes (Sasaki et al., 1981), wheat bran (Shipman et al., 1975), municipal and industrial waste waters (Kobayashi, 1977), and clarified effluents of a biogas plant (Vrati, 1984) have been used. The produced biomass of phototrophic bacteria is a valuable source of animal feed; it is rich in vitamins and in essential and sulfur-containing amino acids (Vrati, 1984) and has been used in plankton production, in the culture of shrimp, and as food for fish and chicken (Kobayashi, 1977; Mitsui, 1979). Addition of phototrophic bacterial cells to the food increased the survival of fish as well as the production and quality of hens' eggs (Kobayashi and Tchan, 1973). With similar success the cell biomass of phototrophic bacteria has been used as fertilizer in agriculture (Kobayashi and Tchan, 1973). It may be used also for the production of hydrogen (Vrati and Verma, 1983; Bolliger et al., 1985), of biotin (Fillipi and Vennes, 1971), and of 5-aminolevulinic acid (Sasaki et al., 1990) and for other purposes.

Under nitrogen starvation, almost all phototrophic bacteria are able to produce molecular hydrogen. This process is mainly due to hydrogen evolution from nitrogenase. A large number

of substrates has been used by different research groups and with different purple nonsulfur bacteria to produce hydrogen (Kumazawa and Mitsui, 1982; Sasikala et al., 1993). Several attempts also have been made to produce hydrogen with immobilized cells of phototrophic bacteria (Francou and Vignais, 1984; Planchard et al., 1989; Vincenzini et al., 1982; von Felten et al., 1985; Weetall et al., 1981). Cells of *Rhodospirillum rubrum* (e.g., immobilized in a packed column) produced hydrogen with lactate as electron donor for 3,000 hours, with an activity loss of 60% after this time (von Felten et al., 1985). During the first days, mean rates of hydrogen production were 18–24 µl H_2 per mg dry weight and hour. Also cell-free systems were applied in hydrogen production (Mitsui, 1975). A recent comprehensive discussion of the technology of hydrogen production from phototrophic purple bacteria is given by Sasikala et al. (1993).

Poly-β-hydroxy-butyrate has been known for long as a storage product of phototrophic purple bacteria. In fact, an array of similar substances collectively termed "poly-3-hydroxyalkanoates" (PHAs) is accumulated, of which poly-3-hydroxybutyrate (PHB) is the most common. The PHAs occur as inclusion bodies of the cells visible in the light microscope and can account for a major fraction of the cell dry weight under appropriate growth conditions. Under strong nitrogen starvation or other conditions that restrict protein synthesis, excess carbon is converted into PHAs. In both *Rhodospirillum rubrum* and *Rhodobacter sphaeroides*, large amounts of storage PHAs can be synthesized under controlled growth conditions. *Rhodobacter sphaeroides* can produce more than 60% of its mass as PHAs of which 98% was PHB and the remainder was poly-3-hydroxyvalerate (Brandl et al., 1991). *Rhodospirillum rubrum* can produce a number of copolymers including an interesting tetrapolymer consisting of C_4, C_5 and C_6 repeating units (Brandl et al., 1989). A flexible polymerase which uses a variety of substrates yielding PHAs with different properties may be useful for the production of biodegradable thermoplastic polyesters of commercial value (Fuller, 1995). A copolymer of PHB and poly-3-hydroxyvalerate is commercially produced using cells of *Ralstonia eutropha*. A recent summary of aspects of biopolymer production by phototrophic purple bacteria is given by Fuller (1995).

Acknowledgement. The calculations for and the preparation of phylogenetic trees by Dr. J. Süling are kindly acknowledged.

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Prokaryotes (2006) 5:65–71
DOI: 10.1007/0-387-30745-1_3

CHAPTER 3.1.2

The Genera *Prosthecomicrobium* and *Ancalomicrobium*

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The genera *Prosthecomicrobium* and *Ancalomicrobium* are heterotrophic prosthecate bacteria, which produce numerous prosthecae per cell extending in all directions. These prosthecae vary in size and number both within and among species. Morphologically *Prosthecomicrobium* and *Ancalomicrobium* resemble one another because of their budding division and multiple prosthecae, but the former group is obligately aerobic whereas *Ancalomicrobium* strains are facultative anaerobes. Images of the type species *Prosthecomicrobium pneumaticum* and *Ancalomicrobium adetum* are shown in Figs. 1 and 2, respectively.

Taxonomy

Phylogenetic analysis of the 16S ribosomal RNA gene sequences of *P. pneumaticum*, *P. enhydrum*, *P. hirschii*, *A. adetum*, and several uncharacterized *Prosthecomicrobium*-like and *Ancalomicrobium* strains demonstrate that these organisms belong to the Alphaproteobacteria (Fig. 3). This group contains other budding and prosthecate bacteria, including representatives of the bacterial genera *Rhodomicrobium* and *Rhodopseudomonas* as well as *Hyphomicrobium* and *Caulobacter*. These findings are consistent with earlier 16S rRNA cataloguing studies on these genera (Schlesner et al., 1989).

Ancalomicrobium comprises a coherent clade within this group. Notably, *Ancalomicrobium* carries out a mixed-acid type fermentation, the products of which are identical to those of *Escherichia coli*, a member of the Gammaproteobacteria (Van Neerven and Staley, 1988). *Ancalomicrobium* is the only member of the Alphaproteobacteria currently known to have a mixed-acid fermentation.

Analyses of the 16S rRNA genes demonstrate that the genus *Prosthecomicrobium* is polyphyletic (Fig. 3) as none of the type strains of the other two species clusters with the type species, *P. pneumaticum*. Three strains, including *P. enhydrum* (ATCC 23634) and two *Prosthecomicrobium*-like bacteria, AP4.6 and P3.12, cluster with *Devosia neptuniae* (LGM 21357), a nonprosthecate organism. SCH75 is a morphologically distinctive organism whose multi-appendaged cells produce a holdfast from one polar prostheca (Schlesner et al., 1989). This representative, along with SCH127, is more closely related to members of the *Mesorhizobium* genus than to other members of *Prosthecomicrobium*. *Prosthecomicrobium hirschii* (ATCC 27832) and SCH71 form deep branches unrelated to any named genera. Finally SCH235 clusters with members of several named genera. This analysis suggests that the genus *Prosthecomicrobium* should be revised taxonomically.

Chemical analysis of phospolipids from different *Prosthecomicrobium* strains indicated that there are at least five distinct groups (Sittig and Schlesner, 1993). Four of these groups are represented in Fig. 3, with members of each group clustering in distinct phylogenetic positions. Additionally, various species and several as yet unnamed strains show little or no intrageneric DNA-DNA hybridization with one another (Moore and Staley, 1976; Schlesner et al., 1989; Chernykh et al., 1990).

To our knowledge, 16S rRNA sequencing of the remaining characterized species (Table 1) *P. litoralum* (Bauld et al., 1983), "*P. polyspheroidum*," "*P. consociatum*," and "*P. mishustinii*," (Vasilyeva and Lafitskaya, 1976; Vasileva et al., 1991) has not yet been reported and will be important in assessing the extent of diversity within the *Prosthecomicrobium* genus.

Reclassification of the bacteria that have been analyzed is required, as they do not cluster with the type species of *Prosthecomicrobium*, *P. pneumaticum*. The named species should therefore be reassigned to different genera.

Habitats

Multi-appendaged bacteria have been reported from a variety of natural habitats. Many strains have been isolated from fresh water (Staley, 1968), brackish and marine water (Bauld et al., 1983; Schlesner et al., 1989), groundwater (Hirsch

and Rades-Rohkohl, 1983), soil (Vasil'eva et al., 1974), and pulp mill aeration ponds (Stanley et al., 1979), where they may occur in high concentrations (Fig. 4). In one report (Bianchi, 1989), what appears to be a species of *Ancalomicrobium* was unexpectedly found in high concentrations in seawater used for shrimp aquaculture. Apparently the long prosthecae make these bacteria resistant to grazing by protozoan predators. Bacteria with multiple appendages have also been reported in the intestinal tracts of insects (Cruden and Markovetz, 1981), possibly because of concentration by feeding.

The multi-appendaged prosthecate bacteria appear to be well adapted for growth in oligotrophic and mesotrophic soil and aquatic habitats. Chemostat studies of *Prosthecomicrobium hirschii* indicate that their maximum growth rates are low regardless of the concentration of carbon source (Semenov and Vasileva, 1986). However, they have a high affinity for certain substrates such as glucose, as well as a low rate of respiration. These data suggest that in environments with low sugar concentrations these bacteria can metabolize efficiently because they have high-affinity uptake systems, which provide them with their selective advantage. Thus, their slow growth and low respiration rates may be advantages in oligotrophic environments where they reside (Semenov and Vasileva, 1986; Semenov and Staley, 1993).

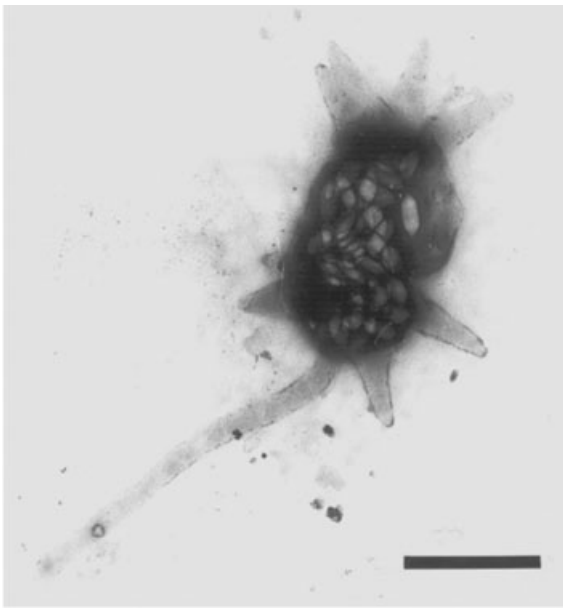

Fig. 1. *Prosthecomicrobium pneumaticum* (ATCC 23633). Transmission electron micrograph showing gas vesicles within the cell, many short prosthecae, and a single long prostheca. Bar = 1.0 μm.

Autoradiographic studies of acetate uptake in pulp mill aeration lagoons by *Ancalomicrobium* indicated that *Ancalomicrobium* was not as important as other bacteria in the metabolism of this substrate (Stanley and Staley, 1977). While their in situ uptake of sugars, which are abundant in this habitat, was not examined, it is clear that they are metabolically active in this environment.

Enrichment and Isolation

These bacteria occur in relatively low concentrations in natural habitats, typically 0.1–1 cell per ml (Staley et al., 1980), although they have been found in concentrations greater than 10^6 cells per ml in pulp mill aeration lagoons (Stanley et al., 1979). Generally, prior to isolation of pure cultures it is necessary to enrich for them. The usual procedure for enrichment entails using low-nutrient media. The most common procedure is to add a sterile peptone solution to a natural water sample to give a final concentration of 0.01% (Houwink, 1951). Schlesner et al. (1989) used a variety of carbohydrates (0.05% glucose,

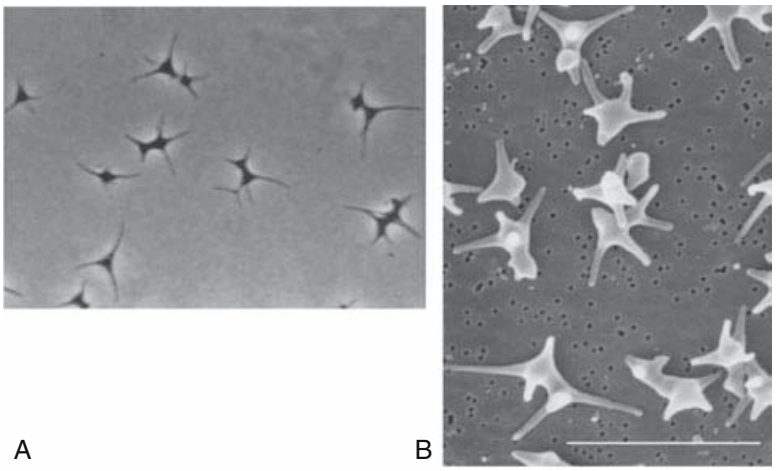

Fig. 2. *Ancalomicrobium adetum* (ATCC 23632). A) Phase contrast image of *A. adetum* cells showing long appendages and including one with a branch and B) scanning electron micrograph. Bar = 5.0 μm.

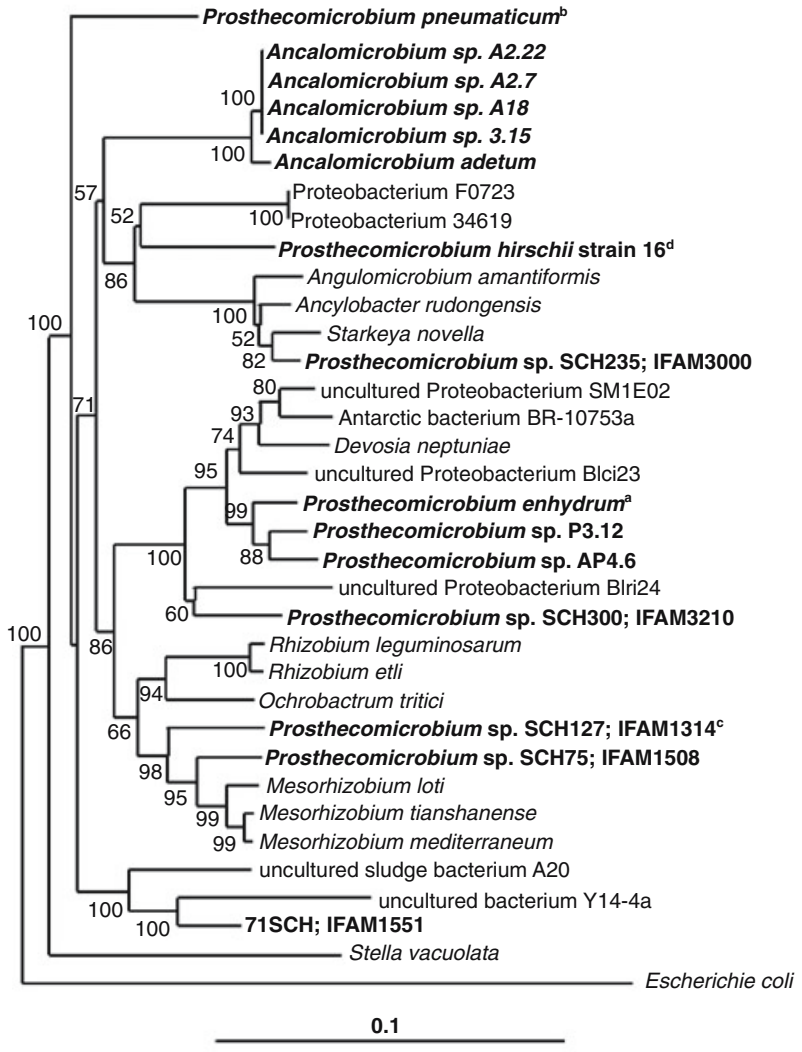

Fig. 3. Phylogeny of 16S rRNA genes from various *Prosthecomicrobium* and *Ancalomicrobium* isolates. Rooted neighbor-joining tree showing evolutionary relationships between various *Ancalomicrobium* and *Prosthecomicrobium* strains (bold). Reference sequences were picked by comparing these to 92,813 rRNA sequences in the RDP-II database using the sequence match tool (Cole et al., 2003), and complete sequences with the highest similarity to the query were used in our tree. Distances were calculated using the Kimura-2-parameter test over a total of 1137 characters. *Escherichia coli* was used as an outgroup. Percentages at nodes, with values greater than 50% shown, indicate bootstrap values for 1000 replications of the dataset; a-d indicate groups classified by their phospholipid composition as examined by Sittig and Schlesner (1993). a—group 1; b—group 2; c—group 3; and d—group 5. Bar represents the 10% 16S rDNA sequence distance.

Table 1. Species of *Ancalomicrobium* and *Prosthecomicrobium*.[a]

Species name	Type strain	Source
Ancalomicrobium adetum	4a (ATCC 23632)	Putah Creek, CA, USA
Prosthecomicrobium pneumaticum	3a (ATCC 23633)	Putah Creek, CA, USA
Prosthecomicrobium enhydrum	9b (ATCC 23634)	Putah Creek, CA, USA
Prosthecomicrobium hirschii	16 (ATCC 27832)	Pond, NC, USA
Prosthecomicrobium litoralum	524-16 (ATCC 35022)	Seawater, Puget Sound, USA
"*Prosthecomicrobium mishustinii*"	17	Soil, Colchis, Russia
"*Prosthecomicrobium consociatum*"	11	Compost, Shermetovo, Russia
"*Prosthecomicrobium polyspheroidum*"		Soil, Russia

[a]Although the species in quotation marks are in culture, their names have not been validly published.

or 0.01% lactate, or 0.1% glucosamine) or 0.25% $CaCO_3$ or 0.1% $NaNO_3$ in their enrichments.

A typical enrichment procedure is as follows: 10 mg of peptone is added to a 150-ml graduated beaker, which is covered with aluminum foil and autoclaved. Alternatively, a volume of an autoclaved peptone solution, equivalent to 10 mg, can be added to the beaker. A fresh- or marine-water sample is collected aseptically in a sterile bottle and 100 ml is transferred to one of the prepared beakers. This is then incubated at room temperature for 1–4 weeks, during which wet mounts are examined periodically using a phase microscope. When numbers of these organisms become appreciable, they may be isolated by streaking portions or spread-plating dilutions (10^{-3} to 10^{-5}) on dilute peptone agar (DPA). Additionally, these bacteria can be isolated from

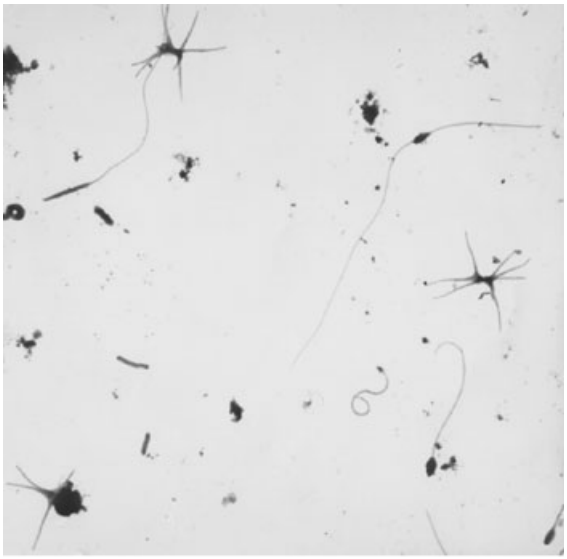

Fig. 4. Electron micrograph of a pulp mill environmental sample showing a high concentration of prosthecate bacteria.

liquid enrichment cultures by passing them through a column containing glass beads and plating eluted fractions (Staley, 1968).

Dilute Peptone Agar (DPA)

Peptone	0.1 g
Hutner's modified salts solution (see below)	20 ml
Vitamin solution (see below)	10 ml
Agar	15 g

Add distilled water up to a total of 1 liter.

Hutner's Modified Salts Solution

Nitrilotriacetic acid	10.0 g
$MgSO_4 \cdot 7H_2O$	29.7 g
$CaCl_2 \cdot 2H_2O$	3.3 g
$NaMoO_4 \cdot 2H_2O$	12.7 mg
$FeSO_4 \cdot 7H_2O$	99.0 mg
Metals "44"	50 ml

First neutralize the nitrilotriacetic acid with potassium hydroxide, then add the remaining ingredients. Adjust the pH to 7.2 with KOH and H_2SO_4. Add distilled water up to a total of 1 liter. Metals "44" contains per 100 ml: ethylene diamine tetraacetic acid, 250 mg; $ZnSO_4 \cdot 7H_2O$, 1095 mg; $FeSO_4 \cdot 7H_2O$, 500 mg; $MnSO_4 \cdot H_2O$, 154 mg; $CuSO_4 \cdot 5H_2O$, 39.2 mg; $CoCl_2 \cdot 6H_2O$, 20.3 mg; and $Na_2B_4O_7 \cdot 10H_2O$, 17.7 mg. Add a few drops of sulfuric acid to prevent precipitation before making to volume with distilled water, and store at 4°C.

Vitamin Solution

B_{12}	0.1 mg
Biotin	2 mg
Calcium pantothenate	5 mg
Folic acid	2 mg
Nicotinamide	5 mg
Pyridoxine HCl	10 mg
Riboflavin	5 mg
Thiamine HCl	5 mg

Add distilled water up to 1 liter and store at 4°C in a dark container.

The DPA plates should be incubated for about two weeks at room temperature prior to examination. Colonies are best located using a binocular dissecting microscope. The most likely colonies are small, have an entire margin, and may be a variety of colors (Table 1). Wet mounts of prospective colonies should be examined with a phase microscope, and when a presumptive colony has been located, it should be restreaked for purification. If the entire colony has been used in preparation of the wet mount, a loopful of the wet mount can be removed and used for streaking. This material may be streaked on DPA again, although a richer medium, MMB (Modified Medium B), will permit more rapid growth.

MMB Agar

Peptone	0.15 g
Yeast extract	0.15 g
Glucose	1.0 g
Ammonium sulfate	0.25 g
Hutner's modified salts solution	20 ml
Vitamin solution	10 ml
Agar	15 g

Add distilled water up to 1 liter. Adjust pH to 7.0–7.5 prior to autoclaving.

In habitats where their concentrations relative to other bacteria are much higher, isolation may be effected by streaking directly onto MMB agar. This practice has been used to isolate strains from pulp mill oxidation pond samples.

Cultivation and Maintenance

For routine cultivation and maintenance, cultures may be grown on MMB agar. Some strains tested seem to grow well on the richer and more easily prepared medium used for cultivation of caulobacters (Poindexter, 1964). This medium contains 0.2% peptone, 0.1% yeast extract, 0.02% magnesium sulfate, and 1.5% agar and is made to volume with tap water. Though higher yields may be obtained with this medium, pleomorphic forms occur with some strains. For example, *Ancalomicrobium adetum* produces high yields on this medium, but cells may form short chains and mini cells with one or two prosthecae. Alternatively a more concentrated version of MMB, termed "Super" MMB, has been found to work well for cultivation of *Ancalomicrobium* strains (Van Neerven and Staley, 1988).

These bacteria can also be grown on a defined medium containing ammonium sulfate as sole

nitrogen source and glucose as sole carbon source. The composition of this medium is as follows:

Defined Medium

Ammonium sulfate	0.25 g
Disodium phosphate	0.0005 M
Glucose	0.25 g
Hutner's modified salts solution	20 ml
Vitamin solution	10 ml

Add distilled water up to a total of 1 liter. For solid medium, add 1.5% agar.

This medium has been useful in ascertaining vitamin requirements using the single-deletion method (Staley, 1968). It has also been of limited use in determining carbon source utilization patterns, although MMB from which glucose has been deleted is generally more useful for that purpose.

Slant cultures maintain viability for at least one month at refrigerator temperatures. Lyophilization is satisfactory for longer periods of preservation. Frozen cultures made using 15% (w/v) glycerol and stored at –70°C have proven to be viable for at least 4 years. Storage of glycerol stocks at –20°C is not recommended.

Identification

Table 2 shows some of the major differences among the four validated and two nonvalidated species of *Prosthecomicrobium* and one species of *Ancalomicrobium* that have been described at this time.

The first step in determining whether an unidentified bacterium is a representative of one of these genera involves examination of a wet mount of a suspected colony under a phase microscope using an oil-immersion objective. Prosthecae on most strains can be detected by ordinary phase microscopy, but if there is any uncertainty, whole cells of the suspect strain must be examined using a transmission electron microscope (TEM). Most species of *Prosthecomicrobium* produce relatively short appendages (i.e., <1.0 μm) under normal growth conditions, but these can readily be seen by TEM. Following initial isolation and preliminary identification on the basis of aerobic growth and morphology, 16S rRNA gene sequencing and phylogenetic analysis may be helpful in the identification of isolates.

Once an isolate has been identified as a member of this group, it should be streaked for purification on MMB medium or PYG medium. To differentiate between the two genera, it is essential to conduct a test to determine whether the strains are able to grow as facultative anaerobes. This can be done on the Hugh-Leifson medium (Hugh and Leifson, 1953), using glucose as a carbon source.

Ancalomicrobium strains are facultative anaerobes whereas *Prosthecomicrobium* are obligate aerobes (Table 1). *Ancalomicrobium* cells have long appendages (Figs. 2a and 2b) when grown in dilute media such as MMB. *Ancalomicrobium adetum* also produces gas vacuoles under certain conditions such as late stationary growth phase. One species of *Prosth-*

Table 2. Characteristics of *Prosthecomicrobium* and one species of *Ancalomicrobium*.

Characteristic	*A. adetum*	*P. enhydrum*	*P. pneumaticum*	*P. litoralum*	*P. hirschii*	*P. polyspheroidum*	*P. consociatum*	*P. mushustinii*
Appendage length (μm)	ca. 2–3	0.5	<1	<1	<1–3	<0.5	<0.25	<0.65
Motility	–	+	–	–	+	+	–	–
Gas vacuoles	+	–	+	–	–	–	–	–
Anaerobic growth	+	–	–	–	–	–	–	–
Optimum salinity of 25%	–	–	–	+	–	–	–	–
Carbon source utilization								
Pyruvate	+	+	–	+	+	–	–	±
Sorbitol	+	–	+	+	–	±	–	–
Maltose	+	+	+	+	–	+	–	+
Lactose	+	+	+	+	–	+	–	+
Rhamnose	+	+	+	+	–	–	±	±
Methanol	–	–	–	–	+	–	±	–
Mol% G+C	70	66	69–70	66–67	68–70	64–67	66–68	64–65
Colony color	White	White/yellow/red	White	White	Pink	White	Gray	Yellow-orange

Symbols: +, present; –, absent; and ±, present occasionally.

ecomicrobium, *P. pneumaticum*, also produces gas vacuoles. All species of *Prosthecomicrobium* reported to date produce cells with short appendages. However, some species of *Prosthecomicrobium*, such as *P. hirschii*, also produce long appendages, so the morphology of individual cells cannot be used for identification.

Prosthecomicrobium cells typically have short prosthecae, although, as mentioned previously, *P. hirschii* also produces long prosthecae as well. Only one species known to date, the type species *P. pneumaticum*, produces gas vesicles (Fig. 1). It is nonmotile.

Two members of *Prosthecomicrobium* are readily identified by their cellular morphology. *Prosthecomicrobium hirschii* has a dimorphic life cycle consisting of a short-appendaged prosthecate cell and a long-appendaged prosthecate cell (Fig. 5). Curiously the dimorphic lifecycle is not like *Caulobacter* spp. in which one cell type must change into another cell type. Instead, either the short-appendaged, motile, or long-appendaged, nonmotile, cell type can give rise to progeny of either cell type (Staley, 1984). The presence of a dimorphic population of cells is a reliable characteristic for classification of an isolate as *P. hirschii*.

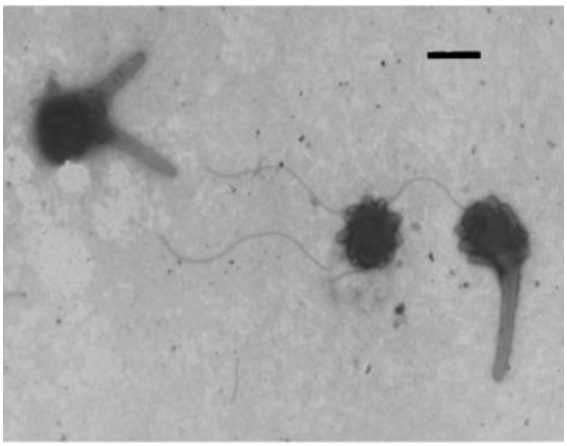

Fig. 5. *Prosthecomicrobium hirschii*. Electron micrograph showing the two typical morphologies of *P. hirschii* cells. Nonmotile cells lacking flagella with long appendages, and motile cells with a single polar flagellum and shorter appendages. Bar = 1.0 µm.

"*Prosthecomicrobium polyspheroidum*" is a long (>3 µm), rod-shaped bacterium with evenly spaced rows of short appendages giving it a "corn cob" appearance. This species as well as "*P. mishustinii*" and "*P. consociatum*" is not yet on the approved lists of bacterial species.

The remaining named species of *Prosthecomicrobium* are similar in morphology. *Prosthecomicrobium enhydrum* is motile and produces short appendages (Fig. 6). *Prosthecomicrobium litoralum* is a nonmotile, short-appendaged organism that inhabits brackish water and therefore has a requirement for moderate salinity. "*Prosthecomicrobium mishustinii*" is nonmotile, with moderately sized appendages. "*Prosthecomicrobium consociatum*" is nonmotile, with short appendages. All of these strains show some variability in the number and length of prosthecae (Fig. 6b).

Morphologically these bacteria resemble green sulfur phototrophic bacteria of the genera *Prosthecochloris* and *Ancalochloris.* Also, *Prosthecomicrobium* spp. resemble another organism, *Verrucomicrobium spinosum*, which has pili at the tips of its prosthecae (Schlesner, 1987). This organism, which has been placed in the genus *Verrucomicrobium*, falls into a completely different 16S rRNA group (Albrecht et al., 1987). Spinate bacteria superficially resemble these bacteria, but their noncellular appendages have many transverse striations and consist entirely of protein (Easterbrook and Coombs, 1976a; Easterbrook et al., 1976b).

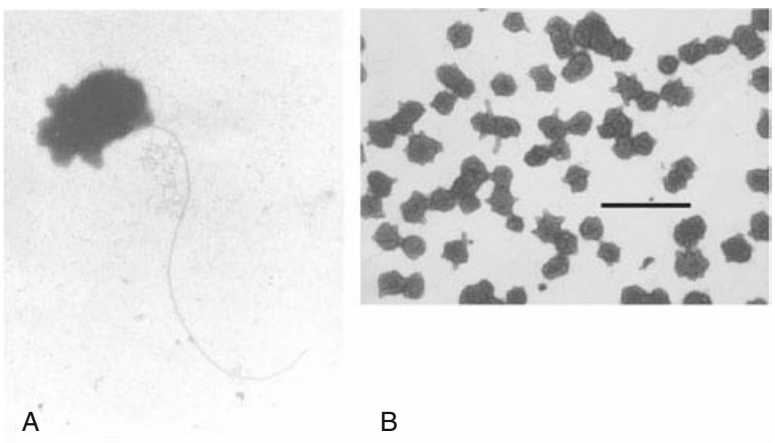

Fig. 6. *Prosthecomicrobium enhydrum*. A) Electron micrograph showing a single cell with a single subpolar flagellum, and B) electron micrograph of a field of *P. enhydrum* cells showing the variability in prosthecae length. Bar = 5.0 µm.

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Prokaryotes (2006) 5:72–90
DOI: 10.1007/0-387-30745-1_4

CHAPTER 3.1.3

Dimorphic Prosthecate Bacteria: The Genera *Caulobacter*, *Asticcacaulis*, *Hyphomicrobium*, *Pedomicrobium*, *Hyphomonas* and *Thiodendron*

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Rationale for Clustering *Caulobacter*, *Asticcacaulis*, *Hyphomicrobium*, *Pedomicrobium*, *Hyphomonas* and *Thiodendron*

The genera treated together here comprise the *dimorphic prosthecate bacteria (DPB)*, in which reproduction regularly results in the separation of two cells that are morphologically and behaviorally different from each other (Fig. 1). One sibling is nonmotile and prosthecate, possessing at least one elongated, cylindrical appendage that is an outgrowth of the cell surface, including the outer membrane, the peptidoglycan layer, and the cell membrane, and that may also include cytoplasmic elements such as ribosomes; such an appendage is a *prostheca* (Staley, 1968). In natural populations, this prosthecate cell is usually also sessile by virtue of adhesive material associated with a cell pole or with the prostheca. The other sibling is flagellated, bearing (typically) one polar or subpolar flagellum, by means of which it is actively motile. This mode of reproduction is unique as a regular feature of a prokaryote reproductive cycle. It is regarded here as a reflection of an ecologic program common to these genera, viz., as a means of dispersing the population at each generation and thereby minimizing competition between siblings for resources.

In bacteria lacking motility, sibling separation in space depends on abiotic forces; if both are sessile, clones accumulate as cell clusters. In most motile unicellular bacteria, both siblings are motile; if they are also chemotactic, both cells will tend to follow the same gradients and so travel together. In the DPB, spatial separation is promoted by the motility of one cell and the immobility of its sibling.

Other prosthecate bacteria differ from those of the genera treated in this chapter either by lacking a motile stage (*Prosthecobacter*, *Filomicrobium*, *Ancalomicrobium*) or by producing motile cells only under certain environmental conditions (*Prosthecomicrobium*, *Rhodomicrobium*). *Thiodendron* is included in the DPB cluster even though its habit has so far been surmised only from polytypic populations in natural samples or enrichments; it bears one or two appendages that appear to be prosthecae, has been seen to form buds on those appendages, and is accompanied by motile cells that may be its own swarmers.*

Throughout the eukaryotic world, there are parallels for the asymmetric cell reproduction that is characteristic of the DPB, but very often with the consequence that only one of the two siblings continues to reproduce while the other differentiates and exhibits diminished or lost potential for subsequent reproduction. More relevant parallels are found at the organismic level, such as among fungi and invertebrate animals that are not freely mobile as adults but produce motile offspring or plants that produce propagules equipped for travel; the analogy to eukaryotes underlies the usage of the terms "mother" and "daughter" to refer to the nonmotile and motile siblings, respectively, of the DPB.

The parallel with dispersal units seems particularly apt as evidence accumulates that the motile siblings of DPB are in an analogously juvenile condition (Krasil'nikov and Belyaev, 1970; Matzen and Hirsch, 1982b; Moore, 1981; Moore and Brubaker, 1976; Moore and Hirsch, 1973; Morgan and Dow, 1985; Pate et al., 1973; Poindexter, 1964, 1981a; Swoboda and Dow, 1979; Wali et al., 1980). In each species so far studied, the motile cell is less actively growing than its prosthecate sibling; in *Asticcacaulis* and *Hyphomicrobium*, the swarmer is also typically considerably smaller than its prosthecate sibling.

This chapter was taken unchanged from the second edition.

* *Thiodendron* isolates have not been reported. The organism is known only from natural samples in which it has been interpreted as a sulfur-oxidizing prosthecate bacterium, probably with a motile stage. The absence of information regarding properties of monotypic populations does not justify a discussion beyond the excellent summary by Schmidt (1981).

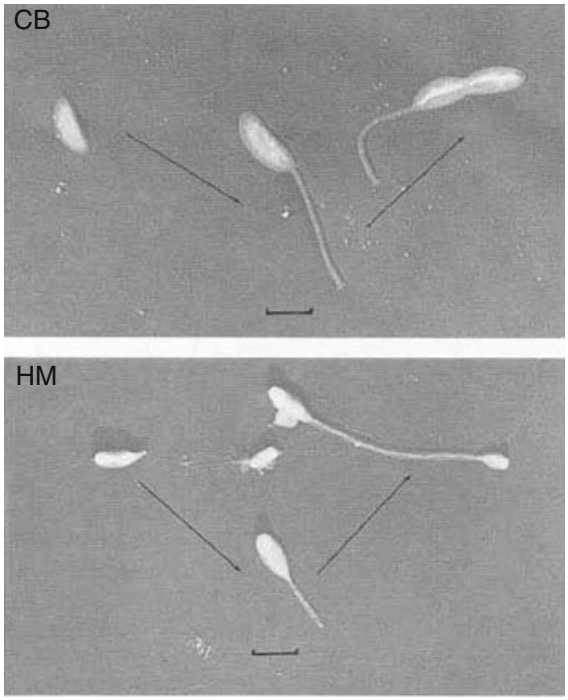

Fig. 1. Caulobacters (CB) and hyphomicrobia (HM). Left to right in each electron micrograph: swarmer, prosthecate cell, and reproducing cell. Transmission electron micrographs, Pt-shadowed cells. Bar = 1 μm.

Growth and progress toward reproduction are initiated in these swarmers only after a period in which their most evident activity is motility. The regulatory mechanisms by which growth and development are postponed have not been elucidated, although lipid, phosphorus, and nitrogen metabolism have been implicated as possibly influential metabolic processes (Chiaverotti et al., 1981; Emata and Weiner, 1983; O'Neill and Bender, 1989; Poindexter, 1984a; Mansour et al., 1980). Attachment is not necessary for the onset of development and growth, but presumably usually occurs prior to development in natural habitats. Although both swarmers and prosthecate cells are adhesive, attachment is probably initiated mainly in the swarmer stage (Hirsch, 1974; Leifson, 1962; Moore and Marshall, 1981; Zavarzin, 1961), since: 1) prosthecate cells are typically found attached, not afloat (although that may be because that is where they *can* be seen); 2) if pili are present in a strain, they occur predominantly or exclusively on swarmers (Schmidt, 1966; Umbreit and Pate, 1978); and 3) in laboratory-cultivated populations, the swarmer is demonstrably more active in initiating attachment (Newton, 1972). Moore and Marshall (1981) have suggested that, like pili, flagella may serve as a means of carrying adhesive material through the electrostatic barrier between cell and substratum, thereby aiding the establishment of stable contact (see Planktonic Versus Sessile Life of Prokaryotes in the second edition).

Thus, like the eukaryotes in which immotile organisms produce motile or transportable offspring, dispersal of DPB populations is the most reasonable interpretation of the major advantage of mobility in the juvenile stage. By fission or by budding, each normal reproductive event in these bacteria produces two siblings: one to grow and one to go. It is consistent with this developmental and reproductive habit that these bacteria exhibit the physiologic properties of oligotrophs (Poindexter, 1981b), most importantly: *tolerance of prolonged nutrient scarcity.* This is the principal physiologic property that can be exploited in their enrichment and isolation and is reflected in their distribution in nature. Once isolated, individual strains may tolerate much higher nutrient concentrations than are useful in their enrichment and isolation, but most isolates exhibit either growth inhibition or loss of control over cell form ("pleiomorphy") upon incubation in media designed for the cultivation of bacteria parasitic on plants or animals (see references in "Cultivation," this chapter). Such nutrient-rich media are generally suitable for the cultivation of copiotrophs, but morphology of those few DPB that are able to grow on such media may be so aberrant in such cultures that they may not be recognizable as DPB.

When cultivated under conditions that allow uniform dimorphic morphology and cell size from one generation to the next, cultivated DPB are morphologically identical to bacteria observed in natural materials. On the basis of the morphology of the reproductive stage, two fundamentally different types of DPB can be distinguished in natural samples, enrichment cultures, and pure cultures: caulobacters (*Caulobacter and Asticcacaulis*) and hyphomicrobia (*Hyphomicrobium*, *Pedomicrobium*, and *Hyphomonas*). Caulobacters reproduce by fission and hyphomicrobia by budding, almost always from the distal tips of prosthecae (hence the usage of the term "hyphae"). *Caulobacter* and *Asticcacaulis* can be distinguished from each other by the site of adhesion of sessile cells to substrata; *Caulobacter* cells adhere by the distal tip of the prostheca (hence "stalk"), while *Asticcacaulis* cells adhere by the cell pole and the prostheca is not adhesive. Among the hyphomicrobia, *Pedomicrobium* cells are distinguished morphologically from those of the other two genera by the production of buds whose long axes are perpendicular to the long axis of the hypha, rather than an extension of the hyphal axis; they also tend to produce more than one hypha per cell, but multiple prosthecae occur in stressed and aging populations of all DPB and are not a dependable morphological trait, particularly in

natural populations. Adhesiveness of a cell pole is typical of hyphomicrobia, but it is not a universal property of isolates under all conditions. The hyphae are not adhesive in any of the budding genera. The reverse orientation of sessile cells is helpful in distinguishing *Caulobacter* from hyphomicrobial cells in natural samples.

In addition to their developmental and reproductive habit and fundamentally oligotrophic physiology, these genera exhibit several other common properties that can be characterized only with pure populations. These properties are not unique to this group; on the contrary, they imply relationships to other eubacterial groups, particularly to pseudomonads, with which they often share their natural habitats. All are oxybiontic and grow in well aerated cultures; a tolerance of low pO_2 is exhibited by *Pedomicrobium* isolates, and a preference for such conditions is seen in *A. biprosthecum*. They seem universally cytochrome-positive and catalase-positive, although there are exceptional reports (e.g., Lapteva, 1977, 1987) of negative tests. Only one species (*C. crescentus*) has been examined for superoxide dismutase (SOD) activity; like certain *Pseudomonas* spp., *C. crescentus* possesses two SODs (Steinman, 1982). The Fe-SOD appears to be cytoplasmic, while the CuZn-SOD is periplasmic (Steinman and Ely, 1990). Unequivocal anaerobic growth is sustained only by *Hyphomicrobium* isolates, by dissimilatory respiration of nitrate to N_2.

All DPB are chemoheterotrophic, and variations in carbon-source preference are useful traits in generic and species differentiation. Ammonium ions are suitable as the sole source of nitrogen for isolates that can be cultivated in defined media; ammonia is assimilated by addition to glutamate or by reductive amination of pyruvate and glyoxylate (Doronina, 1985; Ely et al., 1978). Only *Hyphomicrobium* isolates have been reported able to use nitrate as the sole nitrogen source. In complex media, peptone is universally suitable as a nitrogen source. Poly-β-hydroxybutyrate (PHB) and polyphosphate are stored as reserves and may be detectable microscopically in cells in natural samples.

DPB possess outer membranes, and growing cells stain Gram-negatively. The outer membranes of the genera that have been characterized (*Asticcacaulis* [Jordan et al., 1974]; *Caulobacter* [Agabian and Unger, 1978]; *Hyphomonas* [Dagasan and Weiner, 1986; Shen et al., 1989]) exhibit a higher proportion of high-molecular-weight proteins than do those of other Gram-negative bacteria. Superficial layers of repeating protein subunits ("R-layers") are known in *Caulobacter* (Smit et al., 1981) and possibly in *Hyphomonas* (Dagasan and Weiner, 1986), in which two proteins account for more than half of the outer membrane proteins.

Most isolates are susceptible to antibiotics that inhibit prokaryotic protein synthesis (aminoglycosides, macrolides, tetracyclines), but may be resistant to synthetic antimicrobials such as sulfonamides; high concentrations of quinolones are required for inhibition of growth (J. S. Poindexter, unpublished observations). Any type of inhibitor, including mitomycin C, hydroxyurea, and cell wall synthesis inhibitors, may cause developmental aberrations (Haars and Schmidt, 1974; Koyasu et al., 1983; Moore and Brubaker, 1976; Moore and Duxbury, 1981; Weiner and Blackman, 1973).

Neutral to slightly alkaline reaction is optimal for growth, except for *Pedomicrobium* isolates, which grow well at pH 9.0. Moderate temperatures (20 to 30°C) are also generally optimal, but many isolates grow well at temperatures as low as 5°C, at least as primary isolates, and a few grow at temperatures above 35°C. Many isolates require vitamins, and growth is generally stimulated by B vitamins. When only one vitamin is required, it is biotin or B_{12}, while riboflavin and Ca-pantothenate are stimulatory for certain isolates.

The DNA base composition of bacteria of these genera is in the range of 59 to 67 mol% of GC, except in *A. excentricus* (55%) and *Hyphomonas hirschiana* (57%). RNA sequence analysis has revealed a closer relationship to pseudomonads than to other bacterial groups, although the relationship is "remote" (Stackebrandt et al., 1988); the presence of intracellular nucleases appears to interfere with the application of this analytic technique to phylogenetic interpretation of *Caulobacter* and *Hyphomonas*. Extensive studies of DNA-DNA hybridization have revealed little sequence similarity within the group, or even within a genus, except among isolates derived from the same initial sample, among variants of a single strain, or ~~in~~ only a few instances ~~among~~ isolates of clear phenotypic similarity (Gebers et al., 1984, 1985, 1986; Moore et al., 1978). Lack of DNA-DNA hybridization is not predictive of similarities among other molecules, such as outer-membrane proteins (Dagasan and Weiner, 1986). In the DPB, naturally-occurring plasmids have been detected only among caulobacters (Anast and Smit, 1988; Schoenlein and Ely, 1983). However, pseudomonad plasmids such as RP4 can be propagated and expressed in both *Caulobacter* and *Hyphomicrobium* (Anast and Smit, 1988; Chatterjee and Chatterjee, 1987; Dijkhuisen et al., 1984; Ely, 1979).

Possibly as an aspect of their oligotrophic nature, in the sense that they are not adapted to continual, well-supported growth, continuous

vegetative propagation of DPB results in diversification. This has been a persistent problem with all the genera and is perhaps best exemplified by Mevius' strain B; of the seven strains listed as *Hyphomicrobium aestuarii* in *Bergey's Manual* (Hirsch, 1989), six (MEV-533, MEV-533Gr, EA-617, EN-616, NQ-521 and NQ-528) are laboratory derivatives of one original isolate (Mevius, 1953). Most variations are detected as changes in colony morphology or texture and are presumably cell surface composition variants. This kind of change often occurs early in the purification of an isolate, especially of *Hyphomonas* (Moore et al., 1984). Pongratz (1957) reported one isolate of *H. polymorpha* as PR727 (rough) and PS728 (smooth); all five *Hyphomonas* isolates from the deep-sea vent community were all originally "rough," but two are now described as smooth (Moore and Weiner, 1989) and all have smooth variants (J. S. Poindexter, unpublished observations). Deposition of metal oxides is a trait that may be lost by initially positive isolates (Gregory and Staley, 1982; Tyler and Marshall, 1967a), reducing the usefulness of this characteristic in differentiating genera and species. Temperate bacteriophages also appear in long-cultivated strains (Driggers and Schmidt, 1970; Gliesche et al., 1988; Schmidt and Stanier, 1966), suggesting genetic perturbations during perpetual cultivation.

Despite this long list of similarities, there is no reason at present to regard this set of genera as a phylogenetically-related cluster. It is a group distinguished by its reproductive habit, which seems related to its ecology; its common ecologic program should be of sufficient advantage to have evolved more than once. The physiologic similarities arise largely from the oligotrophic nature of these organisms, which is consistent with a growth habit that promotes dispersal of successive generations.

Taxonomy by nucleic acid composition and sequence similarity has not resulted in a suggestion that these genera should be redistributed among bacterial groups differently from the placement inferred from traditional dependence on Gram-reaction, flagellation, cell morphology, nutrition, cytochrome content, and natural distribution. However, it has so far provided only scant evidence that they should be grouped together. Determination of rRNA cistron similarities revealed affinities among *Hyphomicrobium*, *Hyphomonas*, and *Caulobacter*, as well as between *Hyphomicrobium* and purple bacteria and nonmotile prosthecate bacteria (Moore, 1977). Similarities of rRNA sequences have also been detected among *Hyphomicrobium*, *Pedomicrobium*, and *Filomicrobium* (the last, a genus of nonmotile prosthecate bacteria), although this group appears to comprise a previously unrecognized fourth subgroup of the "alpha" subdivision of purple non-sulfur bacteria (Stackebrandt et al., 1988). Although presently inferred phylogenetic distances within this group seem great, they are smaller than the distance from any non-prosthecate bacteria.

Accordingly, aside from the possibility that the regular production of a motile stage is a late modification or a dispensable property among prosthecate bacteria, it seems appropriate at present to recognize the DPB as a cluster of genera that comprise a probably related subgroup of heterotrophic pseudomonads specialized for distribution in nature as uncrowded, oligotrophic populations.

Distribution of Dimorphic Prosthecate Bacteria

A single term can be used to describe the occurrence of DPB in waters: ubiquitous. This term applies without qualification to *Caulobacter* and is almost as appropriate for hyphomicrobia, as evidenced not just by the frequency of the detection of these two types within natural populations, but also by the relative ease of their enrichment and (with proper attention to their particular physiological traits) isolation. Lapteva (1987) has suggested that caulobacters, as typical aquatic bacteria, should be regarded as probably second only to pseudomonads in breadth of distribution and numbers. These two bacterial groups together may be responsible for the bulk of mineralization of dissolved organic material in aquatic environments, with caulobacters being especially important when nutrient concentrations and ambient temperatures are low (Allen, 1971; Staley et al., 1987).

When detected in unenriched samples, DPB are not found as crowds; even when total microbial density is high, DPB are usually sparse—with notable exceptions such as *Hyphomonas*-like organisms in some deep-sea hydrothermal vent samples (Jannasch and Wirsen, 1981) and *Pedomicrobium* in manganese concretions (Ghiorse and Hirsch, 1982; Marshall, 1980; Sly et al., 1988; Tyler and Marshall, 1967a, 1967b). Similarly, even when DPB are practically alone, as when total microbial counts are low or when they colonize submerged surfaces, they do not exploit the space by filling it with their progeny. Quantitative studies of their occurrence by direct plating, dilution for most-probable-number (MPN) calculation, or direct microscopic enumeration by scanning electron microscopy reveal population densities of 10^3 to 10^5 per ml of water or per gram of soil, detritus, or sediment; 10^6 DPB per such a unit, as reported by Lapteva (1987), is an exceptionally high density. By themselves, the

DPB have not been found responsible for turbidity of natural waters, nor are they detectable by sample odor as are, for example, sulfate-reducing bacteria and streptomycetes; microscopical examination of samples is required for their detection. The only macroscopic evidence of their possible presence is metal (Fe or Mn) oxide deposits on wet surfaces, but whether they cause or just adhere to such deposits remains to be established. Accordingly, "abundant" in referring to these organisms must be read as meaning 10^3 or more per ml or gram, "present" to mean detectable (usually microscopically and often only after enrichment), and "predominant" as relatively unaccompanied, but not necessarily "abundant."

Asticcacaulis is rarely sighted and even more rarely isolated. *Pedomicrobium* distribution is probably wider than evidence currently suggests, since a suitable method for enrichment and isolation of this type of hyphomicrobium has been developed only recently. *Hyphomonas* may be a type restricted to marine environments; its morphologic twin, *Hyphomicrobium*, is found in brackish waters (as well as soils and fresh water), but not marine sources. Distribution is discussed here principally with reference to *Caulobacter* and *Hyphomicrobium*.

Practically any type of seawater from harbors, estuaries, the open ocean, deep-sea hydrothermal vents, storage reservoirs contains *Caulobacter* and may also contain hyphomicrobia, probably *Hyphomonas*. *Caulobacter* may be the predominant form of aerobic chemoheterotrophic bacteria in oceanic samples (Jannasch and Jones, 1960), particularly in Antarctic waters (Takii et al., 1986; Waguri, 1976). DPB also occur in sediments, particularly if algal and/or plant material (including wood) has settled onto the bottom (Austin et al., 1979). DPB are typically found attached to submerged surfaces, whether or not they are detectable in bulk water, and so they settle with such materials.

They are also present in freshwater ponds, lakes, streams, rivers, and reservoirs (reviewed in Poindexter, 1981a), even temporary pools and puddles (Gebers, 1981; Masuda, 1957); in canals and lagoons of various trophic states (Lapteva, 1977, 1987; Staley et al., 1980, 1987; Stanley et al., 1979); in pipelines and water distribution systems, including domestic tap water; in wells (Masuda, 1957; Shah and Bhat, 1968); in home aquaria; in bottled spring water (Gonzalez et al., 1987); and in sewage. In short, if a site has been wet for any length of time, DPB will be present.

In this regard, it should be noted that the bulk of the carbon of some organic-rich sites in nature is insoluble, including plant litter and partly-treated sewage and activated sludge, as well as sediments, soils, and waters polluted with industrial wastes to the point of loss of translucency that is not due to microbial blooms. Such sites may support the growth of oligotrophic bacteria such as the DPB despite their "richness." Polymer-degrading bacteria such as cytophages are typical of such sites; presumably, the DPB subsist on soluble nutrients that are slowly released from insoluble organic materials by such bacteria.

Detection of DPB in sewage is sporadic, although they are known to occur in the highly aerobic type of sewage treatment known as activated sludge (Hartmans et al., 1986; Schmider and Ottow, 1986; Vedinina and Govorukhina, 1988), and phages lytic for DPB can be isolated from sewage effluents (Gliesche et al., 1988; Schmidt and Stanier, 1965). In anaerobic sewage treatment processes, only *Hyphomicrobium* appears to occur commonly, and then specifically in sewage carrying a high nitrate load. *Hyphomicrobium* is strongly favored in such sewage by the addition of methanol, which accelerates the removal of nitrate as N_2 as it selectively favors *Hyphomicrobium* multiplication (Claus and Kutzner, 1985).

Seasonal fluctuations have been characterized only for freshwater caulobacters; other DPB are not sufficiently abundant to allow meaningful quantitative determination of seasonal changes. Lapteva (1977, 1987) observed that caulobacters fluctuate with phytoplankton. Both types of aquatic organisms rise numerically in the springtime, may or may not decline (or descend in the water) during the summer, but definitely do so during the winter. They rise together again (in number and in location in the water, such as a lake) in the springtime, a pattern also noted by Staley et al. 1980. It seems likely that caulobacters respond readily to the release, in early spring, of nutrients immobilized during the winter and released abiotically during "spring turnover." They persist through the summer, but at that season are accompanied by a diversity of later-blooming bacteria.

The only clear limitation on the geographic distribution of DPB in fresh water is temperature; they have not been found in thermal springs.

DPB are found in soils, particularly from nutrient-poor soils such as "podzols."* They are also found in leaf-litter, in frequently-wetted agricultural and lawn soils (Belyaev, 1968; J. S. Poindexter, unpublished observations), and in metal ore deposits (Groudev et al., 1978).

* Podzol: a type of relatively infertile soil found typically in forests and consisting of a thin, ash-colored layer overlying a brown, acidic humus, the organic part of soil, resulting from the partial decay of leaves and other vegetable matter.

DPB are also found in the laboratory: *Hyphomicrobium* in enrichment cultures for nitrifying bacteria (Kingma Boltjes, 1936) and methane-oxidizers (Wilkinson and Hamer, 1972); *Caulobacter* in stock algal cultures (Bunt, 1961; Gromov, 1964; Klaveness, 1982; Li et al., 1984; Zavarzina, 1961); and both in enrichment cultures for N_2-fixing aerobes. Both *Caulobacter* and *Hyphomicrobium* can be isolated from laboratory water baths and distilled water (Callerio et al., 1983; reviewed in Poindexter, 1964). *Hyphomicrobium* is especially easy to detect and to isolate from any appended rubber tubing or plastic outlet of a distilled water reservoir, and *Caulobacter* is isolated from the outlets, the walls of the container, and the air-water interface.

With very few exceptions (*Hyphomonas polymorpha*, isolated from human nasal secretions [Pongratz, 1957]; *C. leidyi*, isolated from millipede hindguts [Poindexter, 1964]; and hyphomicrobia from turbot gills [Mudarris and Austin, 1988]), DPB are not detected in nor isolated from association with animals or decaying animal materials. They have not been reported as agents of food spoilage and are not pathogenic for laboratory animals~~ev~~en those that can persist long enough to stimulate an immune response (Famurewa et al., 1983).

In both soils and waters, DPB (and *Prosthecobacter*; see The Genera Prostheomicrobium, Ancalomicrobium and Prosthecobacter) are especially likely to be encountered as algal epibionts; examining the surfaces of algal thalli is often the most dependable way to detect DPB in a natural sample. Caulobacters are usually attached to algal structures, while hyphomicrobia are more often detected within algal jelly (Geitler, 1965; Hirsch, 1974). These associations are best observed by scanning electron microscopy (SEM) (Fig. 2). In living specimens examined by phase contrast microscopy, they are more difficult to discern; the phase halo of the relatively large algal cells interferes with the visibility of the stalks, while the *Caulobacter* cells may be overlooked waving on their stalks several micrometers away from the algal surface. Even in SEM images, very long stalks may not be traceable to their cells (Fig. 2; see also Tufail, 1987).

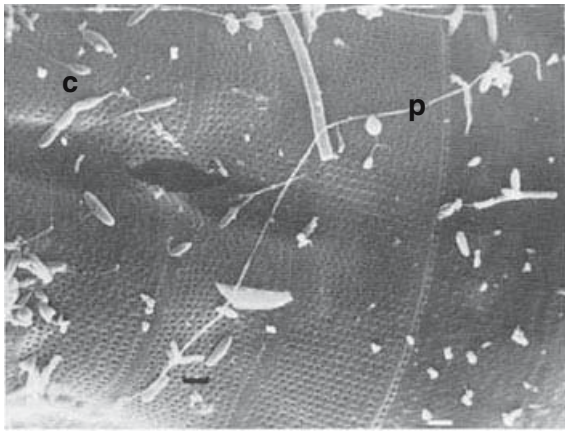

Fig. 2. Prosthecae (p) and prosthecate cells (c) attached to the filamentous diatom *Melosira.* Scanning electron micrograph. Bar = 1 µm.

As algal epibionts, DPB exhibit some preference for association with diatoms (Anderson and Poindexter, 1984; Nemec and Bystricky, 1962; Tufail, 1987). This may reflect any of: a preference of the caulobacters, at least, for siliceous surfaces (glass, sand grains, diatom frustules); strictly spatial coincidence, since diatoms are themselves common as epibionts of other algae and of plants; and environmental factors that favor, in common, the multiplication of these two types of organisms. In the laboratory, when mixed with an algal population, caulobacters attach readily to both phototrophic and heterotrophic diatoms and to algae with nonsiliceous walls (Fig. 3). In natural habitats, caulobacter numbers fluctuate in parallel with phytoplankton in general (Lapteva, 1987; Staley et al., 1987) and they are likely to be found on every kind of alga and on submerged plants as well (Kudryavtsev, 1978). Among the DPB, caulobacters in particular can be isolated from both natural and cultivated algal populations: from soil (Gromov, 1964), water (Gromov, 1964; Lapteva, 1987), and cultures (of *Nostoc* [Bunt, 1961], *Chlorella* [Zavarzina, 1961], *Phormidium, Tribonema, Chlorella,* and *Chlorococcum* [Gromov, 1964], *Anabaena* [Li et al., 1984], and *Cryptomonas* [Klaveness, 1982]).

The principal advantage of attachment to algae is presumed to be an immediate source of soluble organic substances in the algal exudate. During illumination, cyanobacteria and algae also release O_2, an environmental factor found by Lapteva (1987) to be a major positive influence on the occurrence of caulobacter populations. However, O_2 is also potentially toxic, and it has been suggested (Steinman and Eley, 1990) that algal association may have selected for the evolution in caulobacters of a periplasmic SOD in addition to their cytoplasmic SOD (see Steinman, 1982). Since O_2 can be especially damaging to phototrophs, epibiontic heterotrophs such as caulobacters may serve to consume sufficient O_2 to reduce the pO_2 in the immediate environment of photosynthetically active cells (Lupton and Marshall, 1981; Staley, 1971; Stanley et al., 1979), as well as to cycle carbon immediately (algal exudate to CO_2 to alga) through their oxidative metabolism (Allen, 1971). Such considerations suggest mutually beneficial association, not just coincidence.

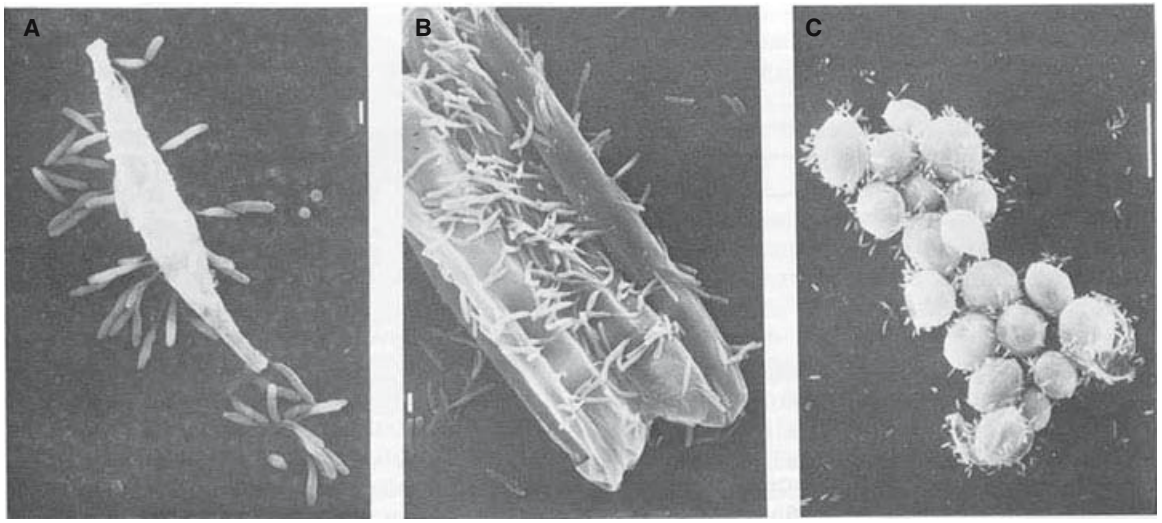

Fig. 3. Two-membered cultures of a marine caulobacter (VC13) attached to: (A) *Phaeodactylum tricornutum,* a phototrophic diatom; (B) *Nitschia* sp., a heterotrophic diatom; and (C) *Oöcystis* sp., a phototrophic green alga. Scanning electron micrographs by K. L. Anderson. Bar = (A and B) 1 μm; (C) 10 μm.

Enrichment and Isolation of Dimorphic Prosthecate Bacteria

Principles and General Procedure

The density of DPB in any natural sample will not be high and typically will be much lower than 10^6 per ml of water or per gram of solid material. Nevertheless, their tolerance of prolonged nutrient scarcity provides a dependable physiological basis for their enrichment. Successful enrichments from water, and from soil suspended in water, result when the sample is allowed to stand undisturbed for one to several weeks. Samples have often proved useful when stored for months and even years, and long-undisturbed samples can be regarded as pre-incubated enrichments.

Nevertheless, it is often more practical to promote microbial multiplication by supplementing the sample with organic nutrients; peptone added to 0.001 to 0.005% (w/v) is a suitable supplement, possibly indicating that microbial development in many natural samples is nitrogen-limited.

Periodic examination, by phase contrast microscopy, of the thin surface film (lifted with a loop, a slide, or a cover slip~~not~~ with a pipet), of growth scraped from the wall of the vessel, or of algal cells (if the culture has been illuminated) will reveal an accumulation of prosthecate cells. Other bacteria present may multiply, but they tend to die off or be consumed by protozoa; the prosthecate bacteria seem refractory to both of these processes.

At a point determined according to the proportion of accumulated prosthecate bacteria, a sample is streaked onto a suitable medium and incubated at room temperature (preferably) or at 30°C. A suitable plating medium for general purposes contains 0.05% peptone, plus possibly a vitamin mixture such as that formulated by Staley (1968), and 1.0 or 1.5% agar. As colonies become visible, they should be marked at two or three days and the plate reincubated. Colonies that become macroscopically visible at four to seven days will include *Caulobacter* and *Asticcacaulis* colonies, which are smooth, circular, and convex with an entire edge. Colonies that become visible only after seven days will include hyphomicrobia; development of *Hyphomicrobium* colonies can be accelerated by placing a flask or bottle of methanol in the incubator with the plates. Colonies of hyphomicrobia are initially hyaline, circular, cohesive, and often crateriform. Streaking on a mineral medium such as 337 (see "Cultivation," this chapter) and incubating in an atmosphere containing methanol vapors will increase selectivity of this step for *Hyphomicrobium*. Supplementation of the medium with 0.02% $MnSO_4$ or $FeSO_4$ (or iron powder or Fe-containing paper clips) may result in metal oxide deposition in the colonies, visible as brownish coloration. Many DPB deposit Fe and/or Mn oxides, at least upon primary isolation, and the presence of such deposits provides a macroscopic indication of possible DPB colonies.

At this point in the isolation of DPB, two major problems may be encountered. First, suitable samples often contain organisms that grow as a film on the surface of agar media; they may grow over and through colonies of DPB and be

carried onto subsequent streak plates. Their spreading can be retarded by the use of 2% agar, but this will also slow the development of DPB colonies. They can be avoided by transferring small, late-arising colonies as soon as possible (with the aid of a dissecting microscope, if necessary) to a secondary plate; the most suitable method is to transfer each colony with a sterile toothpick to a small area on a fresh plate. When growth accumulates in the inoculated patches, samples can be screened microscopically for DPB more conveniently than can the small colonies typical of the primary plates. Repeated restreaking from positive patches, then from isolated colonies, will eventually yield pure populations of DPB.

The second problem arises from the tendency of DPB to attach to other microbial cells, including other bacteria. As a consequence, initial colonies and patches containing DPB are often mixed with other bacteria (see, e.g., Harder and Attwood, 1978). This necessitates careful microscopic observation to avoid overlooking DPB, which may be only a minority in a mixed colony or patch, and repeated restreaking until all colonies on a plate contain only DPB, and only one type of DPB.

Microscopic screening is best accomplished in wet mounts examined with a phase contrast oil-immersion lens. This allows simultaneous evaluation of the presence of prosthecae and of motility. Prosthecae are less than 0.2 μm in diameter in caulobacters and only slightly wider in hyphomicrobia; they are not discernible in an ordinary light microscope. If phase contrast microscopy is not available, a droplet of crystal violet or methylene blue solution added to a wet mount will increase visibility of the prosthecae; the stain can be introduced from the edge of the cover slip after the mount has been examined for motility. Mordanted stains such as those used for visualizing flagella are also suitable for visualization of prosthecae.

Most DPB are adhesive, and in colonies and in pure cultures they tend to adhere to each other in rosettes united by a common mass of holdfast material. Only in *Caulobacter* is the holdfast located at the distal tip of the prostheca, resulting in rosettes in which the cells are at the periphery. In the other genera, adhesive isolates bear holdfast material directly on the cell surface, and cells in rosettes appear crowded while their prosthecae extend away from the cell cluster (Fig. 4). The presence of either type of rosette in colonies on primary isolation plates indicates the possible presence of DPB. Colonies and patches should be pursued to purity if rosettes of cells are present, even if prosthecae are not seen; such clones may eventually prove identifiable as *Asticcacaulis*.

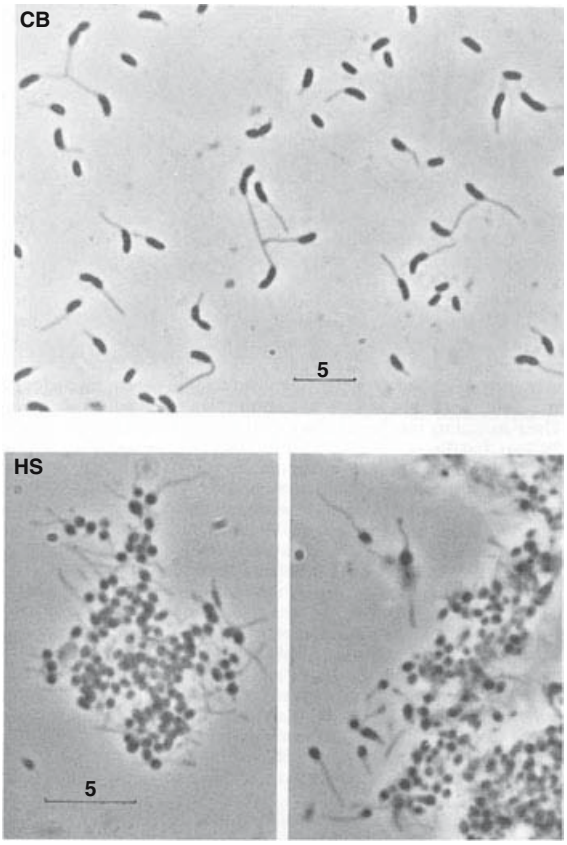

Fig. 4. (a) Caulobacterial (CB) and (b) hyphomicrobial (HS; lower panels) cell clusters formed in pure cultures. Phase contrast microscopy of cells in tannic acid + $HgCl_2$ (CB) or in seawater (HS). Bar = 5 μm.

Final identification of morphologic type should be made on the basis of electron microscopical examination of shadowed or negatively-stained specimens (see "Identification of DPB," this chapter).

The procedure just described is generally suitable for obtaining pure cultures of DPB. More specialized procedures for *Hyphomicrobium* and for *Pedomicrobium* are described below.

It is occasionally possible to isolate DPB without an enrichment step, and if the total microbial count of the sample is low because it was obtained from a strictly oligotrophic habitat, or if the sample is from a long-unused storage reservoir, direct streaking is worth attempting. A few useful guidelines are given below that can improve the development of a useful proportion of DPB and ensure their recognition once isolated.

1. Water samples should not be filtered to remove eukaryotic microbes and particulates. If filtration is for some reason desired, then the filter should also be used as inoculum for a separate enrichment culture. DPB are often more abundant on algal and fungal thalli and on

particles than in the ambient water, and the material on the filter may be a more suitable inoculum than the water.

2. Soil samples should be ground, not just shaken, to break up microbial clumps and to detach prosthecate cells from particles.

3. DPB can be collected on bait placed in the enrichment culture or in situ in the habitat. Glass surfaces such as microscope slides are the easiest to handle. (For simple observation of these organisms, this is the method used by Henrici and Johnson [1935]; somewhat more demanding, because of their physical delicacy, is the use of plastic-coated electron microscope grids [Hirsch and Pankratz, 1970].) To remove DPB, the bait should be rinsed well to remove casually-associated bacteria, then scraped (while wet) with a hard object to remove prosthecate cells. Breaking the prosthecae of caulobacters will not kill the cells, and hyphomicrobia do not attach by their prosthecae. Rubbing with a soft cotton swab will not detach these tightly-adhesive bacteria.

4. Dilution of the water sample or suspension of ground soil or sediment is often helpful. When DPB are present in sufficiently high proportion so that they will persist to higher dilutions than many of the other types present in a sample, dilution~~es~~pecially of samples to be supplemented with nutrients~~re~~duces the number of faster-multiplying bacteria and results in usefully high proportions of DPB after shorter incubation periods.

5. *Above all*, initial streaking must employ dilute media. Colony development will take several days, and colonies will be quite small, but the majority of DPB either do not grow on media containing 0.5% or more of soluble organic material or exhibit extreme pleiomorphy and absence of motility. Prosthecal development, in particular, is aberrant; in addition, cell shape is irregular, lysis is frequent, and viability in the colonies is low. Failure to accommodate this characteristic of DPB is probably the main reason they are so much more frequently sighted in natural samples than isolated from them; filtering them out before enrichment surely accounts for another major loss of DPB from natural samples prior to incubation. In addition, they are not reported in studies employing acridine orange (see, e.g., Kogure et al., 1979) because prosthecae fluoresce very weakly, or not at all, and most of the DPB strains tested either are not inhibited by nalidixic acid or develop aberrantly in its presence (see, e.g., Weiner and Blackman, 1973).

On the other hand, the purity of isolates can be checked by streaking on routine bacteriological "nutrient agar"(0.5% peptone plus 0.3% beef extract) for freshwater and soil isolates (Attwood and Harder, 1972; Gebers and Beese, 1988; Hirsch and Conti, 1964a) and on full-strength Zobell's 2216 (0.5% peptone, 0.1% yeast extract, 0.01% $FePO_4$ in 75% seawater) for marine isolates. Among marine DPB, only *Hyphomonas* will grow readily and with recognizable dimorphology on Zobell's medium, and even their growth is improved by diluting the medium three- to ten-fold (Havenner et al., 1979; Weiner et al., 1980).

Specific Enrichment Procedures for *Hyphomicrobium* and *Pedomicrobium*

Methanol-Nitrate Anaerobic Enrichment for *Hyphomicrobium* This method, developed independently by Sperl and Hoare (1971) and by Attwood and Harder (1972), is the only dependably rapid enrichment method for DPB. It yields a nearly pure population of *Hyphomicrobium* in three days to three weeks; the lower the organic content of the sample, the earlier the *Hyphomicrobium* population will develop (Attwood and Harder, 1972).

A sample of water or sewage (a few ml) or of mud or soil (not more than 1 gram) is added to a mineral medium (such as "337," see below) containing 0.5% KNO_3 and 0.5% methanol. The culture is incubated anaerobically, preferably in a tightly screw-capped vessel. When turbidity and bubbles (of N_2) appear, phase contrast microscopical examination usually reveals a high proportion (often more than 90%) of hyphomicrobia, which can then be purified by streaking on the same medium and incubating either aerobically or anaerobically. Methylotrophic denitrifying DPB isolated by this means, as well as strains isolated initially as aerobes but that are capable (sometimes only after adaptation [Sperl and Hoare, 1971]) of anaerobic growth with methanol and nitrate, have been assigned to four species of *Hyphomicrobium* (*H. vulgare*, *H. facilis*, *H. aestuarii* and *H. zavarzinii* [Hirsch, 1989]); thus, although highly selective for the genus *Hyphomicrobium*, the method yields several species among the isolates.

Humic Gel Selection for *Pedomicrobium* This method yields *Pedomicrobium* without a liquid enrichment step. Samples of soil or manganous deposits (e.g., from pipelines) suspended in water or saline, or of a water sample, are streaked on humic gel agar (for preparation, see Gebers and Hirsch, 1978) containing vitamins (to prevent pleiomorphy of *Pedomicrobium*) and cycloheximide (to inhibit fungi) at pH 5–6 (to inhibit other bacteria). After four to six weeks, yellow-brown cohesive colonies with dense cen-

ters and frayed edges appear. Isolation of *Pedomicrobium* requires vigorous disruption of these colonies (e.g., with the aid of a mechanical homogenizer) in a suspending medium and restreaking, since they are often overgrown by or contain other bacteria. After isolation, more rapid growth can be allowed by cultivation in acetate-yeast extract medium (PSM, below).

The principle underlying this direct isolation is the same as in the general procedure outlined above: during the first few weeks of incubation, other bacteria and fungi (and sometimes also algae and amebae) grow and multiply. *Pedomicrobium* colonies develop as the others decline (Gebers, 1981).

Identification of Dimorphic Prosthecate Bacteria

The genera of DPB are defined and therefore identified primarily on the basis of morphological traits. Accordingly, identification of an isolate as a representative of one of these genera requires microscopical examination, during the growth phase, of pure cultures that meet the following criteria.

1. Uniform dimorphic morphology.
2. Minimal to moderate accumulation of reserve polymers.
3. Not more than 50% of the population accounted for by swarmers.

These criteria are based on the following considerations.

1. Most isolates, particularly of hyphomicrobia, exhibit pleiomorphy even during the growth phase of a culture; all isolates do so in stationary phase. Pleiomorphy can often be reduced by vitamin supplementation of the medium (Gebers, 1981; Gebers and Beese, 1988; Gebers and Hirsch, 1978; Matzen and Hirsch, 1982a; Vedenina and Govorukhina, 1988) and is invariably reduced by the use of media containing not more than 0.2% soluble organic nutrients. "Uniform dimorphic morphology" means all prosthecate cells look alike, all swarmers look alike, and all reproductive cells (the link between the two forms) look alike.

2. DPB accumulate large quantities of carbon or phosphorus reserves when these nutrients are available in excess. Reduction of the carbon or phosphorus source that minimizes such accumulation typically also yields cultures of more uniform morphology and reduces the proportion of "bizarre" forms (Tyler and Marshall, 1967b).

3. Because swarmers of these bacteria arise by cell reproduction and do not themselves reproduce, a swarmer proportion greater than 50% indicates a failure of cell maturation and implies that aberrant development will be observed among the prosthecate cells present (see, e.g., Dow et al., 1983). *A. excentricus*, whose nonprosthecate cells can divide in some media (Larson and Pate, 1975), is an exception to this criterion.

In any pure culture that meets these criteria, there will be three types of cells: prosthecate cells, swarmer cells, and reproducing cells. Their definitive characteristics are best determined by electron microscopy of shadowed or negatively-stained specimens (Fig. 5). The bands typical of (although not universal among; Poindexter,

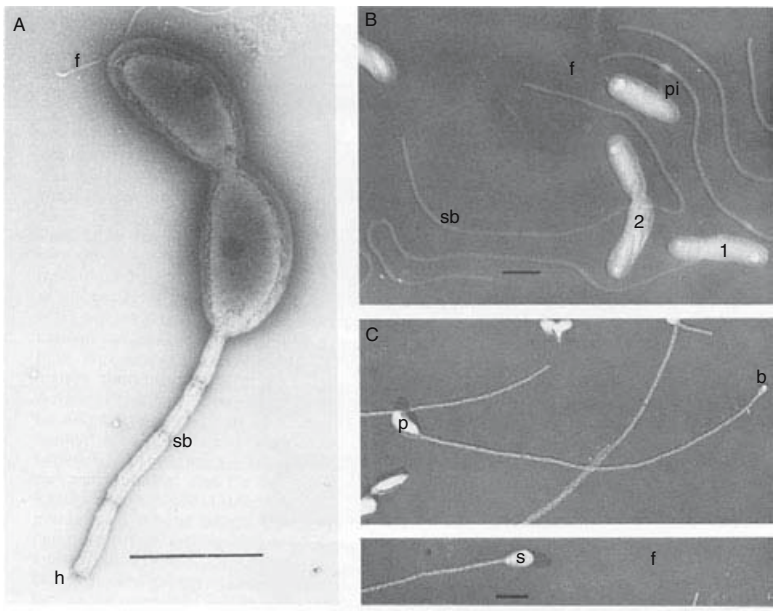

Fig. 5. (A) Negatively stained (K-PTA) *Caulobacter* cell. h, holdfast material; sb, stalk band; f, flagellum (with hook). (B) Pt-shadowed cells of *A. biprosthecum* with one (1) and two (2) prosthecae, and a swarmer with flagellum (f) and prosthecal initials (pi); sb, stalk band. The incipient swarmer of the dividing biprosthecate cell is flagellated. (C; two panels) Pt-shadowed *Hyphomicrobium* sp. cells; p, prosthecate ("mother") cell; b, very young bud; s, incipient swarmer with flagellum (f). Bar = 1 μm.

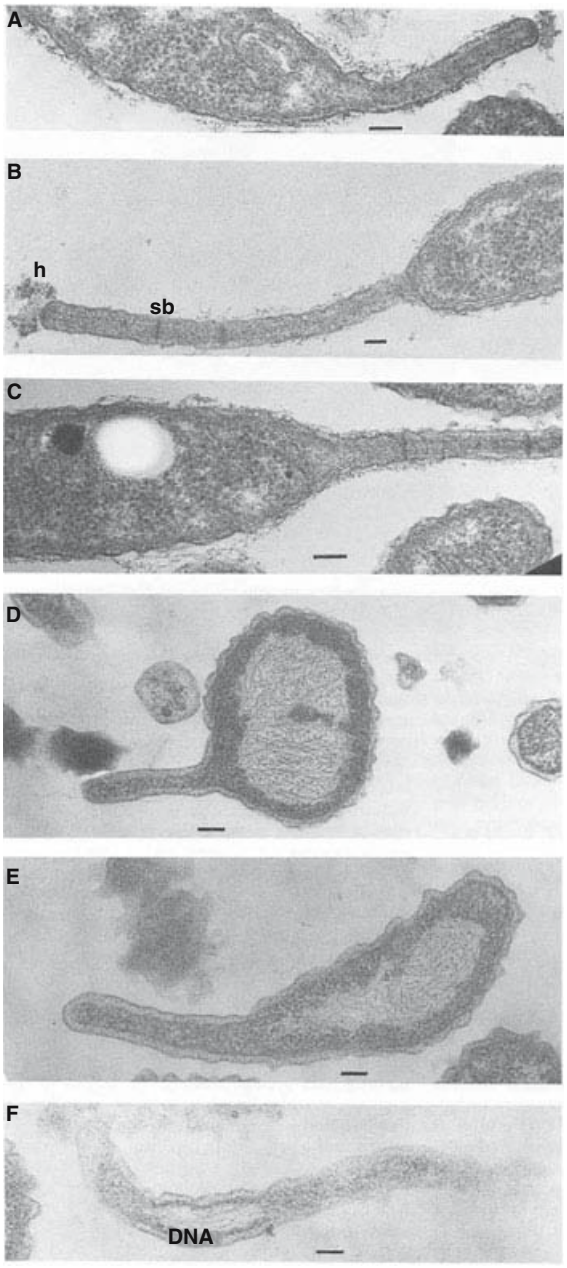

Fig. 6. Sections of prosthecae. (A–C) *Caulobacter* stalks; h, holdfast material; sb, stalk band. (D–F) *Hyphomonas* hyphae, with ribosome density as within the cell body cytoplasm; DNA, fibrils of DNA. Bar = 100 nm.

1989a, 1989b) caulobacterial prosthecae are discernible in such specimens. The identity of a cellular appendage as a prostheca can be established only by examination of thin sections (Fig. 6) that reveal the presence of all three components of the cell envelope: outer membrane, peptidoglycan layer, and cytoplasmic membrane. This technique also distinguishes the caulobacterial "stalk," which typically lacks cytoplasmic components through most or all of its length, from the (wider) hyphomicrobial "hypha," which includes cytoplasmic components throughout its length.

Cell shape is uniform in each developmental stage of each isolate, but variable within each genus. *Caulobacter* cells may be vibrioid, rod-shaped, or fusiform; *Asticcacaulis* cells are only rod-shaped. Known hyphomicrobia are rod-shaped or ovoid to nearly spherical, and incipient buds and swarmer cells may exhibit a shape different from that of mature, prosthecate cells. The morphology of the reproductive cell is distinctive for three of the genera; the fourth form is shared by two genera, as follows.

Caulobacter: approximately equatorial constriction; one pole prosthecate, one pole monoflagellate. There is one prostheca per cell.

Asticcacaulis: unevenly divided by septation; the prosthecate sibling is characteristically longer, with the prostheca subpolar or lateral, and the shorter sibling subpolarly monoflagellate. There may be one or two prosthecae per cell.

Pedomicrobium: the larger, prosthecate cell is the "mother" cell, and the smaller bud arises at the distal tip of the prostheca; as the bud grows, its long axis is perpendicular to the hyphal axis; the bud bears one subpolar flagellum. There may be one, two, or more prosthecae per cell.

Hyphomicrobium and *Hyphomonas*: morphology as in *Pedomicrobium* except that the long axis of the bud is a continuation of the hyphal axis. In *Hyphomicrobium*, the swarmer may possess more than one (polar or subpolar) flagellum. Typically one, rarely more than two prosthecae per cell.

Distinguishing among the genera of hyphomicrobia has long been difficult, particularly because of their tendency to pleiomorphy (Bauld and Marshall, 1971; Bauld and Tyler, 1971; Bauld et al., 1971). However, it is now apparent that this morphology occurs in at least three distinct physiotypes: 1) methylotrophs (now *Hyphomicrobium* [Hirsch, 1989]), among which requirements for organic growth factors are unknown, although vitamins may be stimulatory, and for which nitrate can serve as sole source of nitrogen; 2) non-methylotrophic organisms (now *Pedomicrobium* [Gebers, 1989]) that prefer organic acids as carbon sources, accept ammonia as sole source of nitrogen, and require organic growth factors; and 3) amino-acid-requiring isolates (now *Hyphomonas* [Moore and Weiner, 1989]), all of which are either marine isolates or can be maintained on marine media. Accordingly, distinction between the two genera that have similar morphology in the budding stage requires determination of dependence on amino

acids as macronutrients or of ability to grow with methanol or methylamines as the only organic substrates.

Cultivation of Dimorphic Prosthecate Bacteria

Although media designed to mimic the composition of animal tissues ("routine" bacteriological media) are unsuitable for the cultivation of these chemoheterotrophic bacteria (for example: Attwood and Harder, 1972; Bauld et al., 1971; Gebers, 1981; Gebers and Beese, 1988; Gebers and Hirsch, 1978; Gromov, 1964; Havenner et al., 1979; Hirsch and Conti, 1964a; Kingma Boltjes, 1936; Kudryavtsev, 1978; Lapteva, 1977, 1987; Larson and Pate, 1975; Li et al., 1984; Loeffler, 1890; Poindexter, 1964, 1981a; Weiner et al., 1980), such media are *qualitatively* suitable; they need only be diluted at least three- to ten-fold. The importance of reducing total solute concentrations—principally of organic compounds, phosphate, and ammonium—cannot be over-emphasized. Growth of DPB in "rich" media or in severely unbalanced media is extremely poor if it occurs at all, and the cells are structurally fragile and morphologically aberrant. *Hyphomonas* isolates are the only members of this group that tolerate 0.5% or more of complex organic material, but in such media, viability even of this group is lower than on more dilute media, and the populations exhibit considerable genetic as well as structural and developmental instability.

The importance of employing dilute media was discovered during the first reported isolation of *Caulobacter* (Loeffler, 1890). In a study of methods for staining bacterial flagella, Loeffler encountered "eines höchst merkwürdigen Organismus" in a strongly diluted cabbage ("Kohlrabi") infusion. He purified the organism on Kohlrabi infusion solidified with gelatin, which the organism did not digest. On transfer to a richer, meat extract-peptone-gelatin agar medium, its growth was slower and development of prosthecae (described by Loeffler as thick thread-like appendages) was sparse. On return to the very dilute medium, the organism developed extremely long prosthecae that extended across the microscopic field. The organism is illustrated in Figs. 7 and 8 of Loeffler (1890) as it appeared on the two media. Uncertain of its nature, Loeffler named the organism *"Vibrio* (?) *spermatozoides"*; the cell form and pale yellow color of the colonies suggest that it was the organism known today as *C. vibrioides*. Because of his study, nutrient sensitivity in *Caulobacter* is the longest-known physiologic property of these DPB.

Many DPB are also sensitive to chelating agents such as nitrilotriacetate, ethylenediamine tetraacetate, and citrate and to phosphate, which binds many cations with affinities comparable to those of organic chelators (Hirsh, 1974; Hirsch and Conti, 1964a; Larson and Pate, 1975; Poindexter, 1984b). Thus, if chelators are used, their concentrations must be not more than 1 mM. Similarly, substances added as pH buffers (e.g., phosphate, HEPES, imidazole) are inhibitory or interfere with development at concentrations that provide dependable buffering capacity; imidazole is tolerated at the highest concentration (5 mM), but not by all isolates. Phosphate is provided in some media for some strains at 10 to 20 mM, but development is impaired (as evidenced by the accumulation of swarmers in late exponential and stationary phases), and the cells are relatively fragile. The majority of isolates exhibit requirements for vitamins (biotin, riboflavin, B_{12} or pantothenate) or for organic growth factors that are still unidentified. Pleiomorphy, in particular, is frequent among hyphomicrobia unless vitamins are provided (Gebers, 1981; Gebers and Beese, 1988; Gebers and Hirsch, 1978; Matzen and Hirsch, 1982a; Vedinina and Govorukhina, 1988).

Detailed recipes for isolation and cultivation of DPB are presented below. However, dilute (less than 0.2%) peptone media will support the growth of all DPB (even of methylotrophic hyphomicrobia, which will obtain their principal carbon source from laboratory air [Kingma Boltjes, 1936]). For nonfastidious isolates, a general basal medium should provide (in distilled water or in 50 to 75% seawater) a mixture of amino acids, vitamins, trace minerals (with relatively high proportions of Mg and Ca [Harder and Attwood, 1978; Hirsch and Conti, 1964a; Johnson and Ely, 1977; Poindexter, 1984a, 1984b]), not more than 1 mM phosphate, and ammonia at a molarity not more than twice the molarity of amino acids. A principal carbon source could be added as: a sugar (for instance, glucose, xylose, fructose, or maltose; filter sterilized) for *Caulobacter* and *Asticcacaulis*; methanol or methylamine for *Hyphomicrobium*; acetate or ribose for *Pedomicrobium*; and amino acids for *Hyphomonas*, with a total organic concentration not more than 0.1 to 0.2%. A neutral pH, an incubation temperature of 25–30°C, and aerobic conditions will allow growth of all types of DPB.

Cultivation of *Caulobacter* and *Asticcacaulis*

Isolates from fresh water and soil can be cultivated on dilute peptone-yeast extract medium (PYE). However, prostheca development is somewhat inhibited by the yeast extract, and

morphology is more regular in dilute peptone medium supplemented with $CaC1_2$ (PCa). Marine caulobacters have been cultivated only in complex medium (CPS). The majority of isolates must be cultivated and maintained on PYE, PCa, or CPS. For *C. vibrioides*, PYE must also be supplemented with riboflavin (0.1 μg/ml) to ensure perpetual subcultivation.

Growth in liquid medium occurs as a surface film on stationary cultures and as evenly-suspended turbidity in agitated cultures.

PYE Medium (Poindexter, 1964)

Peptone	0.2%
Yeast extract	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%

Use tap or distilled water.

PCa Medium (Poindexter, unpublished)

Peptone	0.2%
$MgSO_4 \cdot 7H_2O$	0.02%
$CaCl_2 \cdot 2H_2O$	0.015%

Use distilled water.

CPS Medium (Poindexter, 1964)

Peptone	0.05%
Casamino acids	0.05%
Seawater	75–80%

Isolates of *C. crescentus* and *C. leidyi* grow readily in defined HiGg medium (see below), and *A. excentricus* can be cultivated in this medium supplemented with biotin (2 μg/L). Glutamate is not required as a growth factor or as a source of carbon or of nitrogen. Its role appears to be primarily as ammonia acceptor, since *C. crescentus*, at least, lacks glutamate dehydrogenase (Ely et al., 1978); growth rate and yield are maximal when the medium provides equimolar amounts of glutamate and ammonium chloride and glucose as principal carbon source (Poindexter, unpublished observations).

HiGg Medium (Poindexter, 1978)

Glucose (filter sterilized)	5–10 mM
Monosodium glutamate (filter sterilized)	5–10 mM
Phosphate (Na and K salts)	0.1–1 mM
NH_4Cl	5–10 mM
Imidazole	5 mM

Use Hutner's mineral base (Cohen-Bazire et al., 1957), prepared without vitamins.

Cultivation of *A. biprosthecum* in defined medium is dependent particularly on the presence of amino acids. Growth is optimal when five amino acids are provided as well as glucose, ammonia, and biotin (MS-B-AA medium).

MS-B-AA Medium (Larson and Pate, 1975)

Glucose	0.1%
Alanine, glutamate, serine, proline, aspartate	100 mg/ml of each
$(NH_4)_2HPO_4$ (optional)	0.075 mM
$NaH_2PO_4 \cdot H_2O$	0.5 mM
KH_2PO_4	0.5 mM
$MgSO_4 \cdot 7H_2O$	0.01%
Sodium citrate	0.01%
D-Biotin	4 μg/l

Trace salts (prepared at 1,000×; see Larson and Pate, 1975, for preparation); final concentration per liter:

$CaCl_2 \cdot 2H_2O$	10 mg
$CuSO_4$	1 mg
$CoCl_2 \cdot 6H_2O$	1 mg
$FeSO_4 \cdot 7H_2O$	10 mg
$K_2B_4O_7 \cdot 4H_2O$	1 mg
MoO_3	1 mg
$MnSO_4 \cdot H_2O$	10 mg
$ZnSO_4 \cdot 7H_2O$	10 mg

Cultivation of *Hyphomicrobium*

Hyphomicrobium isolates generally do not require organic growth factors and can be cultivated aerobically on defined media that provide a mixture of minerals, including nitrate or ammonia as nitrogen source and C_1 compounds as carbon sources (methanol, methylamine, trimethylamine). Many isolates can also be cultivated anaerobically in mineral media supplemented with methanol and KNO_3 as terminal electron acceptor or with methylamine and thioglycollate. Most isolates do not grow evenly dispersed in liquid media; in stationary cultures, cells accumulate as a pellicle and as a film on the vessel wall, and growth of many isolates is not evenly suspended even in continuously-agitated liquid cultures. All known isolates will grow on freshwater media, and some will also grow on seawater media or in media containing up to 5.5% NaCl. Temperature optima are wide, and growth may occur at 5 to 45°C.

Of many versions of the mineral medium 337," variations relate to quantities of trace salts; optimal quantities vary with the isolate. The following recipe is based on the recipes of Hirsch and Conti (1964a, 1964b), Moore and Hirsch (1972), Moore (1981), and Matzen and Hirsch (1982a) and would seem to be an average composition of general suitability.

Medium 337 General

The following are added per liter of distilled water:

KH_2PO_4	1.36 g
Na_2HPO_4	2.13 g
$(NH_4)_2SO_4$	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2 \cdot 2H_{20}$	2–10 mg
$FeSO_4 \cdot 7H_2O$	2 mg
$MnSO_4 \cdot 7H_2O$ (20 mg, to detect Mn oxidation)	0.4–0.8 mg
$Na_2MoO_4 \cdot 2H_2O$	0.5–2.5 mg
Methanol (or methylamine, 67.5% solution)	5 ml
Vitamin B_{12} or vitamin mixture (Staley, 1968)	
KNO_3 (for anaerobic growth)	5 g

Cultivation of *Hyphomonas*

A defined medium (GAMS) has been developed for one isolate (*Hyphomicrobium neptunium*, now regarded as *Hyphomonas neptunium*), although growth is much slower than in Zobell's marine broth. All other isolates have been cultivated only on complex media, for which one-third strength Zobell's appears to be particularly suitable (Havenner et al., 1979). As with *Hyphomicrobium*, growth is typically clumpy, but smooth variants that grow more evenly dispersed regularly arise in stock cultures of *Hyphomonas* (Pongratz, 1957; Moore and Weiner, 1989; J. S. Poindexter, unpublished observations). All known isolates are marine (Weiner et al., 1985) and do not grow on freshwater media.

GAMS Medium (Havenner et al., 1979)

Glutamate, aspartate, serine, methionine	125 mM of each
Calcium pantothenate	0.26 µM
Seawater	30%

Cultivation of *Pedomicrobium*

Pedomicrobium isolates have not been cultivated on defined media; they may, like most *Caulobacter* isolates, require organic growth factors not yet identified. A generally suitable complex medium will provide an organic acid (acetate, malate, pyruvate, succinate, gluconate) at 10 mM as carbon source, a complex organic mixture (peptone, casamino acids, yeast extract) at 0.05% as nitrogen source, and a mixture of vitamins.

In isolates that deposit oxides of iron or manganese on their cells, this property can be observed macroscopically as yellow or brown coloration in colonies grown on media containing ferrous or manganous salts. Such colonies will develop a blue color when flooded with acidified 2% $K_4[Fe(CN)_6]$ or 0.4% leukoberbelin blue I (see Gebers, 1989). The property of metal oxide deposition may, however, be lost on laboratory cultivation and seems to be a "spasmodic" property in individual isolates of both *Pedomicrobium* and *Hyphomicrobium* (Tyler and Marshall, 1967a).

PSM (Gebers, 1981; Gebers and Beese, 1988)

Sodium acetate	10 mM
Yeast extract	0.05%
Starch (or 0.4% gelatin)	0.2%
Metals 44 (Cohen-Bazire et al., 1957)	1 ml/l
Vitamins (Staley, 1968)	10 ml/l

Adjust to pH 9.0.

PYVM (Gebers and Beese, 1988)

Peptone	0.025%
Yeast extract	0.025%
Vitamins (Staley, 1968)	10 ml/l
Hunter's base (Cohen-Bazire et al., 1957)	20 ml/l
DL-Malate	10 mM

Adjust to pH 7.5.

Maintenance of Dimorphic Prosthecate Bacteria

All DPB can be maintained as vegetative stock cultures on dilute peptone medium of appropriate ionic composition, refrigerated between transfers. They can also be preserved as frozen suspensions, with a cryoprotectant such as glycerol (*Hyphomicrobium*, *Hyphomonas*) or without a cryoprotectant (*Caulobacter*, *Asticcacaulis*). Hyphomicrobia and some *Asticcacaulis* isolates are also dependably preserved by lyophilization, with or without milk; however, this process is not dependable for *Caulobacter* isolates, particularly marine strains. *Hyphomicrobium* and *Pedomicrobium* are tolerant of desiccation and can be preserved as slant cultures in screw-capped tubes stored at room temperature; if dry, they can be revived by rehydration with liquid growth medium.

Ecological Roles and Potential Applications of Dimorphic Prosthecate Bacteria

The DPB exhibit two characteristics that could be exploited in the purification of waters: the property of adhesiveness and the ability to metabolize organic materials available in extremely low quantities. A wide variety of materials are suitable substrata for their attachment, including glass—a durable material available in increasingly burdensome quantities that could support (physically) equally durable populations of bacteria such as *Caulobacter* and *Hypomicrobium* in purification beds for groundwater and other waters bearing low levels of pollutants. Caulobacters have been demonstrated to be capable of rapidly mineralizing agricultural pollutants present in very low concentrations (Grimes and Morrison, 1975). They rise to prominence in enrichment cultures provided with hydrocarbons, including aromatic compounds, as sole carbon sources (Moaledj, 1978; Murakami et al., 1976) and retain the ability to oxidize aromatic compounds after years of cultivation on peptone media (Chatterjee and Bourquin, 1987). Efforts are in progress to expand their catabolic versatility by the introduction of *Pseudomonas* genes (Chatterjee and Chatterjee, 1987) and other plasmids (Anast and Smit, 1988).

Hyphomicrobia can mineralize a variety of pollutants, including aromatic hydrocarbons (Moaledj, 1978), dimethyl sulfoxide and dime-

thyl sulfide (DeBont et al., 1981; Suylen and Kuenen, 1986), methyl chloride (Hartmans et al., 1986), and various alcohols (Köhler and Schwartz, 1982). The unique capability of *Hyphomicrobium* to denitrify sewage supported (metabolically) by methanol, a relatively inexpensive carbon source, has already proved practicable (Claus and Kutzner, 1985). Since anaerobic stages of sewage treatment plants generate methane and at least one isolate of *Hyphomicrobium* is known to utilize this compound (see Hirsch, 1989), it would seem reasonable to expect that denitrification and methane utilization could be combined in *Hyphomicrobium*~~in~~ an isolate or by genetic manipulation (Dijkhuisen et al., 1984)~~th~~ereby eliminating the need for methanol in the denitrification of sewage by *Hyphomicrobium*.

On the other hand, the same properties that would allow exploitation of these organisms probably enable them to foul submerged surfaces; they are particularly likely to become a nuisance in water distribution systems. Although often seen as members of biofouling communities on submerged surfaces, DPB have been regarded as relatively unimportant as pioneers, for two reasons, both based on microscopical studies. First, the microbial cells that appear earliest on experimental surfaces are not typically prosthecate (Corpe, 1978; Corpe et al., 1975; Marshall, 1976). Second, prosthecate bacteria do not predominate numerically at any stage of fouling. The first observation is consistent with the experimental evidence that attachment is initiated principally by the nonprosthecate, swarmer stage (Newton, 1972; Hirsch, 1974; Leifson, 1962; Moore and Marshall, 1981; Zavarzin, 1961), in which stage DPB are not distinguishable from other rods and vibrios by microscopy. The second is predictable, but does not preclude a significant role of DPB in initial conditioning or continued maintenance of the surface as suitable for attachment of other microorganisms and, later, of animals.

Beyond potential assistance from DPB in remedying problems of technological origin, their predictable presence in almost any sample of fresh or sea water and in many types of soils implies that their activities are compatible with~~an~~d possibly of benefit to~~di~~verse microbial communities. At present, the only significant role of which they seem capable is as mineralizers. However, the frequent occurrence of DPB attached to plant and algal remains may reflect their participation in mobilization of detritus as polymer-digesting microbes slowly free organic carbon from insoluble forms. As Lapteva (1987) has pointed out, although DPB may be responsible for the bulk of mineralization only in waters of low organic content, their total contribution in the vastness of the biosphere's waters may be considerable.

Similarly, the high frequency of the occurrence of DPB as algal epibionts may reflect an influence on primary productivity that is not yet appreciated. In quantitative studies of *Pseudomonas* and *Caulobacter* in association with algae (*Gomphonema*, *Cyclotella*, and *Chlorella*), Allen (1971) observed that total productivity~~al~~gal and bacterial~~wa~~s higher in two- or three-membered populations than when algae or bacteria were incubated as monotypic populations. Associations fared especially well when the source of carbon was organic material derived from a macrophyte *(Naxas)*. He inferred that within algal-bacterial associations, carbon could cycle between substratum and epibionts; such association would therefore be of mutual benefit to the associates. This intriguing possibility has not been pursued experimentally.

Nevertheless, the dimorphic habit of caulobacters and other DPB can be viewed as an adaptation particularly appropriate to attachment to living substrata. As new surface was generated by growth and reproduction of the substratum organism, each generation of epibiont would be prepared to relocate (by means of its swarmers) on virgin territory, while the substratum would not be overburdened by dense accumulations of epibionts. Clearly, the possibility that epibiontic prosthecate bacteria are advantageous to their algal associates and could have a direct and positive influence on primary productivity in aquatic (and possibly terrestrial) environments is worthy of exploration. Such a niche would be consistent with both the physiological properties and the unique developmental pattern of the dimorphic prosthecate bacteria.

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Prokaryotes (2006) 5:91–114
DOI: 10.1007/0-387-30745-1_5

CHAPTER 3.1.4

The Genus *Agrobacterium*

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Introduction

The genus *Agrobacterium* is a group of Gram-negative soil bacteria found associated with plants. Many members of this group cause disease on plants. Infections of wound sites by *Agrobacterium tumefaciens* cause crown gall tumors on a wide range of plants including most dicots, some monocots, and some gymnosperms. Infections by *A. rhizogenes* cause hairy root disease. *A. vitis* causes tumors and necrotic lesions on grape vines and is commonly found in the xylem sap of infected plants. Despite the general perception that most of the agrobacteria cause disease, the member of this group most often isolated from soil, *A. radiobacter*, is avirulent.

Phylogeny

Earlier studies using physiological characteristics such as ability to grow on various carbon sources placed the agrobacteria with the rhizobia in the family *Rhizobiaceae*. More recent studies of both 16S rDNA and other chromosomal gene DNA sequence homologies suggest that these two groups of bacteria are indeed closely related (Willems et al., 1993). Both physiological characteristics and 16S rDNA sequence data place these bacteria in the α subgroup of the Proteobacteria. They appear to be closely related to members of the genus *Brucella*.

On the basis of genomic organization the agrobacteria appear to form a unique group within the α2 subgroup of the Proteobacteria (Jumas-Bilak et al., 1998; see also Genetics); Biovar 1 strains and *A. rubi* have both a circular and a linear chromosome. Biovar 2 and 3 strains also have 2 chromosomes, but both appear to be linear. Large plasmids (200–400 kb) are present in most strains. *Rhizobia*, although closely related to agrobacteria, appear to lack the linear chromosome present in biovar 1 agrobacteria.

Genetic experiments suggest that at least some members of the rhizobia are closely related to agrobacteria. The gene order on the circular chromosome appears to be conserved between *Sinorhizobium meliloti* and *A. tumefaciens* (Hooykaas et al., 1982). When plasmids from *Rhizobium phaseoli* were introduced into *A. tumefaciens* strain C58 (a biotype 1 strain; see also Taxonomy), the resulting bacteria were able to form nitrogen-fixing nodules on bean roots. This outcome suggests that all of the chromosomal genes required for the interaction of *R. phaseoli* with plants were present on the *A. tumefaciens* chromosomes (Martinez et al., 1987; see also Genetics, Chromosomal). Along similar lines, the *nodC* gene on the sym plasmid of *S. meliloti* can be induced when this plasmid is present in *A. tumefaciens* but not when the plasmid is transferred to *E. coli*, *Xanthomonas campestris* or *Pseudomonas savastanoi* (Yelton et al., 1987). All of these results suggest that the chromosomal genes of agrobacteria and *rhizobia* are so closely related they can substitute for each other. Sequencing of the genomes of *S. meliloti* and *A. tumefaciens* is currently in progress and should help to elucidate the relationship between these bacteria.

Taxonomy

The genus is divided into species largely based on pathogenic properties, although other physiological characteristics correlate with pathogenic properties. The major species are *A. radiobacter* (nonpathogenic), *A. tumefaciens* (the causative agent of crown gall tumors), *A. rhizogenes* (the causative agent of hairy root disease), and *A. vitis* (the causative agent of tumors and necrotic disease on grapevines). There are also less well studied proposed species such as *A. rubi* isolated from cane galls on *Rubrus* species.

Agrobacteria also have been divided into biotypes (biovars) based on physiological properties. Biovar 1, which includes most strains of *A. tumefaciens*, has no growth factor requirements and will grow in the presence of 2% NaCl. Most strains produce 3-ketolactose. All biovars produce acid from mannitol and adonitol. Biovar 1 bacteria also produce acid from dulcitol,

melizitose, ethanol, and arabitol. Some biovar 1 strains are able to grow at 37°C. However, they may lose the Ti plasmid, which is required for virulence, when grown at this temperature. Biovar 2 includes most strains of *A. rhizogenes*. These bacteria require biotin for growth. They fail to grow in the presence of 0.5% NaCl or at 37°C. Some biovar 2 strains can grow on tartrate, producing alkali. Biovar 3 strains include most *A. vitis* strains. Some authors also include some *A. tumefaciens* strains in this group. Like biovar 1 strains, these bacteria will grow in the presence of 2% NaCl but generally do not grow at 37°C. Both biovar 2 and 3 strains fail to produce 3-ketolactose. Biovar 3 strains can produce alkali from tartrate. Some biovar 3 strains require biotin for growth (Table 2). Selective growth media for various biovars have been reported and are described in the section on isolation of agrobacteria (Table 1). Biovars 1 and 3 contain both strains with wide and others with narrow host ranges (Kerr et al., 1977b).

Habitat

Agrobacteria usually are found in soil in association with roots, tubers, or underground stems. The bacteria also cause tumors from which they can be isolated. Tumors may be prevalent on grafted plants at the graft junction; examples include grapes, roses, poplars, and fruit trees. In some cases, the bacteria can be isolated from the xylem of infected plants. Thus it is often possible to isolate *A. vitis* from the xylem of infected grapevines.

Although agrobacteria are generally isolated from cultivated soils and plants, biovars 1 and 2 can be found in association with roots from uncultivated plants of the natural savanna and tall grass prairie which has never been cultivated (Bouzar et al., 1987). As is the case in most other field studies of agrobacteria, the majority of these isolates were nonpathogenic. Schroth et al. (1971) were able to isolate agrobacteria from almost every soil they tested in California by using selective media and enrichment culture methods. Thus the bacteria appear to be widely distributed regardless of the plants previously grown in the location. However, the number of bacteria pathogenic for a crop grown in a particular location was greater if the same crop had formerly been grown in that location.

Isolation

Agrobacteria can be isolated from soil obtained from the vicinity of infected plants, from galls formed by the bacteria, or, in the case of grapevines, from the xylem sap of infected plants. The bacteria are not numerous in older galls and may be easier to isolate from the surrounding soil than from the tumor tissue.

Agrobacteria grow readily in culture on complex or defined media (Table 1). Nutrient agar (with or without yeast extract [0.5%]) or yeast mannitol agar will support the growth of most strains. Some strains require B vitamins for growth, usually 0.2 mg/liter each of biotin, pantothenic acid and/or nicotinic acid. Many strains, including most *A. rhizogenes* isolates, are sensitive to salt and will not grow on media such as Luria-Bertani agar because this medium contains too much NaCl. The colonies are generally white or slightly cream or pale pink in color. No distinctive pigment is produced. Large amounts of extracellular polysaccharide may be produced on some media, giving the colonies a watery appearance. The bacteria grow at a moderate rate. *A. tumefaciens* will usually require 2 to 4 days to form colonies on complex media. Some strains of *A. rhizogenes* are slow growing and may require as much as 1 week to form colonies on complex media.

Optimal growth temperature for most strains is between 25 and 28°C, although the optimal temperature for plant infection may be lower (22°C).

Selective media may be used to isolate Agrobacteria (Table 1).

Identification

Agrobacteria have been traditionally identified as Gram-negative bacteria that don't produce fluorescent pigment on King's B medium and do produce tumors (or hairy roots) when inoculated onto test plants. The test plants most often used are tomato, sunflower, *Datura* spp., *Kalanchoë daigremontiana* (also called *Bryophyllum*), tobacco, and *Nicotiana glauca* (Figs. 1–4). These plants respond relatively readily and rapidly to inoculation of *Agrobacterium* strains by producing tumors in as few as 10 days. Sugar fermentations and production of ketolactose also have been used in identification of agrobacteria (Table 2). In recent years, lipid and fatty acid profiles have been used to identify both virulent and avirulent agrobacteria (Jarvis et al., 1996; Bouzar et al., 1993a). Polymerase chain reaction (PCR) has also been used in identification and to distinguish pathogenic from nonpathogenic strains. The PCR primers chosen from *vir* genes such as *virD2* (See Genetics) can be used to identify potentially pathogenic strains (Haas et al., 1995). Pathogenic strains have been identified by their ability to grow on different opines, and the

Table 1. Media for growth of agrobacteria.

General Media

Luria Agar (for biovar 1 and some biovar 3 strains)

Tryptone	10g
Yeast extract	5g
NaCl	5g
Water	1 liter
3M NaOH	1ml
Agar	14g

Yeast Mannitol Agar (for all biovars)

Mannitol	10g
Yeast extract	1g
K_2HPO_4	0.5g
$CaCl_2$	0.2g
NaCl	0.2g
$MgSO_4 \cong 7H_2O$	0.2g
$FeCl_3$	0.01g
Water	1 liter
Agar	15g

Adjust to pH 7.0. For biovar 2 add biotin, calcium pantothenate, and nicotinic acid, all at 200µg/liter.

Mannitol Glutamate Agar (for all biovars) (Roberts et al., 1974)

Mannitol	10g
L-Glutamic acid	2g
KH_2PO_4	0.5g
NaCl	0.2g
$MgSO_4 \cdot 7H_2O$	0.2g
Biotin	0.002g
Water	1 liter
Agar	15g

Adjust pH to 7.0 before autoclaving.

H4 Minimal Medium (for biovars 1 and 3; biovar 2 will grow very slowly on this medium) (Matthysse et al., 1976)

NH_4Cl	5g
NH_4NO_3	1g
Na_2SO_4	2g
K_2HPO_4	3g
KH_2PO_4	1g
$MgSO_4 \cdot 7H_2O$	0.1g
Water	1 liter

Dissolve salts in the order given; adjust pH to 7.2; add 10ml of sterile 20% glucose after autoclaving.

AB Minimal Medium

K_2HPO_4	3g
NaH_2PO_4	1g
NH_4Cl	1g
$MgSO_4 \cdot 7H_2O$	0.3g
KCl	0.15g
$CaCl_2$	0.005g
$FeSO_4 \cdot 7H_2O$	0.0025g
Water	1 liter

General Media

Dissolve salts in the order given; adjust pH to 7.2; add after autoclaving 10ml of sterile 20% glucose or sucrose.

Selective Media (Kerr, 1986)*

Selective Medium of Biovar 1*

L(-) Arabitol	3.04g
K_2HPO_4	1.04g
KH_2PO_4	0.54g
NH_4NO_3	0.16g
$MgSO_4 \cong 7H_2O$	0.25g
Sodium taurocholate	0.29g
Water	1 liter
1% Crystal violet	2ml
Agar	15g

Add after autoclaving 10ml of 2% cyclohexamide and 10ml of 1% $Na_2SeO_3 \cong 5H_2O$. On this medium colonies of agrobacteria are white, circular, raised, and glistening. They may become mucoid.

Selective Medium for Biovar 2*

Erythritol	3.05g
K_2HPO_4	1.04g
KH_2PO_4	0.54g
NH_4NO_3	0.16g
$MgSO_4 \cdot 7H_2O$	0.25g
Sodium taurocholate	0.29g
Yeast extract	0.01g
Malachite green	0.005g
Water	1 liter
Agar	15g

Add after autoclaving 10ml of 2% cyclohexamide and 10ml of 1% $Na_2SeO_3 \cong 5H_2O$. On this medium colonies of agrobacteria are white, circular, raised, and glistening. They may turn brown a they age.

Selective Medium for Biovar 3*

Adonitol	4.0g
K_2HPO_4	0.9g
KH_2PO_4	0.7g
NaCl	0.2g
$MgSO_4$	0.2g
Yeast extract	0.14g
Boric acid	1.0g
Water	1 liter
Agar	15g

Adjust pH to 7.2 before autoclaving. After autoclaving add 10ml of 2.5% cyclohexamide, 1ml of 8% triphenyltetrazolium chloride, 1ml of 2% D-cycloserine, and 1ml of 2% trimethoprin. On this medium colonies of agrobacteria have dark red centers with white edges.

*Note that these media are only semi-selective. Other organisms may grow. Additional tests are necessary to positively identify an isolate as *Agrobacterium*.

Table 2. Traits used for identification of biovars of *Agrobacterium*.

Characteristic	Biovar 1	Biovar 2	Biovar 3	*A. rubi*
Growth on selective medium 1A[a]	Yes			
2E[b]		Yes		
RS[c]				Yes
Growth factor requirements	None	Biotin	Biotin, some strains	Biotin, pantothenic acid, nicotinic acid
3-Ketolactose production	Most strains	No	No	No
Growth on 2% NaCl	Yes	No	Yes	Yes
Growth at 37°C	Yes	No	No	Yes
Acid production from mannitol	Yes	Yes	Yes	Yes
Adonitol	Yes	Yes	Yes	Yes
Erythritol	No	Yes	No	No
Dulcitol	Yes	Yes	No	No
Melizitose	Yes	No	No	No
Ethanol	Yes	No	No	No
Arabitol	Yes	No	No	No
Alkali production from tartrate	No	Yes	Yes	No

Data from Kerr (1986).

formation of particular opines by tumors caused by various strains has been used to group these strains. In general, grouping by sugar fermentations, fatty acid profiles, PCR, opine production and utilization, and genome organization all reach similar conclusions so that no one method of identification of agrobacterial species or biovars is preferable.

Preservation

The bacteria can be stored as stabs into vials of nutrient agar (all biovars) or Luria agar (biovars 1 and 3) at room temperature essentially indefinitely (more than 10 years). They can also be stored frozen in 25% glycerol at –70°C. Liquid cultures of biovars 1 and 3 can be spun down, resuspended in phosphate buffered saline containing 1 mM $MgSO_4$, and stored in the refrigerator for approximately 10 weeks.

Physiology

General

Agrobacteria are Gram-negative, nonspore–forming, short rods. They can use glucose as a carbon source, growing aerobically. Table 1 lists agrobacterial growth media formulations and Table 2 lists characteristics of different biovars.

Fig. 1. The stem of a tobacco plant wounded at two places and inoculated with *A. tumefaciens*. The tumors are shown at 6 weeks after inoculation.

Opines: Production and Utilization

Crown gall tumors produce specific substances (often substituted L-amino acids) called opines. The production of opines is catalyzed by enzymes encoded by genes introduced into crown gall tumor cells on the T DNA. These genes are usually expressed constitutively in the tumor tissue. Typical opines result from condensation reactions between compounds already

Fig. 2. Carrot root discs (A) uninoculated, (B) inoculated with *A. tumefaciens*, and (C) and (D) inoculated with *A. rhizogenes*. The discs are shown after 5 weeks incubation.

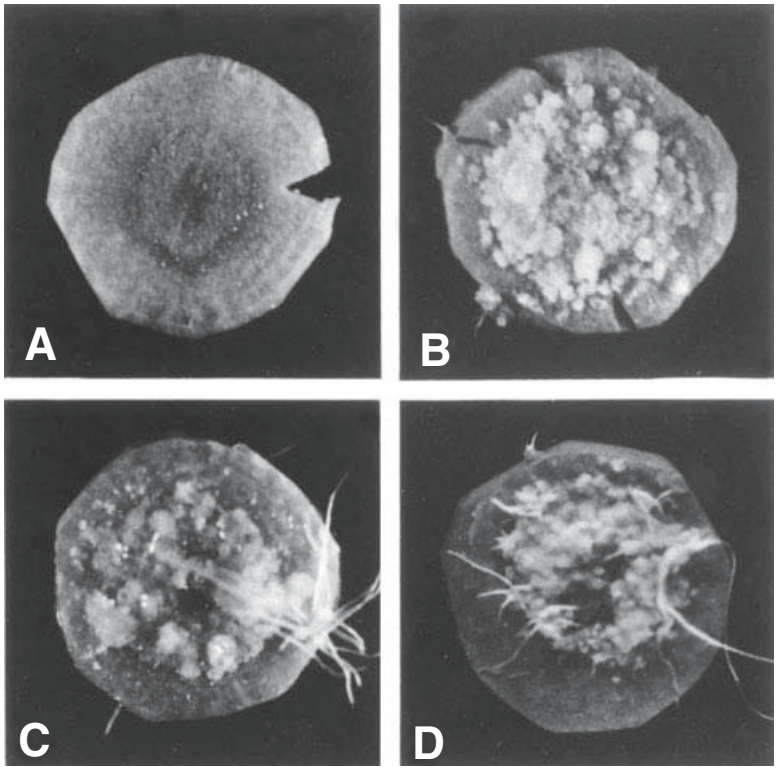

Fig. 3. A leaf of *Bryophyllum daigremontiana* (also called *Kalanchoë daigremontiana*) inoculated with *A. tumefaciens*. The site on the back right was inoculated with a strain lacking the Ti plasmid. Tumors are shown after 4 weeks growth.

Fig. 4. A leaf of *Bryophyllum daigremontiana* (also called *Kalanchoë daigremontiana*) inoculated with *A. rhizogenes*. Note that the roots formed at the wound sites are branching and ageotropic. The leaf is shown 5 weeks after inoculation.

present in plant cells. For example, octopine results from the reaction of the *a*-amino group of arginine and the keto group of pyruvate to form octopine [N_2-(1-D-carboxyethyl)-L-arginine]. The resulting compound (the opine) accumulates in the tumor tissue and can be used by the inciting bacteria as a carbon and nitrogen source. The genes encoding the enzymes for opine utilization are located on the Ti plasmid (pTi) outside the T DNA (the bacterial DNA which is transferred to the host cell) and on the chromosome. In the case of octopine, nitrogen appears to be recovered by the arginase-urease pathway. The genes for these enzymes are chromosomal. The carbon from octopine may be utilized by conversion of arginine to ornithine and then to proline via ornithine cyclase. The gene for this enzyme is located on pTi and, in some strains, a second copy is found in the chromosome (Dessaux et al., 1986). Other opines and their constituent compounds include lysopine (lysine and pyruvate), octopinic acid (ornithine and pyruvate), histopine (histidine and pyruvate), nopaline (arginine and *a*-ketoglutaric acid), nopalinic acid (ornithine and *a*-ketoglutaric acid), agrocinopine A (sucrose-4′-phosphate and arabinose-2-phosphate), agrocinopine C (glucose phosphate and sucrose phosphate), agropine (mannitol and glutamine), cucumopine [4-carboxyl-4-(2-carboxyl)spinacine], leucinopine (leucine and *a*-ketoglutaric acid), succinamopine (asparagine and a-ketoglutaric acid) and vitopine. Ti plasmids generally encode the enzymes for the synthesis of one or more opines in the T DNA and the enzymes for the utilization of the same opine elsewhere on the plasmid. Many Ti plasmids are named and grouped by the opine(s) that the plasmid-induced tumors produce (Dessaux et al., 1998).

Motility and Flagella

A. tumefaciens is motile by means of circumthecal flagella (Fig. 5). Some investigators have also observed polar and lateral flagella. There are four flagellar genes (*flaA*, *flaB*, *flaC* and *flaD*; Deakin et al., 1999). These genes are closely related to each other and to the *flaA* and *flaB* genes of *Sinorhizobium meliloti*. Deletion of the *flaA*, *flaB*, and *flaC* genes results in nonmotile bacteria that are slightly attenuated (tumors are about 70% the size of those induced by wild type bacteria) when inoculated directly into a wound site (Chesnikova et al., 1997). Bacteria, when inoculated into soil surrounding a wounded plant, must be able to move to wound sites on the plant to form tumors and thus must have flagella in order to be virulent. *A. tumefaciens* is chemotactic to a number of substances released by roots including sugars, amino acids and dicarboxylic acids. Genes involved in chemotaxis and motility have been sequenced (Wright et al., 1998; Deakin et al., 1997). These genes have a high level of homology with genes from *S. meliloti* and *Rhodobacter sphearoides*.

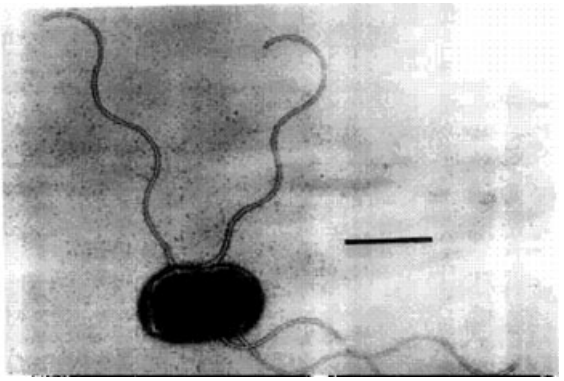

Fig. 5. *A. tumefaciens* as seen in the transmission electron microscope. Note the flagellae. The bar is 1 micron. From Brisbane et al. (1983).

Unique Aspects of *A. vitis*

Tartrate is found in grape sap. Most strains of *A. vitis* are able to degrade tartrate and use it as a carbon source. The genes for the metabolism of tartaric acid often are located on a plasmid, pTAR or pTr. A homologue of the *Pseudomonas ttuC* gene (encoding tartrate dehydrogenase) has been identified on these plasmids. Many of the tartrate plasmids are conjugative in *planta*, allowing their movement between strains (Otten et al., 1995; Salomone et al., 1996).

Genetics

General

Initial research on the agrobacteria suggested the presence of a circular chromosome and several large plasmids. More recent work has identified two chromosomes in *A. tumefaciens*. One is a roughly 3 Mbp circular chromosome that carries most of the known auxotrophic markers, and the other is a linear 2.1 Mbp chromosome to which some auxotrophic transposon mutants requiring adenine, threonine, serine, and pantothenic acid map (Jumas-Bilak et al., 1998; Goodner et al., 1999). Thus both DNA molecules can be considered chromosomes because they appear to contain genes required for normal cell functioning and metabolism. Most strains also have at least one large (approximately 300 kbp) cryptic plasmid, and virulent

strains have a Ti plasmid (approximately 200 kbp). Both the circular and linear chromosomes can be mobilized for conjugation by R68.41, a broad host-range conjugative plasmid (Goodner et al., 1999).

Chromosomal

A. tumefaciens Agrobacteria and *rhizobia* are closely related. The genetic maps of the circular chromosomes in *R. meliloti* and *A. tumefaciens* are very similar (Hooykaas et al., 1982). After *R. leguminosarum* plasmids were introduced into *A. tumefaciens*, the resulting bacteria could induce the formation of nitrogen-fixing nodules on the roots of beans. This finding suggested that all of the chromosomal genes required for the interaction of rhizobia with legumes are already present in the *A. tumefaciens* genome (Martinez et al., 1987). A variety of chromosomal genes from *A. tumefaciens* have been studied. Most of them are involved in the interaction of the bacterium with the plant. A summary of some of these genes is given below. They are grouped as follows: genes involved in exopolysaccharide synthesis, genes involved in attachment to plants, other genes required for virulence, and heat shock genes.

Genes Involved in Exopolysaccharide Synthesis
A. tumefaciens like *S. meliloti* makes a succinoglycan exopolysaccharide, which appears to have the same structure in both species. The pathway and the genes involved (named *exo* genes) for succinoglycan biosynthesis have been determined in *S. meliloti* (Reuber et al., 1993) and appear to be similar in *A. tumefaciens*. Mutations in the genes required for the synthesis of succinoglycan affect nodule formation in *S. meliloti*. However, similar mutations have been reported not to affect the virulence of *A. tumefaciens* (Cangelosi et al., 1987). Succinoglycan is also not required for colonization of tomato roots by *A. tumefaciens*, but mutations in *exo* genes did affect the ability of the bacteria to colonize legume roots (Matthysse, 2000).

In addition to succinoglycan, *A. tumefaciens* makes cellulose (Figs. 6–8). Genes required for cellulose synthesis (*cel* genes) are located on the bacterial chromosome near, but not adjacent to, the *att* genes (Robertson et al., 1988). Five genes organized into 3 operons (*celAB*, *celC* and *celDE*) have been identified. The *celD* and *celE* genes encode putative cytoplasmic proteins. The *celA*, *celB*, and *celC* genes encode putative membrane proteins. The CelA protein has homology to cellulose synthases from *Acetobacter xylinum*, *R. leguminosarum*, *P. fluorescens*, and plants. The C-terminal region of the CelC protein, which may be a glucosyl-transferase, has homology to endoglucanases from *E. chrysanthemi* and *A. xylinum*. The CelB, CelD, and CelE proteins have no significant homology with proteins in the databases (Matthysse et al., 1995). Although *A. tumefaciens* synthesizes some cellulose when grown in culture, the quantity increases markedly when the bacteria are grown with plant cells or roots. The mechanism by which the amount of cellulose synthesized is regulated is unknown. Mutants in *cel* genes are markedly reduced in virulence.

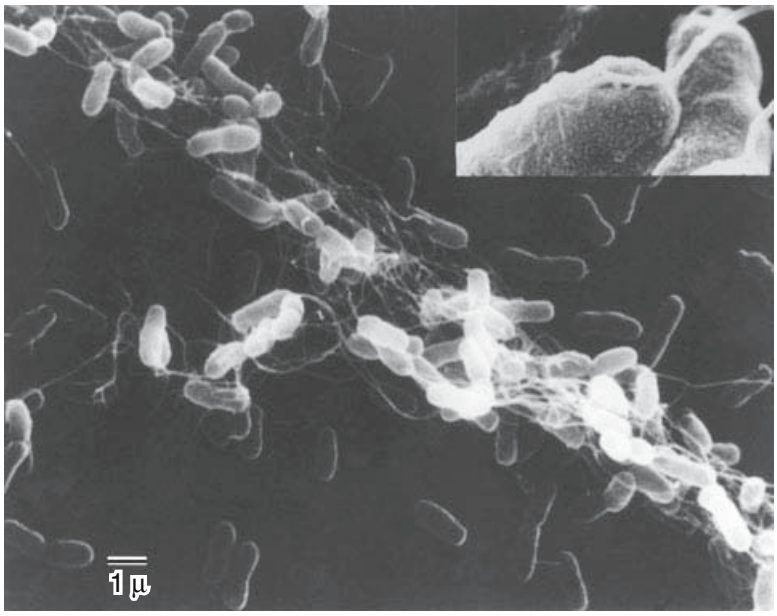

Fig. 6. Scanning electron micrograph showing *A. tumefaciens* grown with plant extract (soytone). The fibrils are cellulose made by the bacterium.

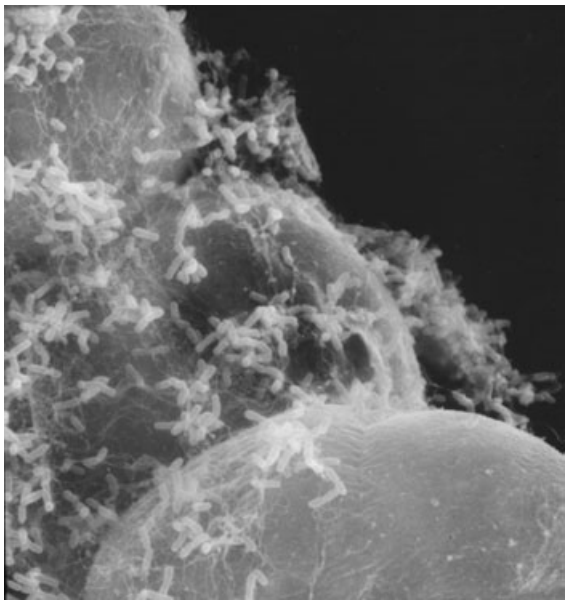

Fig. 7. Scanning electron micrograph of carrot suspension culture cells incubated with *A. tumefaciens* for 18 hours. Note the presence of both individually attached bacteria and clusters of bacteria on the surface of the plant cells. The fibrillar meshwork is cellulose fibrils made by the bacteria. From Matthysse et al. (1981).

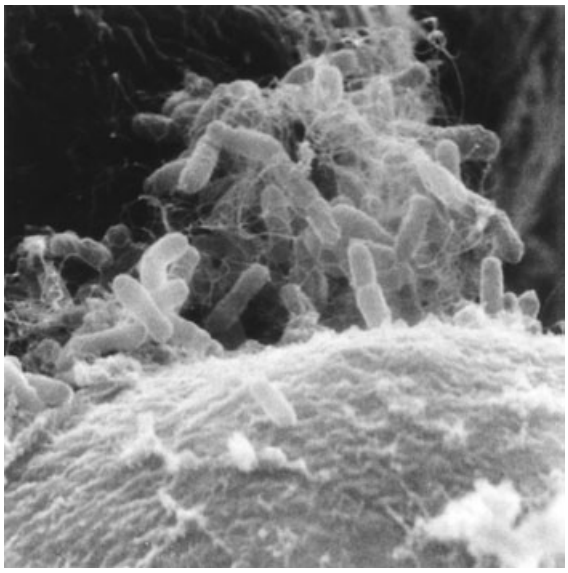

Fig. 8. Scanning electron micrograph of a carrot suspension culture cell incubated with *A. tumefaciens* for 18 hours. Note the presence of both individually attached bacteria and clusters of bacteria on the surface of the plant cells. The large cluster appears to be held together by cellulose fibrils produced by the bacteria. The majority of the bacteria adhering to the plant cell surface appear to be indirectly bound to the plant cell in large aggregates. From Matthysse et al. (1981).

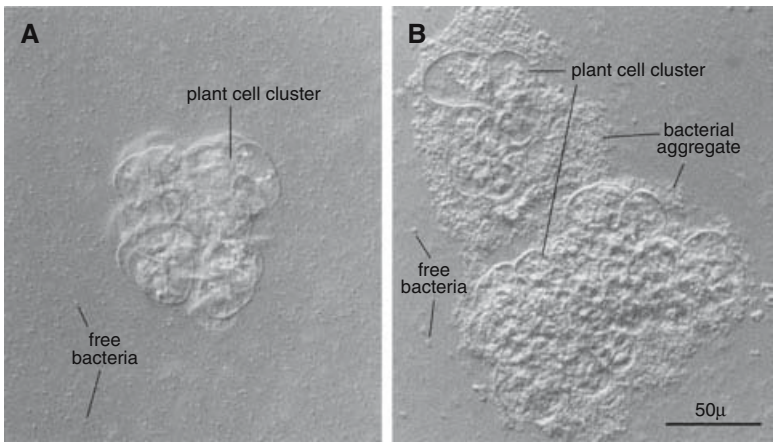

Fig. 9. Light micrographs showing bacteria (with and without attachment genes) incubated with carrot suspension culture cells for 24 hours. (A) A strain of *A. tumefaciens* lacking attachment genes. (B) Wild type *A. tumefaciens*.

Genes Involved in Initial Interactions with the Plant The region of the bacterial chromosome (*att*) in which transposon insertions result in an inability of the bacteria to attach to plants and thus in a lack of virulence is large (more than 35 kb). This region contains more than 32 open-reading frames (ORFs) organized in a minimum of 10 operons (based on the directions of the ORFs; Matthysse et al., 2000; Figs. 9–14). Many of these ORFs have no significant homology to any proteins in the databases. *Att* genes appear to fall into at least two categories: those that can be complemented by conditioned medium (CM) are presumably involved in signaling, and those that are not complemented by CM are presumably involved in the synthesis of the substance(s) that bind the bacteria to the plant surface. The *attA1–E* genes have homology to ABC transporters from a number of bacteria. Conditioned medium complemented the mutations in these genes. The genes are believed to be involved in signaling between the bacteria and the plant

(Matthysse et al., 1996). Conditioned medium also complemented several other *att* mutations. These genes are presumably also required for initial signaling. Conditioned medium did not complement mutations in *attJ*, *attK*, *attN*, *attO*, *attR*, or *attV-Z* genes. *AttR* mutants fail to make an acetylated capsular polysaccharide, which may be involved in binding of the bacteria to the plant surface (Reuhs et al., 1997). Three *att* ORFs products have homology to genes encoding DNA-binding proteins and may be involved in the regulation of gene expression. These ORFs are atrA, attJ and attO (Table 3).

Other known genes (aside from *att* and *cel*, which are required for the initial binding of the bacteria to the plant) appear to be involved in specifying for the general surface structure of the bacteria. These include the *chvAB* genes, which are required for the synthesis of β-1,2-D-glucan and the bacterial response to low external osmotic pressure, and the *pcsA* (*exoC*) gene, which encodes a phosphoglucomutase (Zorreguita et al., 1988; Thomashow et al., 1987; Leigh et al., 1992). Mutations in these genes, which are chromosomal, result in pleiotropic alterations in the bacterial surface. The *chvAB* mutants are avirulent on most but not all host plants. On some plants, they are virulent only at lower temperatures (19° but not 28°C; Banta et al., 1998). No Ti plasmid genes are known to be involved in the initial interactions of the bacteria with the plant.

Other Chromosomal Genes Involved in Pathogenesis Chromosomal genes involved in pathogenesis described elsewhere in this section include *att* genes, which are required for virulence; *cel* genes, which when mutated result in severe attenuation of virulence; *chvAB* genes, which are required for virulence on most hosts at normal (as opposed to cooler) temperatures; the *pscA* gene, which is required for virulence; and the *chvE* gene, which increases the induction

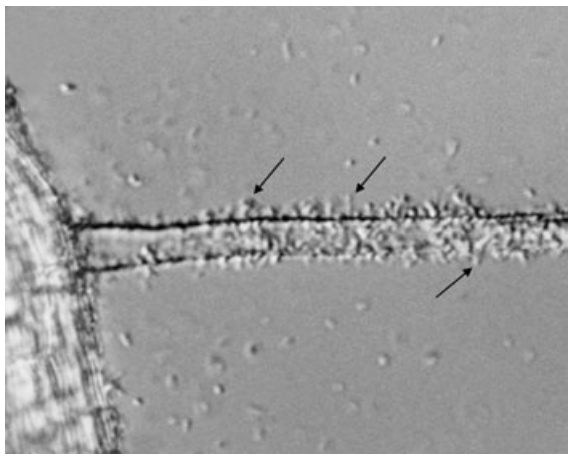

Fig. 10. Light micrograph showing *A. tumefaciens* strain C58 incubated with *A. thaliana* roots. Note the bacteria bound to the root hair surface preferentially in a vertical orientation (arrows). The orientation of bacterial binding varies with the strain examined.

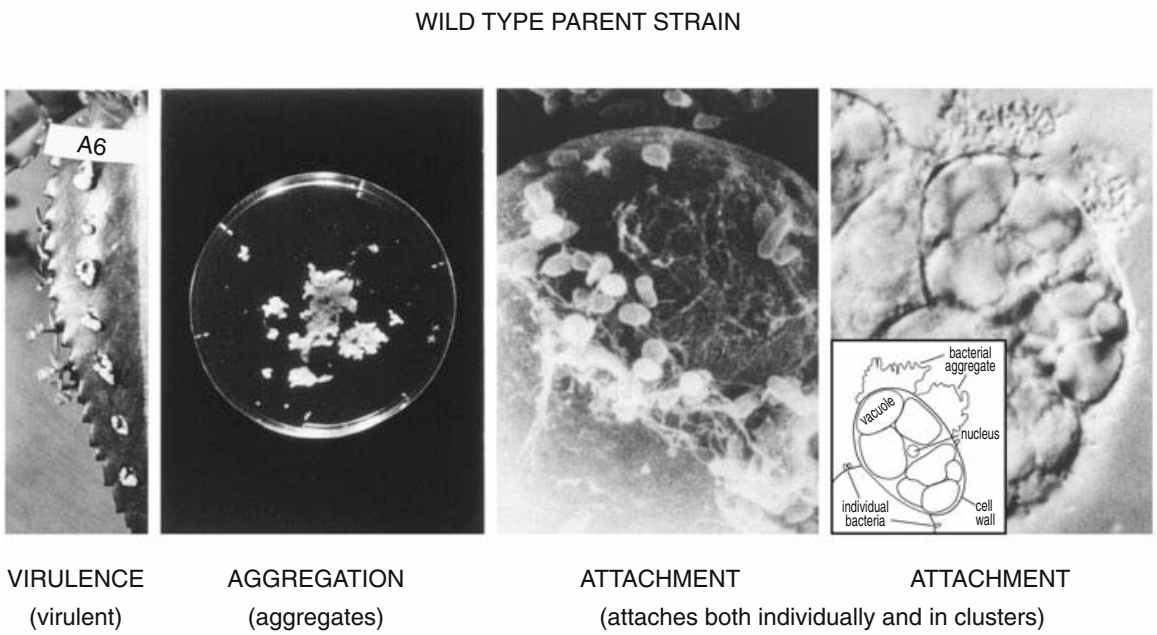

Fig. 11. Interactions of wild type *A. tumefaciens* with plants. From left to right: tumors on a leaf of *Bryophyllum daigremontiana*; aggregation of carrot suspension culture cells; scanning electron micrograph showing attachment to carrot cells; light micrograph showing attachment to carrot cells.

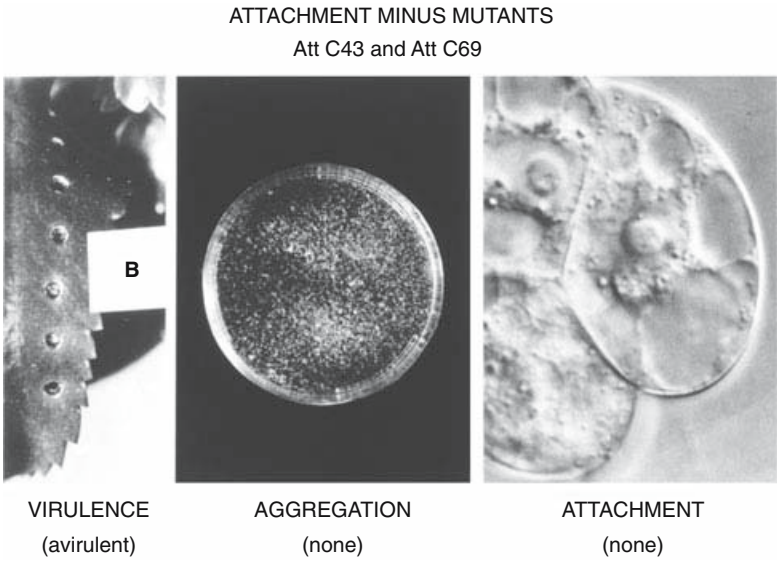

Fig. 12. Interactions of attachment-minus mutant *A. tumefaciens* with plants. From left to right: lack of tumor formation on a leaf of *Bryophyllum daigremontiana*; failure to aggregate carrot suspension culture cells; light micrograph showing lack of attachment to carrot cells. The concentrations of bacteria and plant cells in these incubations were the same as those used in Fig. 11 showing similar incubations of wild type bacteria.

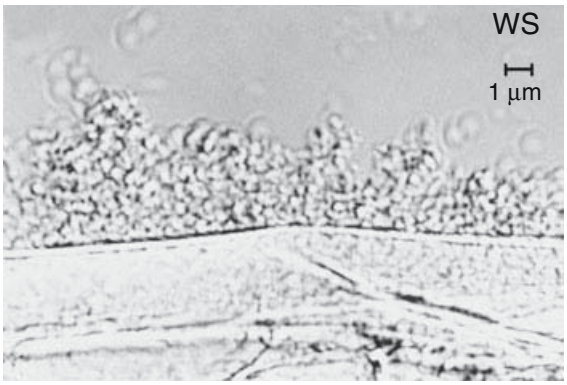

Fig. 13. Light micrograph showing *A. tumefaciens* bound to the surface of a root of *Arabidopsis thaliana*.

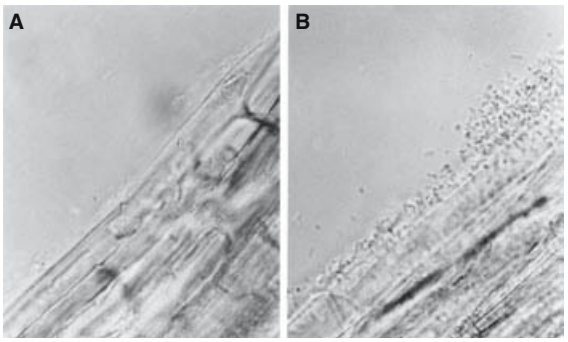

Fig. 14. Light micrographs showing *A. tumefaciens* wild type (B) and nonattaching mutant (A) incubated with tomato roots.

of *vir* genes by acetosyringone. Other chromosomal genes may affect the induction of *vir* genes. The *Ivr* (induction of *vir* genes) mutants are deficient in *vir* gene induction, although the mechanism is not understood (Metts et al., 1991). Some chromosomal genes are involved in *vir* gene induction on specific hosts. Thus some bacterial strains which are virulent on conifers carry a gene (*cbg*) that converts coniferin (which is not a *vir* gene inducer) to coniferyl alcohol (which is a *vir* gene inducer; Castle et al., 1992; Morris et al., 1990). Expression of *virC* and *virD* is regulated by a chromosomal gene *ros*, which encodes a repressor of these genes (Table 3). Ros mutants do not make succinoglycan, suggesting that *ros* is also involved in regulating the expression of *exo* genes. However, Ros mutants are still virulent on all hosts tested (Cooley et al., 1991a; Cooley et al., 1991b; Tiburtius et al., 1996; Hussain et al., 1997).

A two component system, *chvG*/*chvI*, is required for virulence and *vir* gene induction (Table 3). Mutations in these genes have pleiotropic effects; the mutant bacteria are sensitive to detergents and do not grow on complex medium. Because of the severely limited growth of these mutants, it is difficult to assess whether the requirement for *chvG*/*chvI* for virulence is due to a direct requirement for the gene products or to an indirect effect. The signal to which this system responds is unknown (Charles et al., 1993; Mantis et al., 1993).

Mutations in another chromosomal gene, *chvD*, reduce the induction of *virG* by acidic pH and phosphate starvation. This gene encodes a periplasmic ATPase (Winans et al., 1988). One gene, *acvB*, present only on the chromosome in nopaline strains, is required for virulence in these strains. In octopine strains, a second copy of this gene (*virJ*) is located on the Ti plasmid

Table 3. Regulatory genes in *A. tumefaciens* which affect expression of genes involved in the interaction with the host.

Gene	Location	Signals to which gene responds	Mutant phenotype	References
virG[a]	pTi	Acetosyringone and monosaccharides, acid pH, low phosphate	Avirulent, does not express *vir* genes	Winans et al., 1988 Roitsch et al., 1994 Stachel et al., 1985
occR	pTi	Octopine	Can not catabolize octopine, does not express *occ*, *ocd*, *oph*, *msh*, or *traR* genes	Cho et al., 1997
traR	pTi	Homoserine lactone (AAI autoinducer)	Can not conjugate pTi between bacteria, does not express *tra* or *trb* genes	Alt-Morbe et al., 1996 Fuqua et al., 1994 Hwang et al., 1995 Hwang et al., 1994
ros	Chromosomal	Iron?	Succinoglycan (EPS) minus, *virD* and *virC* constitutive	Cooley et al., 1991a Cooley et al., 1991b Tiburtius et al., 1996 Hussain et al., 1997
*chvI**	Chromosomal	? Unknown	Does not grow in complex media, acid and detergent sensitive, avirulent, *vir* gene expression attenuated	Charles et al., 1993 Mantis et al., 1993
atrA	Chromosomal	? Unknown	Avirulent, does not bind to plant	Matthysse et al., 2000
attJ	Chromosomal	? Unknown	Avirulent, does not bind to plant	Matthysse et al., 2000
attO	Chromosomal	? Unknown	Avirulent, does not bind to plant	Matthysse et al., 2000

[a]Member of a two component system. The sensor genes are *virA* and *chvG*, respectively.

(Kalogeraki et al., 1995). In octopine strains, both genes must be mutated to observe a requirement for the gene for virulence.

Heat Shock Genes Two heat shock operons, *groESL* and *dnaKJ*, have been cloned from *A. tumefaciens* (Segal et al., 1995a; Segal et al., 1995b). The *groESL* operon contains an inverted repeat (IR) that appears to be involved in regulation of this operon's expression (Segal et al., 1995a). This inverted repeat is conserved in many bacteria (Segal et al., 1999). It acts as a transcription inhibitor, probably binding a protein repressor (Segal et al., 1996). Expression of the *groESL* operon is regulated not only at the level of transcription, but also at the level of translation by two different systems: the inverted repeat controls the stability of the transcript (Segal et al., 1996), and a temperature-activated RNA processing system specifically cleaves the mRNA of the operon, resulting in differential transcript stability (Segal et al., 1995c). In addition, the *rpoD* gene which encodes the vegetative transcription activator σ70 has been cloned (Segal et al., 1995d). This gene is a heat shock gene in *E. coli*, but it is not part of the heat shock regulon in *A. tumefaciens* (Segal et al., 1995d). A new heat shock promoter that is common to many α-purple bacteria, including *A. tumefaciens*, *Rhizobium meliloti* and *Bradyrhizobium*, has been identified. This promoter is recognized by a heat shock transcriptional activator, a homologue of σ32, which has been cloned from *A. tumefaciens* (Nakahigashi et al., 1995; Segal et al., 1995b).

A. rhizogenes There is relatively little work on the chromosomal genes of *A. rhizogenes*. They are generally believed to be similar to those of the other agrobacteria. However, differences in the binding of biotype 2 *A. rhizogenes* strains to plant cells suggest that at least some of the *att* genes must differ between biotype 1 *A. tumefaciens* and biotype 2 *A. rhizogenes* (Sykes et al., 1988). Differences of biotype 1 and 2 strains in their sensitivity to salt and in the sugars that they are able to metabolize suggest that there may be many differences in chromosomal genes between these strains.

A. vitis Genes are involved in the breakdown of pectin are important virulence factors in *A. vitis*. The genes affecting synthesis of polygalacturonase and endoglucanase are both chromosomal (McGuire et al., 1991). The *pehA* gene has been cloned and sequenced and appears to be closely related to *peh* from *Ralstonia solanacearum* and *Erwinia carotovora* (Herlache et al., 1997). Polygalacturonase mutants are reduced in virulence on grape (Rodriguez et al., 1991).

Ti Plasmid Genes

The *A. tumefaciens* Ti plasmid is about 200 kb. The complete sequences of 3 Ti plasmids are

available. Major groups of genes located on pTi include: the T DNA (the DNA transferred to the host cell), *vir* genes (genes required for, or associated with, virulence), and genes involved in quorum sensing, Ti plasmid conjugation, and opine metabolism.

T DNA The T DNA is the DNA fragment transferred to the host cell whose expression results in tumor formation. Located on the Ti plasmid, T DNA is bounded by 24 base-pair direct repeats referred to as the border sequences. Some Ti plasmids have two segments of T DNA (called TR and TL) with four border sequences. The segments bounded by the border sequences may be transferred individually or as an entire piece carrying both TR and TL. The T DNA carries genes for enzymes for plant growth hormone biosynthesis, for the modification of plant responses to growth hormones, and for the synthesis and secretion of opines. (See Physiology of Opines in this Chapter.)

Two enzymes encoded in the T DNA (tryptophan monooxygenase and indole-3-acetamide hydrolase) carry out the biosynthesis of auxin (indole-3-acetic acid, IAA). Tryptophan monooxygenase (encoded by *iaaM*) converts tryptophan into indole-3-acetamide, and indole-3-acetamide hydrolyase (encoded by *iaaH*) converts indole-3-acetamide into auxin. This pathway of auxin biosynthesis from tryptophan differs from that used by most plants and is the same as that used by some other plant pathogenic bacteria, notably *Pseudomonas savastanoi*, which infects olive and oleander, producing tumors. No DNA transfer is involved in this infection. The *P. savastanoi* genes are expressed in the bacterium, whereas the *A. tumefaciens* genes for auxin biosynthesis, which have eukaryotic promoters, are expressed in the plant host. Host biosynthesis of cytokinins is increased by the expression of the T DNA gene *ipt*, which encodes an isopentyl transferase. These genes encoding enzymes involved in plant hormone biosynthesis have constitutive eukaryotic promoters. Their expression and the resulting overprodution of auxin and cytokinins cause tumor formation. Two other genes carried on the T DNA appear to regulate the response of the plant to hormones, although their mechanism of action is not understood. These are gene 6b in which mutations give rise to large tumors and gene 5 in which mutations have a phenotype only in *iaa*$^-$ mutants. In this background gene 5 mutants produce an increased shoot-like tumor morphology. *Gene 5* encodes an indole lactate synthase. However, the role of indole-3-lactic acid in the plant is unknown (Binns et al., 1998).

The T DNA also encodes enzymes for the synthesis and secretion of opines, peculiar compounds made by the plant only in crown gall tumors. These genes are also preceded by constitutive eukaryotic promoters. It is thought that it is opine production by the tumor cells which results in the advantage to the bacteria in causing tumor formation. The bacteria possess genes for enzymes to metabolize these opines, which they can use as carbon and nitrogen sources. These genes are located outside the T DNA but on the Ti plasmid. Different Ti plasmids encode the enzymes for the production of different opines and their utilization. Ti plasmids and bacterial strains are often referred to by the opine(s) that they produce and use, e.g., octopine or nopaline strains.

The Ri plasmid of *A. rhizogenes* also contains a T DNA. Agropine Ri plasmids carry *iaaH* and *iaaM* genes in their T DNA, but hairy root formation by *A. rhizogenes* is not due to excess auxin production. Other Ri plasmids such as the mannopine and cucumopine plasmids don't contain *iaa* genes. Instead, the *rol* genes, particularly *rolB*, appear to be responsible for the growth of hairy roots (branched, ageotropic roots). There are four *rol* genes, *rolA*, *rolB*, *rolC*, and *rolD*. The mechanism by which they cause root formation and their biochemical activities are not understood, although their sequences are known (the whole of the T_L region of pRi has been sequenced). The *rol* genes appear to modify the hormone receptors or action of growth hormones in the plant. RolB alone is necessary and sufficient to cause full hairy root growth. Fertile plants can easily be regenerated from hairy roots on most species of plants. However, the regenerated plants have an altered morphology due to *rolA*, *rolB*, *rolC*, *rolD*, and ORF13. Ri plasmids contain genes for the synthesis of opines similar to those found on Ti plasmids (Binns et al., 1998).

vir Genes The transfer of T DNA to the host plant is dependent on *vir* genes and the presence of 24 bp direct repeats at the ends of the T DNA (called the border sequences). The particular sequences contained between the border sequences do not influence the DNA transfer. The *vir* genes include *virA* and *virG*, which are a two component system including a sensor for acetosyringone (VirA, an inner membrane protein) and a regulator (VirG, a DNA-binding protein that can be phosphorylated by VirA) which activates the expression of the other *vir* genes (Leroux et al., 1987). In addition to acetosyringone, expression of genes in the *vir* regulon is increased by acid pH and by the presence of monosaccharides. The latter response involves the binding of the sugar by the product of a chromosomal gene, *chvE* (Huang et al., 1990).

ChvE is a periplasmic galactose-binding protein and is required for chemotaxis to several sugars as well as influencing *vir* gene induction. ChvE mutants are avirulent on some but not all plants (Cangelosi et al., 1990; Huang et al., 1990). *VirD1* and *VirD2* encode an endonuclease that cuts the Ti plasmid DNA at the T DNA border sequences (Yanofsky et al., 1986). The *virE2* gene encodes a single-stranded DNA-binding protein (Christie et al., 1988). The *virB* operon encodes a DNA transfer system including a pilus analogous to that used by some conjugative plasmids including pRSF1010, pRP4, and pKM101, as well as to that used by the transport system for *Bordetella pertussis* toxin (Pohlman et al., 1994; Kado, 1994; Zupan et al., 1995; Shirasu et al., 1994; Lessi et al., 1994).

Of the *vir* genes, only *virA* and *virG* are expressed in the absence of inducers. These genes make up a two-component system. VirA, the sensor, is a plasma membrane protein that responds to phenolic compounds such as acetosyringone released by the plant. These inducers are most effective at low pH (5.3–6.0) and in the presence of monosaccharides. The sugars are bound by ChvE, which then interacts with VirA, increasing the response of VirA to the phenolic inducers. Low phosphate concentrations also increase the induction of vir genes. In response to the inducers, VirA autophosphorylates at a histidine residue and then transfers the phosphate to an aspartic acid in VirG. Phosphorylated VirG binds to specific DNA sequences referred to as *vir* boxes located 24 to 72 bp upstream of the *vir* operons and activates the *vir* genes' expression. VirG also binds to *vir* boxes located upstream of *virG* and thus increases its own synthesis (Johnson et al., 1998).

The *virB* and *virD* operons show considerable homology to genes required for conjugation between bacteria and they can mediate the conjugative transfer of other plasmids such as RSF1010. The *virD* operon contains five ORFs. VirD1 and VirD2 bind to the T DNA border sequences and nick the DNA between the third and fourth base of the repeat sequence on the bottom strand. VirD2 is covalently linked via a phosphotyrosine linkage to the 5′ end of the nicked DNA. DNA homology suggests that the border sequences and *virD2* belong to the P-family of conjugal transfer systems, which includes RP4, RK6, and R64. In some Ti plasmids, a sequence is located near the right border outside the T DNA, referred to as overdrive. VirC2 binds to this sequence and increases the efficiency of the formation of single-stranded nicks by VirD2. VirC1 (the other protein endcoded by the *virC* operon) may aid in this process. VirD4 is an integral membrane protein required for transfer of T DNA to host cells. It belongs to the class of TraG-like proteins, which are required for pilus formation. The *virB* operon contains 11 ORFs; nine of these encode membrane proteins. All except *virB1* are required for T DNA transfer. Mutants in *virB1* retain the ability to transfer T DNA but at a hundred-fold reduced efficiency. VirB1 is a secreted protein with an N-terminal region that resembles lysozyme. The remainder of the *virB* genes appears to be involved in the elaboration of a pilin and of a pore for the transfer of T DNA. This structure is formed best at cool temperatures (19–22°C). Sequence and structural studies suggest that VirB2 is the pilin. It may be processed after synthesis to a circular peptide. VirB7 is a lipoprotein and forms a tight complex with VirB4, VirB9, VirB10 and VirB11. VirB4 and VirB11 have ATP-binding sites and possess ATPase activity. The conjugation system encoded by *virD4* gene and the *virB* operon belongs to the group of bacterial conjugation systems that have their highest transfer efficiency on surfaces rather than in liquid suspension. This system is capable of transferring other DNAs as well as T DNA. Using this system, *A. tumefaciens* can transfer pRSF1010 to other bacteria or to plant cells (Johnson et al., 1998).

The *virE* operon contains two ORFs: *virE1* and *virE2*. VirE2 is a single-stranded DNA-binding protein. VirE2 is not required for T DNA transfer, although it is required for efficient tumor formation. VirE2 mutants can be complemented in trans by another *A. tumefaciens* strain, which does not have to contain T DNA, but must contain the *virB* and *virE* operons and *virD4*. Transgenic plants expressing *virE2* can also complement VirE2 mutants. It appears that the VirE2 (and VirF) proteins can be transferred to the host cell independently of the transfer of T DNA. This transfer requires the *virB* operon and *virD4* gene products as well as VirE1. VirE2, a single-stranded DNA-binding protein, may serve to protect the T DNA from degradation in the plant cell. Both VirE2 and VirD2 contain sequences that are similar to plant nuclear localization signals (NLS) and may aid targeting of the T DNA to the plant nucleus. Integration of the T DNA into the plant chromosomal DNA appears to proceed via illegitimate recombination into nonhomologous DNA at random and to involve proteins supplied by the plant rather than the bacterium (Johnson et al., 1998; de la Cruz et al., 1998; Rossi et al., 1998).

VirG regulates the expression of several other Ti plasmid operons. However, the role of these operons in tumorigenesis is unclear. These include *virF*, which is present in octopine Ti plasmids only. *VirF* mutants fail to cause tumors when a low number of bacteria are used in the

inoculation. They also fail to form tumors on some plants, including *Nicotiana glauca*. VirF appears to be transferred to the host cell along with VirE2. The *virH* operon contains four ORFs. Two of these have homology to cytochrome P450 enzymes. This operon is present in octopine and nopaline Ti plasmids and in some Ri plasmids (Deng et al., 1998).

The *virJ* gene is present on the Ti plasmid and in some strains also on the chromosome. The chromosomal copy of the gene is called *acvB*. VirJ may be associated with the T strand DNA (Kalogeraki et al., 1995). The *tzs* gene encodes a cytokinin prenyltransferase, which forms transzeatin. It is present in nopaline, agropine, and mannopine Ti plasmids but not in octopine and succinamopine Ti plasmids. It also is present in some Ri plasmids.

There is evidence to suggest that *virA*, *virC*, *virE*, *virF*, and *tzs* affect the host range of *A. tumefaciens*.

Genes Involved in Quorum Sensing *A. tumefaciens* like many other Gram-negative bacteria has a quorum-sensing system. The genes involved in this system are located on the Ti plasmid and are involved with the bacterial response to opines and with the conjugation of pTi between bacteria. They include *traR* and *traI*, which are homologues of the *luxI* and *luxR* genes of *Vibrio fisherii*. These genes have similar functions to the *V. fisherii* genes; *traI* encodes the enzyme for the synthesis of a homoserine lactone [N-(3-oxo-octanoyl)-L-homoserine lactone], which is the *Agrobacterium* autoinducer (AAI), and *traR* encodes a regulatory protein which binds AAI and DNA. The enzymatic activity of TraI has been demonstrated in vitro; it catalyzes the synthesis of AAI from 3-oxo-octanoyl-ACP (Acyl Carrier Protein) and S-adenosyl-methionine. The TraR protein plus AAI activates several promoters including the *traA* and *traC* promoters (to induce conjugation of bacteria which results in the transfer of pTi between bacteria), the *traI* promoter (a promoter upstream of an operon that includes *traR*) and the *traM* promoter. These observations suggest that the level of TraR in the cell is regulated by positive regulatory feedback loops involving the activation of TraI synthesis to make more AAI and the activation of *traR* expression. There is also apparently a negative feedback loop since TraM is an antagonist of TraR (Winans, 1998; Fuqua et al., 1996).

The expression of *traR* is controlled by *occR* in octopine and by *accR* in nopaline/agrocinopine Ti plasmids (see also Plasmid Conjugation) in response to the presence of octopine or agrocinopine. Thus the genes involved in quorum sensing are only expressed in the presence of the opine made by a crown gall tumor (or by plant cells which have received the opine synthesis genes from pTi). Why quorum sensing should only be necessary in the presence of transformed host cells is unclear (Winans, 1998; Fuqua et al., 1996).

Genes Involved in Plasmid Conjugation to Other Bacteria In addition to transferring a segment of DNA to the host cell (the T DNA), the Ti plasmid can be transferred from one bacterium to another. The genes for this conjugation are distinct from the *vir* genes involved in the transfer of T DNA, although both sets of genes are located on pTi. The *tra* and *trb* genes are required for this process. These genes show homology to the conjugation systems of other plasmids including the 11 *trb* genes of RP4, an IncP plasmid. The *tra* regulon of octopine Ti plasmids is positively regulated in a complicated manner. The *occR* gene product activates the transcription of *traR* in the presence of octopine. The *traR* gene product is homologous to *luxR* and activates the transcription of the *tra* genes in the presence of the *Agrobacterium* autoinducer (AAI), a homoserine lactone which is produced by *traI* (a *luxI* homologue). In nopaline Ti plasmids, AccR regulates the expression of *traR* by acting as a repressor. The inducer is agrocinopine. The result of this regulatory cascade is that conjugation of the Ti plasmid occurs only in the presence of transformed plant cells required to produce opines and a high bacterial population density required to produce a sufficient concentration of the autoinducer. These facts explain the early observations of Kerr et al. (1977a) that strains of *Agrobacterium* could only be induced to transfer the Ti plasmid by conjugation when the donor and recipient strains were inoculated together into a wound site on a susceptible host plant. The advantage to the bacterium in regulating the conjugation of pTi in this manner is unknown (Farrand, 1998).

A. vitis

The genetics and diversity of the Ti plasmids found in strains of *A. vitis* have been studied extensively. Three types of pTi have been characterized: plasmids with one large T DNA that encodes the enzymes for the synthesis of the opines octopine and cucumopine, plasmids with small T DNA that lead to octopine and cucumopine synthesis, and plasmids of the nopaline type Ti plasmids. Some of these Ti plasmids contain two T DNAs. The *vir* regions are conserved among all three types of pTi. One pTi from a vitopine strain (S4) belongs to a different incompatibility group than the other Ti

plasmids belong to (Szegedi et al., 1996). The limited host range of some *A. vitis* strains appears to be due to the *virA* gene since the host range can be extended by replacing this gene with *virA* from *A. tumefaciens* strain A6 (Yanofsky et al., 1985).

The genes of the T region of *A. vitis* include *iaaM*, whose product catalyzes the formation of indoleacetamide (IAM) from tryptophan, and *iaaH*, whose product catalyzes the conversion of IAM into the plant hormone indoleacetic acid (IAA). Wide host range strains also contain the *ipt* gene, which encodes an isopentenyl transferase required for the synthesis of cytokinins (Hoekema et al., 1984). Some limited host-range strains lack the *ipt* gene. Strong expression of *ipt* in grape is toxic to the cells unless *ipt* expression is accompanied by strong expression of the *iaa* genes, particularly *iaaM* (Huss et al., 1990). At least one pTi from *A. vitis* also carries the genes for the metabolism of tartaric acid. In other strains, these genes are carried on a separate plasmid. (See Physiology in this Chapter.)

Ecology

A. tumefaciens

Agrobacteria generally persist for long periods of time in the soil, particularly in the rhizosphere of susceptible host plants. When inoculated onto cherry, the bacteria established populations of about 10^5 CFU per gram of plant tissue and persisted for 2 years (the duration of the experiment). The biocontrol strain of *Agrobacterium* K84 (see Applications) also showed similar population densities and long persistence when inoculated onto several plants. However, bacterial persistence in fallow soil was much reduced; bacterial populations declined from 10^5 per gram soil to 10 in as short a time as 16 weeks. The bacteria could apparently move out from the inoculated rhizosphere and were recovered as far as 40 cm from the original plants (Stockwell et al., 1993). In other studies, agrobacteria were recovered from a fallow soil. The isolates were roughly evenly split between biotypes 1 and 2. About 1/3 of the isolates were pathogenic. All of these pathogenic strains belonged to biotype 1 (Bouzar et al., 1993b). It is often difficult to recover virulent *A. tumefaciens* from galls. In some cases this appears to be due to loss of virulence of bacteria growing with host plants. Belanger et al. (1995) found that when virulent strains isolated from apple were reintroduced onto to axenic apple plants, most of the bacteria recovered were avirulent. This was due to alterations in the pTi *vir* genes. The mutant strains grew faster in the presence of the plant than their virulent parents did and thus came to dominate the bacterial population.

Opines are thought to provide nutrition for *A. tumefaciens* and *A. rhizogenes* located near the transformed plant cells (Dessaux et al., 1993; Guyon et al., 1993). In at least some cases, opines can be transported from their site of synthesis to the rest of the plant and may be found in root exudates. Thus the production of opines at the site of infection may well influence the composition of the rhizosphere community at distant sites (Savka et al., 1996). Utilization of opines is not limited to agrobacteria. Several isolates of soil pseudomonads and coryneform bacteria have been shown to be able to use opines as carbon and energy sources (Beaulieu et al., 1988; Tremblay et al., 1987).

A. vitis

The fate of the bacteria on grapes colonized with *A. vitis* has been followed for 2 years after planting. The bacteria were still present and virulent although the grapes did not have galls or other obvious symptoms of disease (Burr et al., 1995). When the maintenance of bacterial populations was compared in infested soils planted with oats and those planted with grapes, higher population levels were maintained in the presence of grapes (Bishop et al., 1988).

Noncultivated grapes (*Vitis* spp.) have been examined for the presence of *A. vitis*. The bacteria were detected in association with more than half the plants examined. Interestingly the bacterial isolates, which appeared to be diverse as judged by DNA fingerprinting, were largely nonpathogenic (Burr et al., 1999). *A. vitis* is also routinely found in healthy grapes where it can cause gall formation at sites of injury. The bacteria may be found in the phloem or in the tissue just below the bark. Higher bacterial populations are usually recovered from susceptible as compared to resistant cultivars. Freezing injury may facilitate the spread of the bacteria in the plant (Burr et al., 1999).

Disease

General

Infections of wound sites in dicotyledonous plants by agrobacteria lead to the development of crown gall tumors or hairy roots. These abnormal growths result from the transfer of DNA sequences (the T DNA) from the bacterial tumor-inducing (pTi) or root-inducing (pRi) plasmid to the host plant cell. The T DNA sequences become incorporated into plant chromosomes and are maintained and expressed in the plant cell. They code for enzymes involved in

the synthesis of the growth hormones, auxin and cytokinin. It is the overproduction of these hormones that results in the growth of a tumor.

A. tumefaciens

The steps identified in tumor formation by *A. tumefaciens* include bacterial chemotaxis to wound sites in plants (Loake et al., 1988; Ashby et al., 1987); an exchange of signals between the plant and the bacteria (Matthysse, 1994); initial bacterial attachment to the surface of plant cells (Matthysse, 1986); induction by phenolics (including acetosyringone) released from the wounded plant of bacterial *vir* genes encoded on the Ti plasmid (Stachel et al., 1985); the formation of a pilus encoded by the *virB* operon (Fullner et al., 1996); the transfer of the T DNA sequences from the bacterium to the plant cell mediated by *vir* gene products (and possibly the products of other genes; Klee et al., 1983); the integration of the T DNA into plant chromosomes (Chilton et al., 1977); and the expression of the T DNA in the plant cell, leading to increased auxin and cytokinin production, resulting in uncontrolled growth of the cell to form a crown gall tumor (Binns et al., 1988; Akiyoski et al., 1982; Figs. 15–19). Other genes expressed in the plant cell are included in the T DNA, most notably the genes encoding the opine biosynthetic enzymes (Tempe et al., 1984). The bacterial Ti plasmid contains the genes required for the ability to use these opines as carbon and nitrogen sources and thereby support bacterial growth. The benefit of tumor formation to the bacterium is thought to be explained by crown gall-opine production and the subsequent use of opines as substrates for bacterial growth.

Because a wound is required for the interaction of the bacteria with the host, the sites most often infected are the sites of graft junctions. Thus crown gall occurs most often on fruit trees, grapes, roses, and other plants that are routinely grafted. However, disease can be observed on plants that are not grafted. Agrobacteria are found in the soil and in the rhizosphere and easily infect wounds on the roots or at the base of the stem (the crown of the plant).

A. rhizogenes

Two other less well-known species of agrobacteria have a similar pathogenic mechanism. *A. rhizogenes* causes the formation of hairy roots on a wide variety of dicots. These roots are caused by the transfer of DNA from the bacterium to the host, which contains genes (*aux* and *rol*) that lead to the formation of ageotropic branched roots rather than tumors (White et al., 1985; Amselm et al., 1992). Aside from the difference in the T-DNA of the plasmid, this bacterium differs from *A. tumefaciens* in its ability to grow on various carbon sources and in its low tolerance to NaCl (Kerr et al., 1977b). These traits presumably reflect differences in the bacterial chromosomes. Insofar as is known, the mechanism of pathogenesis of *A. rhizogenes* is similar to that of *A. tumefaciens*. The Ri plasmid contains vir genes that are analogous to those of pTi, and the steps in DNA transfer described above for *A. tumefaciens* appear to be similar for *A. rhizogenes* (White et al., 1985).

A. vitis

Agrobacterium vitis causes the formation of tumors on stems of grapes. On grape roots it causes necrotic lesions (Burr et al., 1987). This necrosis results in part from the enzyme polygalacturonase, which is secreted by the bacteria. Other agrobacteria appear to lack this enzyme (McGuire et al., 1991; Herlache et al., 1997). Although some strains of *A. vitis* have a broad host range in the laboratory, in nature *A. vitis* appears to be largely a pathogen of grapes. The bacteria are capable of invading the vascular system of the plant and multiplying in the xylem without any apparent symptoms; indeed, apparently most grape plants have *A. vitis* resident in the xylem (Burr et al., 1995). Unlike *A. tumefaciens*, these bacteria are able to use tartrate (which is found in grape xylem in higher concentrations than in most other plants) as a carbon source (Crouzet et al., 1995). When an infected grape stem is wounded, tumors develop at the wound site. The mechanism of DNA transfer appears to be similar to that of *A. tumefaciens*. The Ti plasmid of *A. vitis* carries *vir* genes which are similar to those of *A. tumefaciens* except that some limited host-range strains lack *virC*. The T DNA is also similar to that of *A. tumefaciens* except for the lack of cytokinin biosynthesis genes in some limited host-range strains (Bonnard et al., 1989; Otten et al., 1994; Canaday et al., 1992; Paulus et al., 1991; Otten et al., 1993). There is little information about the interaction of either *A. rhizogenes* or *A. vitis* with prospective hosts prior to the induction of *vir* genes and the initiation of T-DNA transfer.

Control of Disease Caused by Agrobacteria

Biocontrol of Crown Gall

Biological control of some strains of *A. tumefaciens* can be achieved by dipping the planting material in a suspension of *Agrobacterium* strain

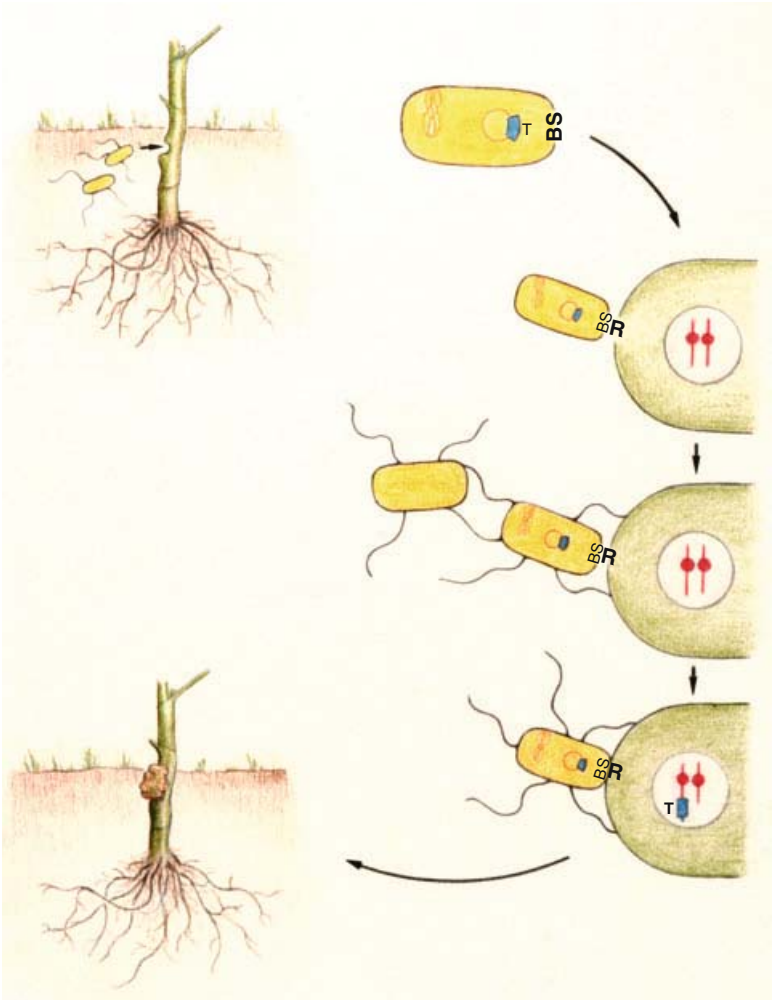

Fig. 15. Drawing showing the steps in the interaction of *A. tumefaciens* with a wounded plant leading to tumor formation. *A. tumefaciens* can be found in the upper layers of the soil. The bacteria are chemotactic to substances released from plant roots and wound sites. The bacteria (yellow) migrate to the wound site and bind to the surface of a plant cell (green). Substances from the plant induce bacterial cellulose synthesis, and the bacteria form aggregates on the plant surface held together with cellulose fibrils (only 2 bacteria are shown in the drawing for simplicity). The *vir* genes are induced and the bacteria transfer a piece of plasmid DNA, the T DNA (blue), to the host cell, where it becomes integrated into the host cell chromosomes. The expression of genes contained in the T DNA (which have eukaryotic promoters) leads to the overproduction of growth hormones in the plant cell and the resulting growth of a tumor. The tumor cells also produce opines which the bacteria use as a source of carbon and energy.

K84. These bacteria can also be applied to graft junctions during the grafting process. Strain K84 makes antibiotic-like compounds called agracins, which kill many strains of *A. tumefaciens*. A genetically engineered derivative of K84, K1026, is often used because it is unable to transfer resistance/immunity to agracins to the target pathogens. Strain K84 produces two agracins (K84 and K434). Agracin K84 only inhibits the growth of *Agrobacterium* strains carrying nopaline/agrocinopine type Ti plasmids. It is not effective against *A. vitis*. Agracin 434 is effective against biotype 2 strains. Strain K84 contains three plasmids: pAgK84 which encodes agracin K84 production, pAgK434 which encodes agracin K434 production and immunity to agracin K434, and pAtK84b which is a deleted pTi and carries nopaline catabolism and resistance to agracin K84. This last plasmid is self-transmissable (Clare et al., 1990). The structures and mechanisms of action of agracins K84 and K434 have been determined. There is some evidence that strain K84 may possess some biological control activity in addition to the production of the two agracins. However, currently this evidence is incomplete.

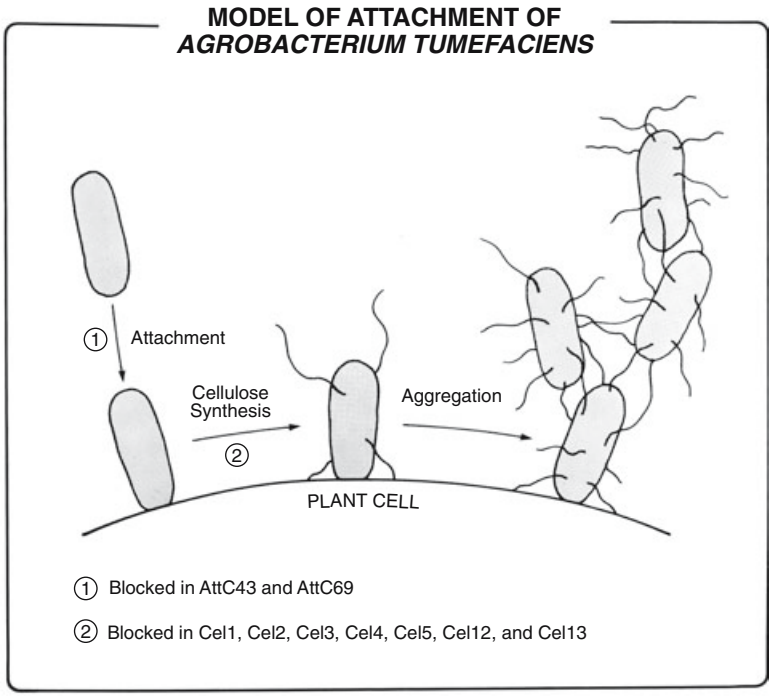

Fig. 16. Drawing showing the steps in bacterial aggregate formation on the surface of a plant cell. The bacteria are chemotactic to substances released by the plant cell. In response to substances released by the plant, the bacteria make and secrete a substance required for bacterial attachment to the plant surface. This step is blocked in *att* mutants, which can be complemented by conditioned medium. The bacteria then bind to the plant surface in a loose fashion and can be removed by shear forces such as water washing or vortexing of cultures. This step requires an acetylated capsular polysaccharide made by the bacteria. The bacteria elaborate cellulose fibrils. These fibrils bind the bacteria tightly to the plant surface. Shear forces can no longer remove the bacteria. The fibrils also trap other bacteria and these new bacteria are induced to form cellulose fibrils that in turn entrap additional bacteria, resulting in the formation of a large bacterial aggregate on the plant cell surface. Strong shear forces can remove the outer portions of these aggregates but the bacteria at the base of the aggregate are tightly bound to the host cell. Aggregate formation is blocked in *cel* mutants.

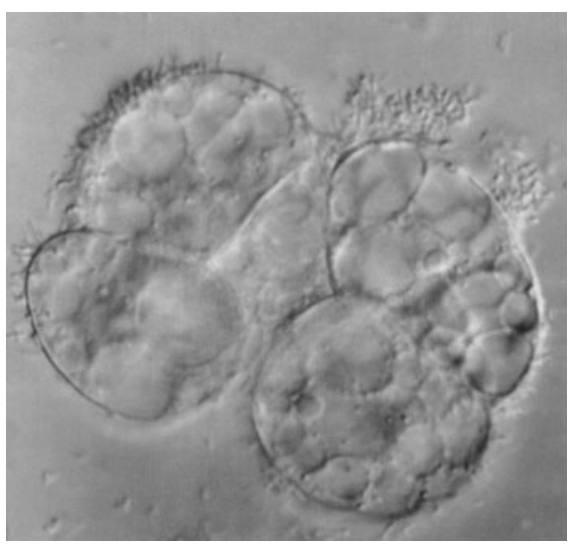

Fig. 17. Light micrograph showing *A. tumefaciens* bound to the surface of carrot cells. Note the presence of both individually attached bacteria and bacteria aggregates on the plant cell surface. From Matthysse (1987).

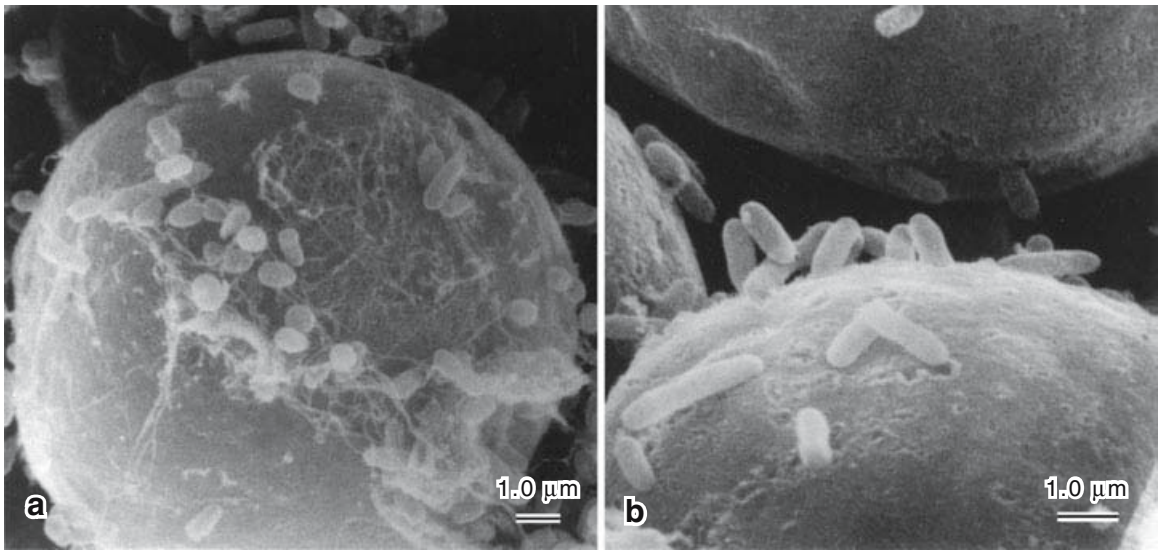

Fig. 18. Scanning electron micrographs showing *A. tumefaciens* bound to the surface of carrot cell protoplasts. A: wild type bacteria. The fibrils are composed of cellulose made by the bacteria. B: cellulose-minus bacteria. Note the absence of fibrils. The bacteria are still able to bind to the plant cells. From Matthysse (1983).

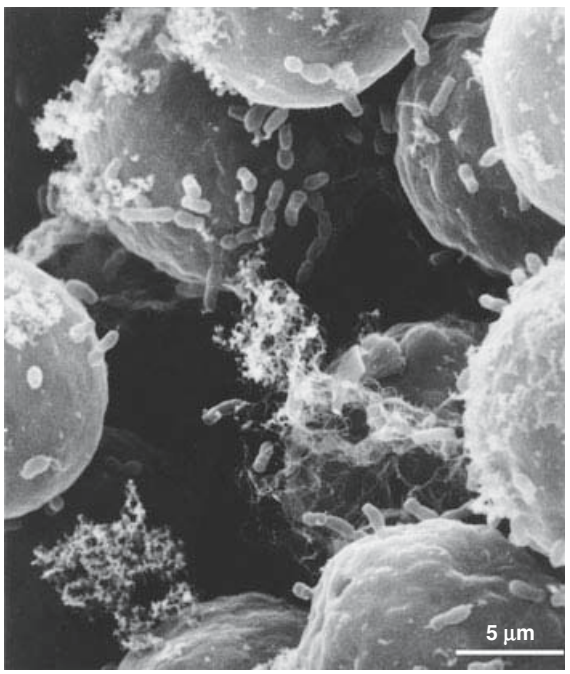

Fig. 19. Scanning electron micrograph showing *A. tumefaciens* on the surface of carrot cells. Both individually attached bacteria and bacterial aggregates held together by cellulose fibrils can be seen.

CONTROL OF *A. VITIS* Crown gall disease on grape has proven to be difficult to control. This is largely because of the persistence of the bacteria in plants that show no symptoms of disease. Management of the disease on grape combines the selection where possible of relatively resistant cultivars with cultivation practices designed to limit the growth of the bacteria (Burr et al., 1998). Cuttings also can be indexed for the presence of the bacteria by examining callus tissue formed at their base. Liquid can be forced through the vascular tissue and plated on semi-selective medium to detect *A. vitis*. In addition, PCR can be used to detect the presence of various bacterial virulence genes in DNA extracted from cuttings (see Identification). Propagation of vines from cultured shoot tips will often give rise to plants that are bacteria free.

Numbers of bacteria carried by grapevines can be reduced by submerging the vines in hot water prior to planting. However, this treatment does have the potential to injure the buds on the vine.

Biological control of *A. vitis* on grape is currently being studied. If *A. vitis* is found largely in the vascular tissue of asymptomatic grapes, then treatment of cuttings with external bacteria as biocontrol agents may fail to kill the internal bacteria. *A. vitis* is not susceptible to biocontrol by *A. radiobacter* K84 unlike *A. tumefaciens*. This may be due in part to the fact that the bacteria are not killed by the agracins produced by K84 and its derivatives (Burr et al., 1998).

Applications

Genetic Engineering

The ability of *A. tumefaciens* and *A. rhizogenes* to transfer genes to plant cells, where they are

stably integrated into the host chromosome(s) and expressed, has made these bacteria extremely useful in genetic engineering of plants. Transfer of T DNA to plants is not dependent on the sequences contained within it. Instead it is dependent on the border sequences which surround it. In addition to the border sequences, which must be located adjacent to the DNA to be transferred, other Ti plasmid and chromosomal genes are required. On the Ti plasmid these are the vir genes. The chromosomal genes required are numerous and not all of them are identified or characterized. Thus the general strategy used to transform plant cells is to clone the genes that are to be introduced along with a selectable marker, such as the *npt2* gene (which gives resistance to kanamycin and G418) or a hygromycin resistance gene, in a plasmid vector (which is a shuttle vector and will replicate in both *E. coli* [for ease of cloning] and agrobacteria). If it is important to be certain that the cloned genes are expressed only in the plant host, an intron can be included in the genes to prevent expression in the bacteria. Eukaryotic promoters and stop signals are placed before and after the gene(s) to be introduced. Once the plasmid carrying border sequences and the genes to be introduced into the plant has been constructed, it is transferred from *E. coli* into *A. tumefaciens* or *A. rhizogenes*. The *Agrobacterium* strain must contain the chromosomal genes required for virulence and a modified Ti plasmid that contains the *vir* region but has the border sequences and the T DNA deleted.

The resulting *Agrobacterium* strain is then used to infect plant tissue, often tissue culture cells or organs such as leaves or shoot meristems. The plant tissue and the bacteria are incubated together long enough for the cloned DNA to be transferred, expressed and integrated into host cell chromosomes (usually a few days). The bacteria are then killed with an antibiotic (such as cefotoxime) to which they are susceptible and the plant cells resistant, and the transformed plant material is treated to select for the marker contained in the transferred DNA. Success rates vary with the plant material used, the particular plasmid construct, the *Agrobacterium* strain, and the protocol employed. Some species of plants, notably legumes and many monocots including grasses, are more difficult to transform than are other plants such as tomatoes. This technique has been used commercially to construct many new lines of crop plants (Birch, 1997; van Wordragen et al., 1992).

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Prokaryotes (2006) 5:115–140
DOI: 10.1007/0-387-30745-1_6

CHAPTER 3.1.5

The Genus *Azospirillum*

ANTON HARTMANN AND JOSE IVO BALDANI

Historical Aspects

The growth of a spirillum-like bacterium in nitrogen deficient malate- or lactate-based media, which had been inoculated heavily with garden soil, was first observed by Beijerinck in 1922. In contrast to carbohydrate-based media, organic acid-based media supported growth of the spirillum without overgrowth by other nitrogen-fixing bacteria like *Azotobacter* and *Clostridium*. Beijerinck found that partially purified cultures of the spirillum exhibited increases in nitrogen at the expense of malate, whereas cultures lacking the spirillum failed to show such increases. In general, cells cultured in sugar media were plump, curved rods containing many lipoidal droplets which sometimes distorted the shape of the cells. On malate or lactate agar, the cells tended to be thinner and straighter, while in dilute bouillon they had a distinct spirillum shape with one or more helical turns. Because of the ease of cultivation on salts of organic acids, as well as the spirillum shape exhibited under certain conditions, Beijerinck considered the organism to be a member of the genus *Spirillum* and to be a bridging organism linking the genus *Spirillum* with the genus *Azotobacter*. He initially named the organism "*Azotobacter spirillum*," but later renamed it "*Spirillum lipoferum*" (Beijerinck, 1925). Later studies of *S. lipoferum* by Schröder (1932) also failed to demonstrate N_2 fixation by pure cultures, and the organism was forgotten for many years except for a few scattered reports. In 1963, Becking isolated an organism resembling *S. lipoferum* that showed incontestable nitrogenase activity (Becking, 1963). Ten years later, F. Favilli identified diazotrophs isolated from Italian soils as *Spirillum lipoferum* and showed their N_2-fixation capacity in pure culture, using a liquid medium containing small amounts of yeast extract (Döbereiner, 1990). The frequent isolation of this organism all over the world and especially from grass roots in the tropics must be attributed to the introduction by Johanna Döbereiner of N-free semisolid malate medium, in which bacterial growth can occur submerged at different depths (Döbereiner and Day, 1976a). This medium permits efficient enrichment of these bacteria because they can grow on molecular nitrogen (N_2) as sole N-source under microaerobic conditions. In a detailed taxonomic study, Tarrand et al. (1978) named these DNA homology group II bacteria "*Azospirillum lipoferum*" because this group seemed to correspond in more ways to Beijerinck's description of *S. lipoferum*, particularly with regard to growth with glucose or mannitol and to the formation of spirillum-shaped cells under certain conditions (Krieg and Döbereiner, 1984). Other species of the genus *Azospirillum* (e.g., *A. amazonense*, *A. irakense*, *A. halopraeferens* and *A. doebereinerae* and a diversity of other microaerobic diazotrophs) could be isolated subsequently from roots of diverse plants and from different (e.g., salt affected) soils using modified semisolid media (see below, Table 2). The scientific enthusiasm and competence of Dr. Johanna Döbereiner, who died in October 2000, have stimulated greatly basic and applied research on *Azospirillum* and the now known high diversity of other plant-associated microaerobic nitrogen-fixing bacteria (Hartmann et al., 2000). Detailed physiological, ecological and molecular genetic studies, mostly on *A. brasilense*, made *Azospirillum* one of the best studied rhizosphere bacteria.

Taxonomy Aspects

Azospirillum spp. are members of the α-subclass of Proteobacteria, which harbors a large number of plant-associated and symbiotic bacteria, such as *Rhizobium*, *Bradyrhizobium*, *Agrobacterium* or *Gluconacetobacter*. Using methods based on DNA and rRNA analyses, it became apparent in 1978 that the two *Azospirillum* species known at that time, *A. brasilense* and *A. lipoferum*, form a consistent DNA/DNA homology group within the RNA-superfamily IV (Tarrand et al., 1978; De Smedt et al., 1980). *Spirillum* spp., *Aquaspirillum* spp. and *Herbaspirillum* spp. could clearly be separated from the genus *Azospirillum* in the

following years by DNA-based molecular and physiological methods.

In a detailed 16S rDNA-based molecular phylogenetic study, Stoffels et al. (2001) demonstrated that the now known seven species of *Azospirillum* as well as *Skermanella* and *Rhodocista* form a cluster. *Azospirillum brasilense*, *A. lipoferum*, *A. doebereinerae*, *A. largimobile* and *A. halopraeferens* constitute one subcluster, while *A. irakense*, *A. amazonense* and *Rhodocista* form a second subcluster (Fig. 1). *Skermanella* is reported to form a third subcluster (Stoffels et al., 2001). The DNA G+C content for these species is in the range of 64–71 mol%. The 16S rDNA sequence similarity between the different species is in the range of 93.6–96.6% within one subcluster and 90.2–90.6% between the species members of the two subclusters. Accordingly, on the basis of different more or less conserved sequence stretches of the 16S rDNA, it was possible to create a set of oligonucletide probes with different degrees of specificity: from the whole cluster (probe AZO440a+b) to subcluster (AZOI-665) and individual species levels (Stoffels et al., 2001). For identification and in situ localization purposes, these probes can be applied in fluorescence in situ hybridization (FISH) of fixed bacterial cells. Since these probes were designed to identify different hierarchical levels, their application in a nested approach allows a very reliable identification (Fig. 2). *Azospirillum* spp. have also been investigated by 23S rDNA analysis (Kirchhof and Hartmann, 1992; Kirchhof et al., 1997b). These studies reported a comparable phylogenetic analysis of the genus *Azospirillum* with a set of oligonucletide probes, which could partially be used for whole cell hybridization but were more appropriate for filter hybridization. Since the data set of the 16S rDNAs is much bigger than that of the 23S rDNAs, the reliabilty of 16S rDNA-derived probes is better and can be reaffirmed or checked against ever-expanding databanks using, e.g., the ARB-software (Strunk and Ludwig, 1997). The 16S-rRNA-directed probes were also designed by Kabir et al. (1995), but at the present stage of knowledge, these probes have considerably limited specificity. They also cannot be used in FISH analysis. In addition to the phylogenetic analysis based on 16S ribosomal genes or diagnostic oligonucleotide probes (Stoffels et al., 2001), a number of other molecular taxonomic tools (e.g., random amplified polymorphic DNA [RAPD] markers [Fancelli et al. 1998] and restriction fragment length polymorphism [RFLP] analysis [Han and New, 1998]) have been used for the identification of *Azospirillum* spp. These approaches allow the rapid molecular identification at the species level. The RFLP-analysis of the whole genome with rarely cutting restriction enzymes followed by pulsed field gel electrophoresis can be used for strain specific identification (Gündisch et al., 1993). These and related studies have also shown that the 16S rDNA genes of *Azospirillum* spp. occur in multiple replicons (Gündisch et al., 1993; Caballero-Melado et al., 1999).

Habitats and Ecology of the Organisms

Azospirilla have a worldwide distribution and occur in large numbers (up to 10^7/g) in

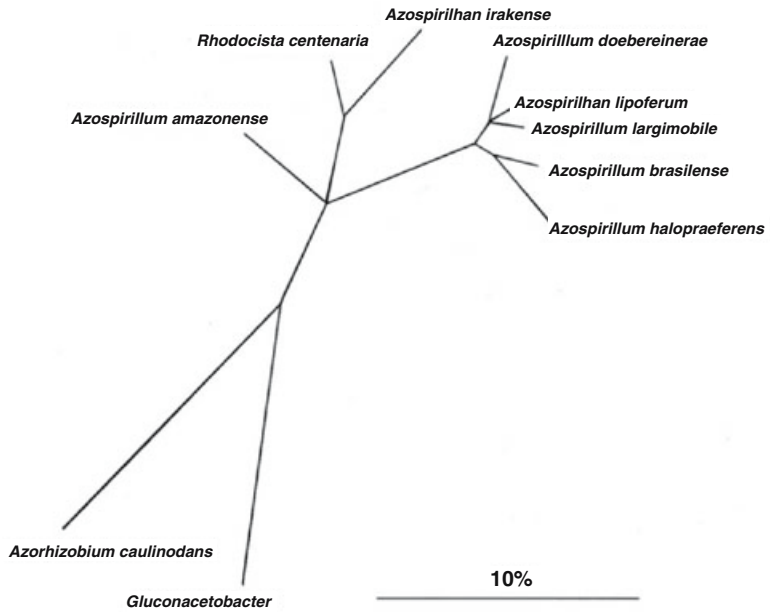

Fig. 1. 16S rDNA phylogenetic tree of *Azospirillum* (consensus tree). For the calculation of the phylogenetic tree, almost complete 16S rDNA sequences of the *Azospirillum*/*Rhodocista*-cluster and closely related members of the α-Proteobacteria were used. Only sequence positions that are represented in more than 50% of the members of the α-Proteobacteria were used for the calculation. The phylogenetic tree is based on "maximum likelihood" analysis, and the topology of the tree was checked with "maximum parsimony" and "distance matrix" analyses. A multiple bifurcation indicates that no exact relative branching order could be determined. From Eckert et al. (2001).

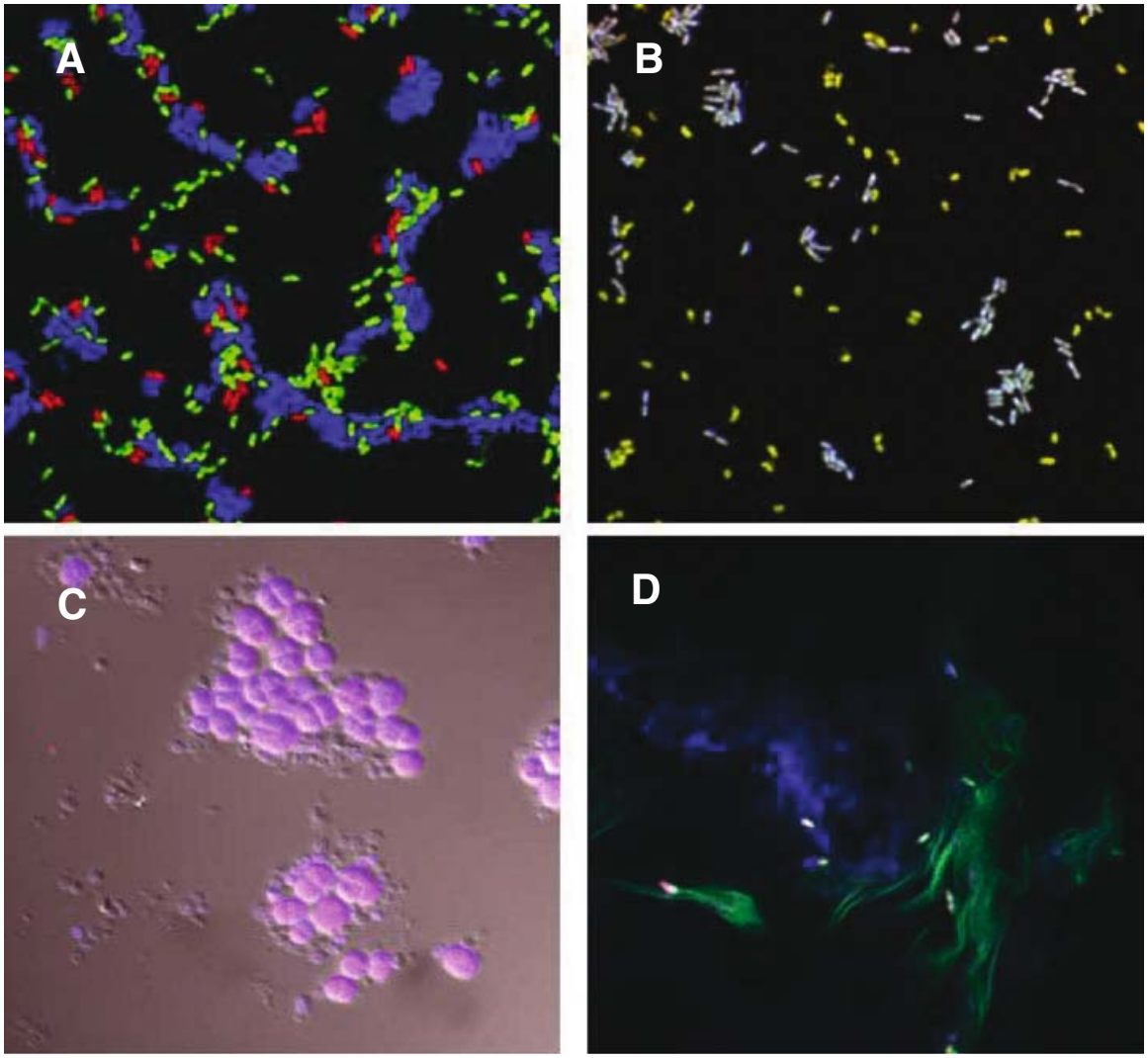

Fig. 2. Application of 16S rRNA-targeted fluorescence-labelled oligonucleotide probes. Multiple application of fluorescence in situ hybridization (FISH) and confocal laser scanning microscopy (CSLM) was used to identify *Azospirillum* species at the single cell level. A) Differentiation in an artificial mixture of *A. lipoferum* DSM 1691, *A. brasilense* DSM 1858 and *A. amazonense* DSM 2878 using the FISH technique. Cells of *A. brasilense* are stained blue by specific binding of the Cy5-labelled probe Abras1420; *A. lipoferum* cells are labelled red by specific binding of the Cy3-labelled probe Alila1113; and *A. amazonense* cells are stained green by specific binding of the FLUOS-labelled probe Aama1250. B) The "top to bottom" approach is a nested application of phylogenetic probes in whole cell identification of an environmental isolate. A mixture of the isolate GSF B3 (from banana roots) and *A. lipoferum* DSM 1841 was simultaneously hybridized with the nested probe set AZO440a+b-Cy3, specific for the *Azospirillum-Rhodocista-Skermanella*-cluster, AZOI-655-FLUOS, specific for the species cluster *A. lipoferum*, *A. brasilense*, *A. largimobile*, *A. doebereinerae* and *A. halopraeferens*, and the probe Abras1420-Cy5, specific for *A. brasilense*. Cells of the isolate GSF B3 are identified by all three probes as *A. brasilense*, resulting in a white staining of the cells, while the *A. lipoferum* cells appear yellow, since only two probes (AZO440a+b-Cy3 and AZOI-665-FLUOS) are bound. C) Whole cell identification of Skermanella parooensis cells with different morphological appearance. The paraformaldehyde-fixed culture was simultaneously hybridized with the Cy3-labelled probe Sparo84, specific for *S. parooensis*, and the Cy5-labelled probe AZO440a+b, specific for the *Azospirillum-Skermanella-Rhodocista*-cluster. The phase contrast image was superimposed with the two CSLM-panels by image analysis. The oligonucleotide probes allow the detection of both the unicellular and multicellular phases of growth. D) In situ detection of *A. brasilense* Cd at the root surface of an inoculated wheat seedling grown in a hydroponic system with quartz sand. A fixed root sample was hybridized with the FLUOS-labelled probe EUB338, the Cy3-labelled probe ALF1b and the Cy5-labelled probe AZO440a+b. *Azospirillum brasilense* cells are binding all three probes and yield a white image, while autofluorescent plant cell wall material appears green and blue. From Stoffels et al. (2001).

Table 1. Occurrence of *Azospirillum* spp.

Plant host	Country	References[a]
Azospirillum brasilense* and *Azospirillum lipoferum		
Forage grasses and Gramineae	Brazil	Baldani and Döbereiner, 1980
Forage grasses	Central and South America, and South Africa	Tyler et al., 1979
Orchard plants	India	Subba Rao, 1983
Legumes and vegetables[b]	Various countries	Bashan and Holguin, 1997[b]
Fruit trees	Brazil	Weber et al., 1999
Kallar grass	Punjab/Pakistan	Reinhold et al., 1987
Cactaceous plants	Mexico	Mascarua-Esparza et al., 1988 Bashan and Holguin, 1997
Major cereals (e.g., dry land rice)	India	Kavimandan et al., 1978 Subba Rao, 1981
	Egypt	Hegazi et al., 1979
Paddy rice	India	Kulasooriyra et al., 1981 Nayak and Rao, 1977
	Phillipines	Watanabe et al., 1979
	France	Bally et al., 1983
Paddy rice/aquatic weeds	India	Nayak et al., 1979
Lake or pond water	Australia	Skerman et al., 1983 Falk et al., 1986
C4-energy crops (*Miscanthus* and *Spartina*)	Germany	Kirchhof et al., 1997 Stoffels et al., 2001
Azospirillum doebereinerae		
Miscanthus	Germany	Eckert et al., 2001
Azospirillum largimobile		
Lake or pond water	Australia	Skerman et al., 1983 Dekhil et al., 1987
Azospirillum halopraeferens		
Kallar grass (*Leptochloa fusca*)	Punjab/Pakistan	Reinhold et al., 1987
Azospirillum amazonense		
Forage grasses	Brazil	Magalhaes et al., 1983
Sorgum, rice, wheat, maize	Brazil	Magalhaes et al., 1983 J. I. Baldani (unpublished data)
Sugar cane	Brazil, Hawaii, Thailand	Döbereiner, 1990
Palm trees (*Bactris gasipeas*)	Brazil (Amazon)	Falk et al., 1985
Pineapple and banana trees	Brazil	Weber et al., 1999
Azospirillum irakense		
Rice	Iraq	Khammas et al., 1989
Fresh water pond	Germany	Winkelmann et al., 1996
Rhodocista centenaria [Rhodospirillum centenum]		
Hot spring	Australia	Skerman et al., 1983
Wastewater	Vietnam	D. Kleiner (personal communication)

[a]Selected citations.
[b]Review.

rhizosphere soils and in association with the roots, stems and leaves of a large variety of different plants (Table 1). Both *Azospirillum brasilense* and *A. lipoferum* could be isolated from 30–90% of soil and rhizosphere samples collected all over the world (Döbereiner et al., 1976b).

In field-grown maize, azospirilla occur on the surface of roots, in the outer cortex, in the inner cortex and in the stele (Patriquin and Döbereiner, 1978; Döbereiner and Baldani, 1979). Infection of the inner cortex and stele occurs in the absence of a dense bacterial colonization or collapse of outlying tissues. Paraxylem vessels can be completely plugged with the bacteria. Infection occurs initially in root branches and spreads longitudinally into main roots. In monoaxenic cultures of pearl millet and guinea grass, azospirilla are found within the mucigel layer of roots and become firmly attached to root hairs. The bacteria enter the roots through lysed root hairs and void spaces created by epithelial desquamation and lateral root emergence (Umali-Garcia et al., 1980). Azospirilla have been observed in intercellular locations within the middle lamella of

root tissues and also intracellularly, sometimes in very large numbers (Patriquin et al., 1983).

The above observations of root colonization were mostly based on nonspecific staining techniques, and in situ identification has rarely been performed. However, these findings were confirmed for the *A. brasilense* strains Sp245 and Sp7 inoculated into wheat plants when their colonization patterns were evaluated using specific fluorescent oligonucleotide probes and confocal laser scanning microscopy (SCLM; Aßmus et al., 1995), strain-specific monoclonal antibodies (Schloter and Hartmann, 1998), or *nifH-gus* fusions (Van de Broek et al., 1993). There was a high predominance of *A. brasilense* in the root hair zone as compared with the root tips of wheat plants. *Azospirillum brasilense* strain Sp245 was repeatedly detected in the interior of root hairs, whereas *A. brasilense* strains Sp7 and Wa3—both isolated from rhizosphere soil—colonized predominantly the rhizoplane (Aßmus et al., 1995). Strain Sp245, already known to be a potential colonizer of the root interior (Baldani et al., 1986), could be found in intercellular spaces of the root parenchyma and inside cortex and parenchyma cells, with the point of emergence of lateral roots being the probable infection site (Aßmus et al., 1997). It has been observed that microcolonies are formed in the intercellular spaces (apoblast) of wheat roots inoculated with strain Sp245 but not with strains Sp7 and Wa3 (Schloter and Hartmann, 1998). However, the plant genotype may influence the extent of root colonization. Inoculation with a *gusA*-labelled derivative of strain Sp245 demonstrated that the bacteria initially concentrate in the root-hair zones and at sites of lateral root emergence. Its spread to the other parts of the root was dependent on the status of the nitrogen and carbon sources present in solution (Van de Broek et al., 1993). Although the process of association between *Azospirillum* and the host plant is still not completely resolved, a model has been suggested (Del Gallo and Fendrik, 1994; Fig. 3). In soil, the relevant properties of azospirilla, its behavior, and its relationship with soil microorganisms have recently been reviewed (Bashan, 1999).

Because of the ability of most strains of *Azospirillum* to colonize both the surface and in some cases the root interior including stems and leaves, these bacteria were grouped with the so-called "facultative endophytic" diazotrophs. This term was suggested to distinguish them from the

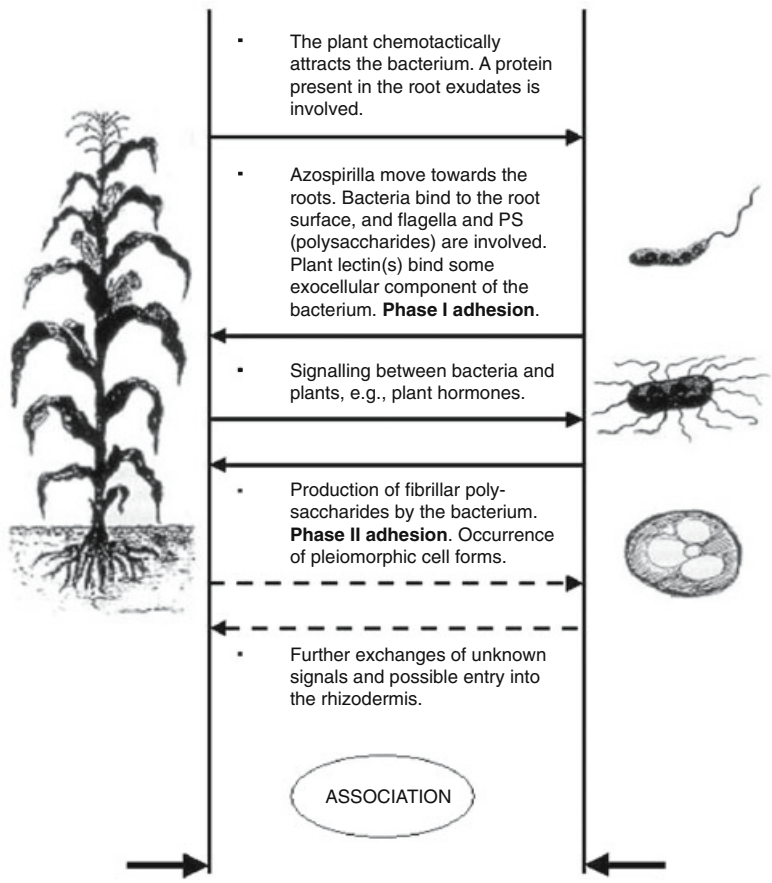

Fig. 3. Working model of the interaction of *Azospirillum* with plant roots. Modified from Del Gallo and Fendrik (1994).

"obligate endophytic" bacteria, which include *Gluconacetobacter diazotrophicus*, a bacterium found colonizing sugarcane plants endophytically (Döbereiner, 1992).

Evidence is accumulating that roots of maize, sorghum and *Phaseolus vulgaris* inoculated with *Azospirillum* have increased respiration rates (Okon et al., 1986; Sarig et al., 1992; Vedder-Weiss et al., 1999). Inoculated roots showed higher specific activities of housekeeping enzymes between the second and third week after inoculation (Fallik et al., 1988). Membrane proton efflux in wheat roots was also changed after inoculation, which might be coupled to the observed increased mineral uptake (Bashan et al., 1989). It seems that in the presence of *Azospirillum*, the hydrolysis of conjugated phytohormones and flavoids (e.g., by β-glucosidases) in the root tissue is stimulated, thus releasing these compounds in the active form (Volpin et al., 1996). Stimulation of root activities and development finally provides *Azospirillum* with enhanced possibilities for colonization and thereby increases its population in the rhizosphere (Vedder-Weiss et al., 1999; Dobbelaere et al., 2001).

Soon after its rediscovery in the mid 1970s, an application of *Azospirillum* as inoculant of crop plants had been suggested for the benefit of cereal and forage grasses, and the first trial of this application seemed very promising (Smith et al., 1984). *Azospirillum's* physiology, mode of association with the roots, and effects on crop plants were reviewed in the light of its potential agronomic application (Okon, 1982; Okon, 1985; Okon and Labandera-Gonzalez, 1994b). Although a substantial impact of nitrogen fixation due to the inoculation with *Azospirillum* was never proven, a consistent plant growth stimulatory effect was evident, leading to improved crop yields. Enhanced proliferation of the root system and root hair development as well as altered cell arrangements in the root cortex caused by *Azospirillum* inoculation was frequently demonstrated (Fallik et al., 1994). This root proliferation effect causes increased root surface activity, leading to enhanced specific uptake of mineral nutrients, microelements and water (Sarig et al., 1988).

Isolation Procedures

The isolation of *Azospirillum* spp. is based on the use of N-free semisolid media containing agar (1.75 g/liter). These microaerobically nitrogen-fixing (diazotrophic) bacteria are selectively enriched because they can grow with N_2 as nitrogen source. Because azospirilla do not harbor powerful oxygen-protective mechanisms for the oxygen-sensitive nitrogen-fixing system, they are unable to grow on N_2 as sole N-source in N-free agar plates or liquid media, as is the case for azotobacters or other strictly aerobic diazotrophs. Microaerobic diazotrophs are aerotactic, and as a result, the nitrogen-fixing population collects in zones of reduced oxygen concentration (Zhulin and Armitage, 1993). There, they form a thin pellicle or veil that moves upwards as it becomes thicker (Döbereiner and Pedrosa, 1987). Thus, the bacteria remain within the zone of an oxygen gradient where the respiration rate of the cells is in equilibrium with the oxygen diffusion rate. To permit this, the consistency of the medium must be perfectly correct so as to enable the bacteria to move through the medium on the one hand, while supporting the pellicle on the other. The agar concentration for such a semisolid medium actually has to vary with its pH, with the brand of the agar, or with the time of sterilization and must therefore be adjusted carefully for each medium formula. A medium with the proper consistency must be just thick enough not to flow upon gentle inclination of the test tube or vial. Two additional general characteristics of these media are important: the amount of medium in a vial, which should be large enough to make a layer at least as high as it is wide, and the addition of bromthymol blue indicator, which helps to make growth characteristics more readily apparent.

The composition of the various media used for the isolation of microaerobic diazotrophs is summarized in Table 2. Test tubes or 7- to 10-ml serum vials containing the semisolid media are inoculated with root or soil dilutions (10^{-5} to 10^{-7}) or directly with small root pieces or soil particles. After characteristic pellicles have formed, N_2 fixation is checked by acetylene reduction activity, and active cultures are transferred to new vials containing the same medium. As soon as a new pellicle is visible, the cultures are streaked out on agar plates containing the same medium with yeast extract (20 mg/liter) added. The small amount of yeast extract permits the growth of small colonies on the surface of plates. Characteristic individual colonies are then transferred again to N-free semisolid media, and those that grow well are streaked out on potato agar for final purification.

Isolation of *Azospirillum lipoferum*, *A. brasilense* and *A. doebereinerae*

These three species can all be isolated using the same semisolid medium (NFb or JNFb) and by exactly the same procedure (see above, Table 2). After 1–2 days of incubation at 35°C, very characteristic veil-like pellicles appear that grow into

Table 2. Four media used for the isolation and cultivation of *Azospirillum* spp.

Ingredients[a]	Semi-solid NFb medium[b]	Semisolid LGI medium[b]	Semisolid modified NFb medium	Potato agar[c]
DL-Malic acid	5.0g	—	5.00g	2.5g
Sucrose	—	5.0g	—	2.5g
K_2HPO_4	0.5g	0.2g	0.13g	—
KH_2PO_4	—	0.6g	—	—
$MgSO_4 \cdot 7H_2O$	0.2g	0.2g	0.25g	—
NaCl	0.1g	—	1.20g	—
$CaCl_2 \cdot 2H_2O$	0.02g	0.02g	0.25g	—
$Na_2MoO_4 \cdot 2H_2O$	—	0.002g	—	—
Na_2SO_4	—	—	2.40g	—
$NaHCO_3$	—	—	0.22g	—
Na_2CO_3	—	—	0.09g	—
K_2SO_4	—	—	0.17g	—
Minor element solution[d]	2ml	—	2ml	2ml
Bromthymol blue solution 0.5% in 0.2N KOH	2ml	2ml	—	—
Fe-EDTA, 1.64%	4ml	4ml	4ml	—
pH-value (adjusted with KOH)	6.8	6.0	8.5	6.8
Vitamin solution[e]	1ml	1ml	1ml	1ml
Agar	1.75g	1.75g	1.75g	15g

[a]Dissolve ingredients in distilled water up to 1 liter.
[b]Ingredients should be added to the medium in the stated order.
[c]Fresh potatoes (200g) are peeled and cooked for 30min and filtered through cotton before other ingredients are added.
[d]$CuSO_4 \cdot 5H_2O$, 0.4g; $ZnSO_4 \cdot 7H_2O$, 0.12g; H_2BO_3, 1.4g; $Na_2MoO_4 \cdot 2H_2O$, 1.0g; $MnSO_4 \cdot H_2O$, 1.5g; and distilled H_2O, 1000ml.
[e]Biotin, 10mg; pyridoxol·HCl, 20mg; and distilled H_2O, 100ml.

dense, white, paper-like, undulated pellicles. These pellicles are just under the medium surface and are easily recognized within the blue medium (use of malic acid leads to a pH increase and a change in medium color). The bacteria should be checked microscopically in wet mounts, where quite characteristic forms and movements of the different species may be seen. *Azospirillum brasilense* cells are very motile, characteristically wiggling rods (0.6–1.7 μm in diameter and 2.1–3.8 μm in length) with poly-β-hydroxybutyrate (PHB) granules; *A. lipoferum* appears either as longer cells moving in a worm-like fashion or as nonmotile large polymorphic involution forms. Cells of *A. doebereinerae*, recently isolated from roots of the C4-plant *Miscanthus sinensis* (Eckert et al., 2001), are motile curved rods or S-shaped, 1.0–1.5 μm wide and 2.0–30 μm long. After growth overnight in liquid medium, cells are small and vibrioid, whereas after prolonged growth in semisolid Nfb medium the cells are very long and their movement is winding or snake-like. Colonies of *A. brasilense*, *A. lipoferum* or *A. doebereinerae* on Nfb agar are similar in appearance, being small, dense, and white or gray. On potato agar, they are first smooth and grayish, later becoming pinkish and structured.

Isolation of *A. amazonense*

Semisolid LGI medium (Table 2) is inoculated as described above and incubated 2–4 days. The medium turns yellow in enrichment cultures but remains greenish in pure cultures. Veil-like pellicles form, as with *A. brasilense*, but these grow into thick surface pellicles after a week. *Azospirillum amazonense* can use malate and starts to grow in Nfb medium, but, owing to its sensitivity to neutral and alkaline pH, the pellicle soon disintegrates and the bacteria die. Cells in LGI medium are very motile, helical or vibrioid, somewhat smaller than *A. brasilense* (0.8 × 2 μm), with very pronounced PHB granules. In media with combined N sources, the cells contain no or only very small PHB granules, and the cells are thinner. Colonies on LGI plates are small, dense and white; on potato agar, they are characteristically white and flat with elevated borders (saucers). In modified potato agar without malate, growth continues until luxuriant white, structured colonies are formed.

Isolation of *A. halopraeferens*

Semisolid NFb medium is modified by adding additional salts and $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$ and NaCl in higher concentrations to meet the moderate halophilic/halotolerant requirement of this species; in addition, the pH is elevated to 8.5 (Table 2). The medium is inoculated as described above and incubated at 41°C. The cells are very similar in appearance to *A. amazonense*. Isolation, as decribed by Reinhold et al. (1987), is best performed by suspending parts of the pellicle in modified Nfb medium, which is then used to seed

molten, cooled (45°C), soft, modified NFb (0.8% agar) medium; then, the medium is poured into Petri plates. Small, white colonies are visible within the agar after one week.

Isolation of *A. irakense*

Rhizosphere soil or roots of rice plants are crushed, suspended in sterile distilled water, diluted and inoculated into semisolid NFb medium. The intermediate purification steps follow basically those for *A. lipoferum*. The final purification is performed on the modified semisolid AAM medium, defined as follows (g/liter): K_2HPO_4, 2.0; KH_2PO_4, 6.0; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2 \cdot 2H_2O$, 0.026; $Na_2MoO_4 \cdot 2H_2O$, 0.002; $FeCl_3$, 0.01; NH_4Cl, 1.0; and distilled water, 1000 ml. For semisolid medium, 1.9 g of agar/liter is added and the NH_4Cl is omitted. For solid medium, 15 g of agar/liter is added. The carbon sources are sterilized separately (i.e., DL-sodium malate) or filter sterilized (i.e., sucrose) and added aseptically to the minimal medium to give a final concentration of 0.03 M. The AAM-medium also contains 4 ml/liter of a 1.64% solution of FeEDTA and the final pH is adjusted to 6.5. Subsurface pellicles are streaked out on the modified AAM agar plates. Colonies formed after 4 days are translucent, glistening, convex with a regular margin, and 1 mm diameter.

Isolation of *A. largimobile*

One drop of fresh lake water is spread onto the surface of lake water agar (LWA) plates, dried and incubated for up to 8 days at 28°C. Colonies grown on LWA for 72 h are 1–2 mm in diameter, colorless, translucent, low convex, round with an entire edge and smooth surface, and readily coalesce. These colonies, containing unicellular or multicellular forms, are further purified on LWA agar, where very active motile cells containing refractile granules appear 4–8 h after incubation. These motile cells have a striking resemblance to cells of *Beijerinckia* species. Although *A. largimobile* shows these peculiar characteristics of multicellular forms, strains of this species are able to grow and fix nitrogen in semisolid NFb medium and growth in many ways resembles that of *A. lipoferum*.

Cultivation of Azospirilla

For rapid multiplication, the *Azospirillum* spp. can be grown in liquid media (compositions given in Table 2 [omitting the agar]) to which a combined nitrogen source has been added (NH_4Cl [1 g/liter] or yeast extract [0.4 g/liter]). Alternatively, complex media such as nutrient broth (NB) or 1/2 DYGS medium (D,L-malate [1 g/liter], yeast extract [2 g/liter], glucose [1 g/liter], glutamate [1.5 g/liter], peptone [1.5 g/liter], $MgSO_4 \cdot 7H_2O$ [0.5 g/liter]; Rodrigues Neto et al., 1986) can be applied. In such media, with rapid stirring or shaking, cell concentrations of 10^8 per ml are reached after 24–48 h. To stabilize the pH at the desired value (Table 2) upon prolonged growth, the addition of 50 mM MOPS (3-[N-morpholino] propanesulfonic acid) buffer (pH 6.8) or MES (2-[N-morpholino] ethane-sulfonic acid) buffer (pH 6.0) is recommended. Alternatively, the phosphate buffer concentration can be increased, as in JNFb, but this may reduce the growth rate. Alternatively, the *Azospirillum* minimal medium of Okon et al. (1976), which also contains high phosphate levels, can be used.

The production of cell mass of nitrogen-fixing *Azospirillum* in liquid medium requires additional measures. Best results are obtained in fermentors where the pH is automatically controlled, and the dissolved pO_2 is maintained at 0.02–0.04 atm (Song et al., 1985; Hartmann and Hurek, 1988c). Nitrogenase activity (acetylene reduction activity, ARA) of such cultures can be checked by incubating in small vials where the gas-liquid interface and the pO_2 in the gas phase are adjusted to optimal pO_2 conducive to nitrogenase activity. N_2-dependent growth can also be obtained in Erlenmeyer or other flasks that are closed with perforable rubber stoppers and where the pO_2 in the gas phase is fixed by air injections according to the oxygen reading of an electrode; however, rapid mixing of the culture has to facilitate the diffusion of oxygen into the liquid phase. In such vials, the optimal pO_2 in the gas phase is difficult to define because it changes with the cell density, liquid/gas interface position, shaking rate, and culture volume. Depending on the species, cultures can be started at a pO_2 of 0.01–0.04 atm with slow shaking, but once logarithmic growth is achieved, such cultures can fix N_2 and grow under vigorous shaking at atmospheric pO_2 or even above (Volpon et al., 1981). An alternative for growing cells with derepressed nitrogenase is therefore to start such batch cultures with 10–20% inoculum or to add a starter dose of combined nitrogen in the range of 1 mM NH_4Cl or 50 mg/liter of yeast extract. This small amount of combined nitrogen is used up in the first phase of cultivation, after which efficient N_2-fixation is initiated in an actively respiring culture that removes excess oxygen to a level conducive for nitrogen fixation. Exact concentrations of such starter doses must be tested for each specific culture conditions. To ensure best conditions for nitrogenase activity, it is advisable to measure the dissolved oxygen concentrations in the culture liquid continously with an autoclavable Clark-

type oxygen electrode or a similar device and to adjust the dissolved oxygen accordingly to 0.2 kPa by adding small doses of air or oxygen manually or by using a regulated automatic device (Hartmann and Burris, 1987; Hartmann and Hurek, 1988c).

Preservation of Cultures

Routine preservation of *Azospirillum* spp. is possible on potato agar (Table 2) or well buffered NB-agar within tightly closed tubes or under mineral oil. Cells from a freshly grown culture are transferred with a wire into the depth of a small vial filled with nutrient agar. After incubation of about 2 days, sterile mineral oil is added in a layer of about 1 cm. *Azospirillum amazonense* must be stored on sucrose medium because an increase of pH over 6.5 kills the bacteria within a few days. Also, *A. lipoferum* is relatively sensitive to alkaline media. Storage of the cultures for many years at –80°C or in liquid N_2 is also possible after adding 50% glycerin or dimethylsulfoxide (DMSO) to an exponentially growing culture. The cells can also be preserved by lyophilization according to the following protocol (Döbereiner, 1995). The cultures are grown to late log-phase in the following medium: K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; K-DL-malate (for *A. brasilense*) or glucose (for *A. lipoferum*), 5 g; yeast extract, 0.4 g; and 1 liter of distilled water. The cells must then be collected by centrifugation and resuspended to a dense cell suspension with 10% sucrose solution containing 5% peptone. Then, 0.1 ml portions are transferred into lyophilization ampoules, which are frozen and lyophilized according to the procedures recommended for *Rhizobium* spp. (Vincent, 1970).

Identification

Azospirillum species as described by Krieg and Döbereiner (1984) are curved plump rods, 0.8–1.0 × 25 μm in size, with PHB granules, which in N_2-fixing cells can reach 50% of the cell dry weight (Okon et al., 1976). The cells are normally Gram negative but Gram variability has been observed in *A. brasilense* (Tarrand et al., 1978). Cells are motile with a single polar flagellum. Numerous lateral flagella of shorter length are formed in *A. lipoferum* and *A. brasilense* on soft nutrient agar where swarming is observed (Hall and Krieg, 1983).

Nonmotile cyst-like forms, or C-forms, occur in *A. lipoferum* and *A. brasilense* on nutrient-deficient media (Tarrand et al., 1978), and fructose favors their formation (Berg et al., 1979; Sadasivan and Neyra, 1985). In association with grass roots in monoxenic culture or in tissue cultures, the C forms have been suggested to serve for the protection of nitrogenase against O_2 damage (Berg et al., 1979; Umali-Garcia et al., 1980). C-forms are more resistant to desiccation than are vegetative cells, and therefore they were also suggested to be true cysts (Sadasivan and Neyra, 1985). Cyst-like cell forms of *A. brasilense* occurring in the rhizosphere of wheat were shown to bind fluorescently labeled rRNA-targeted oligonucleotide probes (Aßmus et al., 1995), and this binding indicates a high ribosome content and thus high physiological activity of these cell forms.

Azospirilla have a typically aerobic metabolism, with O_2 as the terminal electron acceptor. In *A. brasilense* and *A. lipoferum*, NO_3^- can replace O_2 in respiration, and under alkaline conditions N_2O and N_2 are formed (Stephan et al., 1984). Under these conditions, anaerobic growth and N_2 fixation have been shown to occur (Nelson and Knowles, 1978; Scott et al., 1979). Weak fermentative ability has been observed with *A. lipoferum* grown on glucose or fructose (Tarrand et al., 1978). The favored carbon substrates for all *Azospirillum* species are organic acids such as L-malate, succinate, pyruvate, and *trans*-aconitate. Fructose and galactose are also used by all species. The reason for the use of sucrose for isolation and cultivation of *A. amazonense* is its sensitivity to the alkaline pH caused by growth in media with salts of organic acids. The main differential characteristics of the *Azospirillum* species are summarized in Table 3.

Azospirillum spp. have been examined by DNA/DNA and RNA/DNA hybridization studies (Falk et al., 1986; Reinhold et al., 1987), which indicate that they are closely enough related to be included in one genus. The results of 16S rDNA sequence similarity studies confirm this conclusion (Xia et al., 1994; Eckert et al., 2001; Stoffels et al., 2001; Table 4). They demonstrate clearly that *A. amazonense* and *A. irakense* are in a separate subcluster together with *Rhodocista*.

Azospirillum lipoferum

Azospirillum lipoferum, Tarrand et al., 1978, syn. *Spirillum lipoferum* (Beijerinck, 1925) is the type species. Cells in young cultures and in sugar media are as described for the genus. The characteristic pleomorphism of this species is observed in organic acid-containing media, which turn alkaline following growth. The long spirilla or filaments, as well as large, deformed involution forms remain viable and the cell walls seem to remain unchanged. Colonies on potato agar become visible after 48 h and are small,

Table 3. Main physiological characteristics of *Azospirillum* spp.

	Azospirillum doebereinerae	*Azospirillum lipoferum*	*Azospirillum largimobile*	*Azospirillum halopraeferens*	*Azospirillum brasilense*	*Azospirillum amazonense*	*Azospirillum irakense*
Carbon utilization tests[a]							
N-Acetylglucosamine	–	+	+	n.d.	–	d	+
D-Glucose	d	+	+	–	d	+	+
Glycerol	+	+	+	+	+	–	–
D-Mannitol	+	+	+	+	–	–	–
D-Ribose	–	+	+	+	–	+	d
D-Sorbitol	+	+	+	–	–	–	–
Sucrose	–	–	n.d.	–	–	+	+
Acid formation[b] from							
Glucose	d	+	+	–	–	–	–
Fructose	+	+	+	+	–	–	–
Biotin requirement	–	+	–	+	–	–	–
Optimum growth temperature (°C)	30	37	28	41	37	35	33
Optimum pH-value for growth	6.0–7.0	5.7–6.8	n.d.	6.8–8.0	6.0–7.8	5.7–6.5	5.5–8.5
Occurrence of pleomorphic cells	+	+	+	+	–	–	+

Symbols: –, less than 10% of the investigated strains showed positive response; +, more than 90% of the investigated strains showed positive response; d, between 11 and 89% of the investigated strains showed positive response; and n.d., not determined.

[a]Assayed using API®.

[b]Assayed using API® 50 anaerobe.

Table 4. 16S rDNA-similarity of *Azospirillum* species type strains.

	A. lipoferum	*A. largimobile*	*A. brasilense*	*A. haloprae-ferens*	*A. irakense*	*A. amazonense*
A. doebereinerae	96.6	96.6	95.9	93.6	90.6	90.2
A. lipoferum		97.5	97.2	94.8	90.6	90.7
A. largimobile			95.3	93.9	89.7	89.8
A. brasilense				94.6	90.3	91.3
A. halopraeferens					89.8	90.9
A. irakense						91.3

whitish, and smooth. After one week, the colonies turn light pink and become dry and wrinkled and are characteristically indented into the agar. This characteristic colony type occurs only when the medium becomes alkaline. When malate is replaced by a sugar substrate (glucose or fructose), large, white and gummy colonies are formed. Growth in liquid medium with a combined N source and heavy shaking results in rapid growth with extensive clumping of the cells. Microscopic examination of the clumps shows predominantly C-forms embedded within a polysaccharide matrix.

Azospirillum lipoferum can grow lithoautotrophically with H_2 (Pedrosa et al., 1982) owing to the presence of a hydrogenase (uptake hydrogenase) as well as its ability to form ribulose-1,5-bisphosphate carboxylase (Rubisco). Under these conditions, it can be considered as an aerobic, hydrogen-oxidizing bacterium. The type strain is strain ATCC 29707^T/DSM 1691^T (strain 59b of Tarrand et al., 1978), and additional reference strains are ATCC 29708 (strain RG20a), ATCC 29709 (strain SpBr17), and ATCC 29731 (strain SpRG6). Physiological characteristics useful for routine identification are the polymorphic cells in alkaline malate medium, the ability to use glucose, and a requirement for biotin. The species-specific 16S rRNA binding oligonucleotide probe Alila1113 together with the subcluster probe AZOI-665 (Stoffels et al., 2001) enables a fast and reliable identification of *A. lipoferum* by whole cell fluorescence in situ hybridization (FISH; Amann et al., 1995).

Azospirillum doebereinerae

Azospirillum doebereinerae is the most recently discovered *Azospirillum* species (Eckert et al., 2001). Until now, it has only been isolated from the C4-plants *Miscanthus sinensis* cv. *giganteus* and *M. sacchariflorus*, which are cultivated as "renewable energy" and fiber plants. *Azospirillum doebereinerae* is pleomorphic and forms

small (1.0–1.5 μm) vibrioid cells in neutral media, but long (2.0–30 μm) snake-like moving cells in alkaline semisolid NFb or JNFb media. It is very closely related to *A. lipoferum* and *A. largimobile*, but can be distinguished from these species by the inability to use *N*-acetylglucosamine and D-ribose (Table 3). Unlike *A. lipoferum*, *A. doebereinerae* is able to grow without biotin. The type strain is DSM 13131^T (strain GSF71^T) and the additional reference strain is DSM 13400^T (strain Ma4). Using the species-specific oligonucleotide probes Adoeb94 or Adoeb587 together with the subcluster probe AZOI-655, *A. doebereinerae* cells can rapidly and reliably be identified by FISH (Stoffels et al., 2001).

Azospirillum largimobile

Azospirillum largimobile is the former *Conglomeromonas largomobilis* subsp. *largomobilis* (Skerman et al., 1983) and was transferred to the genus *Azospirillum* as *Azospirillum largomobile* on the basis of the results of 16S rDNA similarity and DNA-DNA-hybridization (Dekhil et al., 1997). The name was subsequently corrected to *Azospirillum largimobile* by Sly and Stackebrandt (1999). It can be identified by its characteristic multicellular forms on lake water agar. In contrast to *A. lipoferum*, it has no biotin requirement (Table 3). The reference strain is ACM 2041^T. On the basis of 16S rDNA-directed oligonucleotide probes, *A. largimobile* cannot be distinguished from *A. lipoferum* by FISH, because it also binds the probe Alila1113 (Stoffels et al., 2001).

Azospirillum brasilense

Cells of *Azospirillum brasilense* (Tarrand et al., 1978) are, as described for the genus, indistinguishable from young cultures of *A. lipoferum* but remain very motile and vibrioid even in older alkaline cultures. All strains are Gram negative when cultured in nutrient broth, but they may become Gram variable on nutrient agar. Colonies on potato agar containing malate are indistinguishable from *A. lipoferum* but remain unchanged when malate is replaced by glucose because glucose is not used. This was shown to be due to an inability to transport glucose into the cell (Goebel and Krieg, 1984; Martinez-Drets et al., 1984) and not to the lack of glycolytic enzymes. Bright red mutants occur spontaneously, especially of the type strain Sp7. It was shown that the red pigments are carotenoids exerting some oxygen protective effect (Hartmann and Hurek, 1988c). The type strain is ATTC 29145^T/DSM1690^T (strain Sp7, isolated from grass rhizosphere soil in Rio de Janeiro), and other reference strains are ATCC 29710 (the red mutant Cd of Sp7) and ATCC 29711 (strain Sp35 isolated from grass roots in Rio de Janeiro). Routine identification can be made by the lack of growth on glucose and the presence of motile cells in old alkaline cultures. A rapid identification using the species-specific oligonucleotide probe Abras1420 together with AZOI-655 is possible. To optimize the specificity, the application of the unlabeled competitor probe Abras1420C is recommended (Stoffels et al., 2001).

Azospirillum halopraeferans

Azospirillum halopraeferans (Reinhold et al., 1987) has been found only in association with Kallar grass (*Leptochloa fusca*) in Pakistan and has not been isolated from inside roots or from any other plant. It shows a remarkable adaptation to the extremely arid environment of saline soils, having a pH optimum of 8.0 and a temperature optimum of 41°C even for N_2-dependent growth and a salt requirement of 0.25% NaCl. Cells are very similar in appearance to those of *A. amazonense* but can become wider in alkaline media (1.2 μm). They are motile by one polar flagellum, and cells move in a vibrioid or helical fashion; few cells are motile in very alkaline media. As with the other species, it is oxidase- and urease-positive and can assimilate and dissimilate NO_3^-, but anaerobic NO_3^--dependent growth has not yet been shown. The type strain is DSM 3675^T (strain Au4), and additional reference strains are LMG 7107 (strain Au2), LMG7109 (strain Au5), and LMG7112 (strain Au10). Routine differentiation from the other *Azospirillum* spp. is possible by its relatively high heat tolerance of nitrogenase activity and salt requirement. Additional differential characteristics are shown in Table 3. *Azospirillum halopraeferens* can be rapidly identified by FISH using the species-specific oligonucleotide probes Ahalo1115 or Ahalo1249 together with the cluster probe AZOI-655 (Stoffels et al., 2001).

Azospirillum amazonense

Cells of *Azospirillum amazonense* (Magalhães et al., 1983; Falk et al., 1985) are slightly smaller than those of the two first species (0.9 × 3–4 μm) and contain large PHB granules in N_2-grown cultures. Cells grown with combined nitrogen are much narrower in diameter and have no, or small, PHB granules. Only a single polar flagellum is observed, and there is no swarming on soft nutrient agar. In semisolid NFb medium with an initial pH of 6.8, growth starts with the veil-like pellicle, which however disintegrates once the pH rises above 7.0 (Magalhães et al., 1983). Good growth is observed in LGI medium con-

taining sucrose or glucose (where the pH does not increase). Typical for N_2-fixing cultures in semisolid LGI medium is the formation of a thick surface pellicle with the medium remaining green (pH between 6.0 and 6.5). The optimal pH for growth is between 5.8 and 6.8, and viability is lost rapidly in cultures which reach higher pH. Optimum temperature is the same as for the two first species, 36°C, and no growth occurs below 25 or above 39°C (Magalhães et al., 1983). Nitrate is assimilated but only slightly or not at all dissimilated (by a few strains only), and no denitrification or NO_3^--dependent anaerobic growth occurs. The type strain is DSM 2787^T (strain Y1); DSM 2789 (strain Y6) and DSM 2788 (strain Y2) are two additional reference strains. Additional characteristics are summarized in Table 3. Characteristics useful for routine identification are the white, flat colonies with elevated borders (about 5 mm in diameter) on potato agar containing malate and sucrose, good growth without acid production on sucrose, and the characteristically motile, smaller cell form with large PHB granules. The application of the species-specific oligonucleotide probe Aama1250 enables a reliable and fast identification using the FISH technique (Stoffels et al., 2001).

Azospirillum irakense

Azospirillum irakense, isolated from roots of rice plants (Khammas et al., 1989) but also from pond water (Winkelmann et al., 1996; K. Malik, unpublished observation), closely resembles *A. amazonense* in its physiological characteristics, especially because these two species are the only azospirilla that efficiently utilize sucrose (Table 3). In addition, Khammas and Kaiser (1991) demonstrated pectinolytic activity in *A. irakense* isolates. The type strain is DSM 11586^T (strain KBC1) and the additional reference strains are LMG 10655 (strain KAC5) and LMG 10655 (strain KAC5). *Azospirillum irakense* cells can be specifically identified using the phylogenetic oligonucleotide probes Airak1423 or Airak985 via the FISH technique (Stoffels et al., 2001).

Rhodocista centenaria and *Skermanella parooensis*

On the basis of 16S rDNA sequence similarity and DNA-DNA hybridization results, these two species fall in the same cluster as the *Azospirillum* spp. *Rhodocista centenaria* are nitrogen-fixing, thermotolerant anoxygenic cyst-forming phototrophic bacteria, which were originally named "*Rhodospirillum centenum*" (Favinger et al., 1989). They were reclassified as a separate photosynthetic bacterial genus (Kawasaki et al., 1992). The type strain is DSM 9894^T. However, *Rhodocista* is affiliated to the *Azospirillum* subcluster together with *A. amazonense* and *A. irakense* (Xia et al., 1994) and 16S rRNA from *Rhodocista* hybridizes with the cluster probe AZO440a+b (Stoffels et al., 2001). Using the 16S rRNA phylogenetic probe Rhodo654, *Rhodocista* can be rapidly and specifically identified with the FISH technique (Stoffels et al., 2001).

Skermanella parooensis (formerly *Conglomeromonas largomobilis* subsp. *parooensis*) is a non-nitrogen-fixing organism isolated from freshwater (Skerman et al., 1983; Dekhil et al., 1997). Only one isolate exists. Like in *A. largimobile*, growth of *Skermanella parooensis* occurs in unicellular and multicellular phases. Unicellular phase cells are rod-shaped with rounded or tapered ends, while multicellular conglomerates arise from single cells when they lose motility. They become optically refractile and reproduce by multiplanar centripedal septation. Under suitable conditions, conglomerates dissociate into single cells (Skerman et al., 1983; Dekhil et al., 1997). The type strain is *R. centenaria* ACM 2042^T. DNA from both cell forms gave hybridization signals with rRNA-directed fluorescently labeled specific oligonucleotide probe Sparo84 and the cluster probe AZO-440a+b (Stoffels et al., 2001). This clearly demonstrates that both types are active cell forms which belong to the *Azospirillum* cluster.

Physiology

Profound knowledge of the physiology of these microaerobic nitrogen-fixing bacteria plays an important role for their successful isolation from all over the world using simple semisolid N-free media (Table 2). Other microaerobic diazotrophs associated with plant roots comprise the α-subgroup Proteobacteria (*Gluconacetobacter diazotrophicus* [Gillis et al., 1989], *G. johannae* and *G. azotocaptans* [Fuentes-Ramirez et al., 2001]), as well as the β-subgroup Proteobacteria (*Herbaspirillum* with now three diazotrophic species [Kirchhof et al., 2001], *Azoarcus* spp. [Reinhold et al., 1993], and several diazotrophic *Burkholderia* spp. [Gillis et al., 1995; Estrada-De Los Santos et al., 2001; V. L. D. Baldani et al., submitted]). In the following paragraphs, selected physiological properties which have direct implications for the ecological performance and the application potential of *Azospirillum* are described. Diverse physiological aspects of *Azospirillum* were also reviewed by Hartmann and Zimmer (1994b) and Steenhoudt and Vanderleyden (2000).

Plant Root Colonization

In the rhizosphere, a gradient of exuded nutrients exists, stimulating microorganisms in the surrounding soil. Motility and chemotaxis enable the bacteria to move towards the roots, where they profit from the root exudates as carbon and energy sources. Survey of plant-inducible genes in *A. brasilense* grown in the presence and absence of plant root exudates identified an acidic 40-kDa protein, SbpA (Van Bastelaere et al., 1999). This protein, very similar to the ChvE-protein of *Agrobacterium tumefaciens*, is involved in the uptake of D-galactose and functions in the chemotaxis of *A. brasilense* towards several sugars (D-fucose, L-arabinose and D-galactose). Another important factor of directed movement of *Azospirillum* is oxygen gradients. This aerotactic behavior is necessary to guide the bacteria to optimal niches for microaerobic nitrogen fixation. The sensory system for spatial oxygen and redox gradients characterized in *A. brasilense* is unique (Grishanin et al., 1991; Zhulin et al., 1996).

Chemo- and aerotaxis need an efficient motility apparatus. *Azospirillum brasilense* genes required for the motility in liquid medium, on surfaces, and for general chemotaxis are located on the chromosome or on a 90-MDa plasmid (Onyeocha et al., 1990; Croes et al., 1991). All *Azospirillum* species possess plasmids of sizes from 4 MDa to over 300 MDa (Onyeocha et al., 1990). A 90-MDa plasmid has been commonly observed in *A. brasilense* and *A. lipoferum*. Deletion mutations in the 90-Mda plasmid (p90) of *A. brasilense* Sp7 enabled the identification of three loci involved in motility: Mot1, Mot2 and Mot3. While Mot1 and Mot2 are involved in the synthesis or functioning of lateral flagella, Mot3 deletion mutants lack both polar and lateral flagella. The use of these *A. brasilense* motility mutants in colonization assays directly demonstrated that bacterial chemotaxis was needed for the initiation of wheat root colonization at the root hair zone (Vande Broek et al., 1998). The structural gene encoding the flagellin of the lateral flagella (*laf1*) in *A. brasilense* Sp7 has been characterized (Moens et al., 1995a). Using a monospecific polyclonal antibody against the polar flagellum and a translational *laf1*::*gusA* fusion, it was demonstrated that blocking the rotation of the polar flagellum induces expression of the structural gene of the lateral flagellum (Moens et al., 1996). The structural protein of the polar flagellum, Fla1, is a glycoprotein that is suggested to act as a plant root surface adhesin (Moens et al., 1995b). The attachment of *Azospirillum* to plant roots has been shown in short-term in vitro binding assays as a biphasic process (Michiels et al., 1991). In a first step, the bacteria adsorb to the roots as single cells in a rapid, weak and reversible manner. The polar flagellum is involved in this step. The adsorption phase is followed by an anchoring phase in which bacterial aggregates form and are firmly and irreversibly anchored to the roots. Probably, the anchoring step is dependent on bacterial extracellular polysaccharide production (Del Gallo and Fendrik, 1994; Van de Broek and Vanderleyden, 1995; De Troch and Vanderleyden, 1996). Also fibrillar material has been reported in the anchoring of *Azospirillum* to the root surface (Umali-Garcia et al., 1980). Extracellular polysaccharide production has also been related to the process of flocculation of *Azospirillum* cells (Burdman et al., 1998). A spontaneous mutant of *A. brasilense* Sp7, impaired in surface polysaccharide production and flocculation, was altered in the colonization pattern of wheat roots as compared to the wildtype strain (Katupitiya et al., 1995). The regulatory gene *flcA* was demonstrated to control the production of capsular polysaccharides, flocculation, and colonization pattern of wheat roots (Pereg-Gerk et al., 1998). In addition, the *rpoN* gene, involved in the control of nitrate reduction, nitrogen fixation, and cell motility, also controls the colonization process of wheat roots by *A. brasilense* Sp7 (Pereg-Gerk et al., 1998). An interesting observation was made with a motile, laccase-negative strain (4B) and a nonmotile laccase-positive strain (4T) of *A. lipoferum*, isolated from the rhizosphere of rice (Alexandre et al., 1996). Upon inoculation of rice roots with *A. lipoferum* 4B, a two-step phenotypic transition was observed yielding spontaneously nonmotile laccase-positive variants under extremely low oxygen concentrations (Alexandre and Bally, 1999).

Wheat germ agglutinin (WGA) has been proposed as a signal molecule in the association of *Azospirillum* and wheat roots (Antonyuk et al., 1993). Nitrogen fixation, excretion of ammonium, and increase of indole-3-acetic acid (IAA) biosynthesis of *A. brasilense* strain Sp245 were found to be stimulated by WGA. A similar increase of nitrogen fixation in the presence of WGA has been demonstrated in *A. lipoferum* (Karpati et al., 1999). However, it remains unclear which structures of the cell surface of *A. brasilense* are responsible for WGA-binding. In addition, there are reports of lectin-like proteins on the cell wall of *Azospirillum* spp. that might be involved in the recognition and colonization of root surfaces (Castellanos et al., 1998). A major outer membrane protein of about 42 kDa has been identified in *A. brasilense*. It was proposed that under certain growth conditions, outer membrane proteins interact with exopolysaccharides (EPS), leading to aggregation and flocculation (Burdman et al., 1999).

In the search for *Azospirillum* genes relevant for interactions with plants, sequence homologies have been detected with the *A. tumefaciens chv* gene (Raina et al., 1995) and *Rhizobium meliloti nod* and *hsn* genes (Delledonne et al., 1990; Vielle and Elmerich, 1990). These genes are essential for attachment and tumor formation of plant cells or for nodule formation, respectively. The *A. brasilense chvB* gene could complement a *chvB* mutational defect in *A. tumefaciens* with respect to tumor formation. Therefore, the *chvB*-gene product of *A. brasilense* might also participate in root adsorption of *Azospirillum*. Interestingly, the *nodPQ* genes of *A. brasilense* are located on the 90-MDa plasmid, whereas the *nodG*-homologous gene is chromosomally located (Vielle and Elmerich, 1990; Vielle and Elmerich, 1992). However, the functions of these genes in the *Azospirillum*-plant interaction remain unclear.

Surface polysaccharide structures and the corresponding synthesis genes, which are also relevant for the surface interaction with plant roots, have been investigated via random Tn*5* mutagenesis and genetic complementation of *R. meliloti exo* mutants. *Azospirillum brasilense* produces cell bound capsular (CPS) as well as loosely attached extracellular (EPS) calcofluor-binding polysaccharides (Del Gallo et al., 1989; De Troch et al., 1992). Calcofluor-negative mutants lose the ability to anchor to wheat roots (Anc^-) but retain wildtype adsorption capacity (Ads^+), indicating that a surface polysaccahride is involved in anchoring (Michiels et al., 1991). Through complementation of *R. meliloti exoB* and *exoC* mutants, two *A. brasilense exoB* loci (*exoB1* and *exoB2*) and one *exoC* locus were isolated (Michiels et al., 1988). The *A. brasilense exoB* loci are functionally homologous to *R. meliloti exoB*, while the *A. brasilense exoC* locus could not complement for normal nodule formation. Interestingly, the *exoB1* and *exoC* genes are located at the p90 plasmid of *A. brasilense* Sp7.

Nitrogen Fixation

Azospirillum spp. can convert atmospheric nitrogen into ammonium under microaerobic conditions at low nitrogen levels through the action of the nitrogenase complex. Most of the genetic and biochemical work on nitrogen fixation by *Azospirillum* has been carried out with *A. brasilense*. An *A. brasilense nifHDK* operon, encoding both nitrogenase components, was first isolated on the basis of sequence similarity with the *Klebsiella pneumoniae nifHDK* genes (Quiviger et al., 1982). A number of additional *nif* and *fix* genes involved in processing of and electron transport to the nitrogenase complex, iron-molybdenum (FeMo) cofactor biosynthesis as well as regulation of nitrogen fixation are known (for review, see Steenhoudt and Vanderleyden, 2000). Except for the separately transcribed *nifA* and *nifB* genes, they all are located in the major 30-kb *nif* gene cluster on the chromosome (Liang et al., 1991). Only one report gives evidence of an alternative nitrogenase system in *A. brasilense* (Chakraborty and Samaddar, 1995).

Azospirillum fixes nitrogen only under microaerobic conditions. The optimum level of dissolved oxygen concentration is in the range of 0.2 kPa oxygen, but species- and strain-dependent differences exist in oxygen tolerance (Hartmann et al., 1985; Hartmann et al., 1988d). Mutants with increased carotenoid concentrations were shown to have slightly increased oxygen tolerance (Hartmann and Hurek, 1988c). A shift in dissolved oxygen concentration to 2.0 kPa caused an immediate inhibition (switch off) of nitrogenase activity, which can be recovered when the oxygen is shifted back to optimum levels. No covalent modification of nitrogenase reductase was found in this situation (Hartmann and Burris, 1987). Exposure of cells to higher oxygen levels caused an irreversible inhibition and destruction of the oxygen labile nitrogenase complex. A shift to anaerobic conditions also caused a switch-off in nitrogenase activity which was found to be accompanied by a covalent modification of the nitrogenase reductase in *A. brasilense* and *A. lipoferum* (Hartmann and Burris, 1987). The posttranscriptional regulation of nitrogen fixation is mediated through ADP-ribosylation of nitrogenase reductase (nifH-protein) in *A. brasilense* and *A. lipoferum*, but not in *A. amazonense* (Song et al., 1985; Hartmann et al., 1986). As was originally found in *Rhodospirillum rubrum*, the switch-off mechanism in *A. brasilense* and *A. lipoferum* consists of two enzymes: the dinitrogenase reductase ADP ribosyl-transferase (DraT) and the dinitrogenase reductase activating glycohydrolase (DraG; Fu et al., 1989b). In the presence of high ammonium or low oxygen concentrations, DraT catalyzes the transfer of an ADP-ribose of NAD to Arg 101 of one subunit of dinitrogenase reductase, whereby the covalently modified nitrogenase enzyme is inactivated. When ammonium concentrations are low (and/or oxygen is present), this inactivation is reversed by the action of DraG, which removes the ADP-ribosyl moiety (Zhang et al., 1992). DraT and DraG themselves are both subject to posttranslational control (Fu et al., 1990; Zhang et al., 1993). DraT is inactive under nitrogen-fixing conditions and is activated following a negative stimulus. DraG is active under nitrogen fixation conditions and is inactivated by a negative stimulus. *Azospirillum brasilense ntrBC* mutants escape nitro-

genase switch-off by ammonium. The primary effect of *ntrBC* mutations probably involves changes in DraG enzyme activity (Liang et al., 1992; Liang et al., 1993). It was shown that *draG* and *draT* are involved in the anaerobic and ammonium switch-off of nitrogenase activity and that these responses are regulated differently through the cellular nitrogen control (ntrB/C system; Zhang et al., 1994). Therefore, different environmental stimuli (NH_4^+, anaerobiosis) use independent signal transduction pathways to affect the reversible ADP-ribosylation system.

An alternative, noncovalent mechanism of posttranslational regulation of nitrogenase activity in response to ammonium, which exhibits slower and only partial inhibition of nitrogenase activity, has been demonstrated in *A. amazonense* that lacks the DraT/G-system and in mutant strains of *A. brasilense* harboring a nitrogenase reductase that cannot be ADP-ribosylated (Hartmann et al., 1986; Zhang et al., 1996). It is suggested that the regulation of electron flow to nitrogenase (Hochman et al., 1987) or other regulatory circuits involving respiratory components could be acting on the reversible switch-off at high oxygen conditions. Under nitrogen-fixing conditions, hydrogen metabolism is increased in *A. brasilense* (Chan et al., 1980). It has been demonstrated that hydrogen supports nitrogen fixation in carbon starved cultures of *Azospirillum* by an intracellular localized uptake hydrogenase (Fu and Knowles, 1988; Fu and Knowles, 1989a).

For general nitrogen regulatory control, NtrA and the transcriptional activator NifA are required in *Azospirillum* as they are in other Gram-negative nitrogen-fixing bacteria. Upstream of several *A. brasilense nif* genes, σ^{54}-type promotors and upstream activating sequences (UAS) typical for the binding of NifA have been found. In *A. brasilense*, the regulatory protein NifA is constitutively expressed, but the level of expression is reduced at conditions incompatible with nitrogen fixation (Liang et al., 1992). In addition, NtrC is required for optimal nitrogen fixation and maximal expression of *nifA* transcription. Since in *A. brasilense glnB* mutants (affecting regulatory P_{II}-protein) are *nif* minus, the general nitrogen regulatory cascade is also involved. Most interestingly, two distinct P_{II}-genes (P_{II} and P_Z), which are structurally similar but functionally different, were described in *A. brasilense* (de Zamaroczy et al., 1996; de Zamaroczy, 1998).

Ammonium Uptake and Assimilation

Under nitrogen-limiting conditions, *Azospirillum* express an ammonium-scavenging energy-dependent transport system, which takes up traces of NH_4^+ (Hartmann and Kleiner, 1982). Ammonium, which tends to leak out through the membrane by ammonia (NH_3) diffusion, is reutilized in this way. Recently, the *A. brasilense amtB* gene encoding this N-regulated ammonium transporter was characterized (Van Dommelen et al., 1998). Mutants in *amtB* are impaired both in $^{14}CH_3NH_4^+$ uptake and in growth at low ammonium concentrations. Since growth is not completely abolished in the *amtB* mutant at low ammonium concentration, the existence of a second NH_4^+-transport system was suggested (Van Dommelen et al., 1998). The *amtB* promotor region is characterized by a σ^{54} consensus sequence, and transcription requires the NtrBC two-component regulatory system (Milcamps et al., 1996). The second *A. brasilense* P_{II}-like protein (P_Z, encoded by *glnZ*) was shown to negatively regulate (methyl)ammonium uptake. Since P_Z is not required for NtrC-dependent regulation in *A. brasilense*, it is proposed that this protein is involved in modulating AmtB activity (de Zamaroczy, 1998). Aspects of nitrogen regulation in *Azospirillum* have recently been reviewed in more detail by Steenhoudt and Vanderleyden (2000).

Ammonium is assimilated through the glutamine synthetase/glutamate oxoglutarate aminotransferase (GS/GOGAT) pathway under nitrogen-limiting conditions. At high levels of ammonium or at ample supply of nitrogen through readily metabolizable amino acids, the glutamate dehydrogense (GDH) pathway is operating. Interestingly, NH_4^+ is excreted by *A. lipoferum* and *A. amazonense* when amino acids like glutamate or aspartate are used as sole carbon and nitrogen sources and acetylene reduction (nitrogen fixation) is operating at a reduced level (Hartmann et al., 1988d). In contrast, *A. brasilense* continues to fix nitrogen at high rates in the presence of high levels of external amino acids, and ammonium is not excreted (Hartmann et al., 1988d). Under nitrogen-limiting conditions, ammonium was never found to be released by *Azospirillum* wildtype cells under free-living (culture) conditions, if they still are supplied with a suitable carbon/energy source. Mutations in *glnA* (glutamine synthetase) and *amtB* (ammonium transporter) were reported to result in a less stringent regulation of nitrogen fixation by external ammonium levels, and these mutants release ammonium (Turbanti et al., 1988; Christiansen-Weniger, 1992). Inoculation of wheat plants with *amtB*-mutants resulted in an increased N-uptake by the plants as compared to the inoculation with the wildtype bacterium in 2,4-dichlorophenoxyacetic acid (2,4-D)-treated wheat plants in model experiments (Christiansen-Weniger et al., 1991; Sriskandara-

jah et al., 1993). In addition to assimilatory nitrogen metabolism, most azospirilla can denitrify under anaerobic conditions (Bothe et al., 1981). A possible role of nitrate reductase of *Azospirillum* in the nitrate assimilation of wheat plants was emphasized by Ferreira et al. (1987).

Production of Plant Growth Regulating Substances

The observed plant growth promotion by inoculation with *Azospirillum* can probably be explained in many cases by the action of plant growth regulatory substances produced by the bacterium. Three types of plant hormones can be detected in the supernatant of *Azospirillum* cultures: auxins (Tien et al., 1979; Lambrecht et al., 2000), and in smaller amounts, cytokinins and gibberellins (GA_3; Bottini et al., 1989). The application of an indolepyruvate decarboxylase (IpdC) mutant, producing only 10% of the indole acetic acid (IAA) level of the wildtype, strongly suggested that auxin production by *Azospirillum* plays a key role in the stimulation of plant root growth (Dobbelaere et al., 1999). GA_3 was found in an free acid form in roots of seedlings inoculated with *A. lipoferum* (Fulchieri et al., 1993). In plants with inhibited GA-biosynthesis, GA_3 could only be detected after these plants were inoculated with *Azospirillum* (Lucangeli and Bottini, 1997). Roots of *Azospirillum*-inoculated maize seedlings were found to have higher amounts of both free and bound IAA and indole-3-butyric acid (IBA) than control plants had (Fallik et al., 1989). Using a high-pressure liquid chromatography (HPLC) method with high separation power, a whole array of IAA-related compounds could be identified in *Azospirillum* cultures (Lebuhn and Hartmann, 1993; Lebuhn and Hartmann, 1994). It is not clear, however, whether the relative higher amounts of free IAA and GA_3 in root tissue of inoculated plants (i) are derived from substances directly excreted by *Azospirillum*, (ii) are due to changes in plant hormone metabolism caused by the bacteria, or (iii) are due to higher respiration rates of inoculated roots demanding more glycosidic residues from hydrolyzed hormonal conjugates, thus freeing IAA and GA_3.

After inoculation with *Azospirillum*, a stimulation of root growth is frequently observed with an optimum level of about 10^7 bacteria per plant (Okon and Labandera-Gonzalez, 1994b). The bacterial phytohormones probably provoke the detected changes in root morphology after inoculation. *Azospirillum* inoculated plants exhibit an enhanced root branching and surface area, which in turn can explain enhanced mineral uptake and water status of inoculated plants (Kapulnik et al., 1985; Sarig et al., 1988; Fallik et al., 1994). In certain *A. brasilense*-wheat associations, root hair branching and bacterial attachment were correlated with plant growth enhancement in model systems (Jain and Patriquin, 1984). The involvement of IAA in the interaction of *A. brasilense* and *Panicum miliaceum* roots has been emphasized by Harari et al. (1988). The diminished plant growth promotion effects after inoculation with *Azospirillum* mutants with reduced IAA production support the view that auxin-like substances could be key signals mediating the plant growth stimulation effect (Barbieri et al., 1986; Dobbelaere et al., 1999). The isolation of mutants that completely lack IAA biosynthesis has been unsuccessful so far because of the presence of multiple pathways leading to IAA. Tryptophan (Trp) is generally regarded as a precursor of IAA because the addition of this amino acid to cultures of *Azospirillum* results in higher IAA production (Hartmann et al., 1983). Three main pathways are known for the conversion of tryptophan into IAA: the indole-3-pyruvic acid (IPyA) pathway, the indole-3-acetamide (IAM) pathway, and the tryptamine (TAM) pathway (Costacurta and Vanderleyden, 1995). On the basis of ^{3}H-Trp and ^{3}H-IAM feeding experiments, the existence of an additional Trp-independent pathway was suggested; when no exogenous Trp was added, 90% of the IAA was formed in a Trp-independent process (Prinsen et al., 1993). Biosynthesis of IAA without tryptophan as precursor has been shown in plants, but this is unusal in bacteria (Wright et al., 1991).

The first pathway for the production of IAA starting from Trp involves the oxidative decarboxylation of Trp into IAM and the subsequent hydrolysis of IAM to obtain IAA. The genes encoding these enzymatic activities (Trp-2-monooxygenase [TMO] and indole-3-acetamide hydrolase) are cloned and sequenced in *A. tumefaciens* and *Pseudomonas syringae*. The occurrence of an IAM-dependent pathway for IAA biosynthesis in *Azospirillum* was proposed upon the detection of TMO activity in crude cell extracts from *A. brasilense* and the observation of partial homology of an *A. brasilense* genomic DNA fragment with the *iaaM* gene of *P. syringae* (Bar and Okon, 1993). The second Trp-dependent pathway operating in *A. brasilense* has been identified as the IPyA pathway, involving the transamination of Trp to yield IPyA, followed by a decarboxylation reaction to form indole-3-acetaldehyde. The subsequent oxidation reaction occurs spontaneously and yields IAA. The gene for the key enzyme in this pathway in *A. brasilense*, indole-3-pyruvate decarboxylase, has been isolated and sequenced (Costacurta et al., 1994). DNA hybridization experiments sug-

gested that the corresponding sequence is widespread in *A. brasilense*, *A. lipoferum* and *A. halopraeferens*, but absent in *A. irakense*. An *A. brasilense ipdC* knockout mutant was found to synthesize less than 10% of the level of the wild-type IAA production, indicating that IPyA decarboxylase is a key enzyme for IAA biosynthesis in this bacterium (Prinsen et al., 1993). Further physiological aspects of IAA-production in *A. brasilense* were studied by Iosipenko and Ignatov (1995). Interestingly, it was shown that the presence of auxins upregulates the indole-3-pyruvate decarboxylase gene in *A. brasilense* (Vande Broek et al., 1999). In *A. lipoferum*, Tn*5*-mutants affected in indole acetic acid biosynthesis are available (Abdel-Salam and Klingmüller, 1987).

Recently, it was demonstrated that *A. brasilense* transconjugant strains, expressing the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase gene, showed enhanced plant growth promotion in tomato and canola plants, but not in wheat (Holguin and Glick, 2001). ACC-deaminase cleaves the ethylene precursor 1-aminocyclopropane and thus can reduce too high levels of ethylene inhibitory for plant growth. The differential effect on plant species can be explained by different levels of sensitivity to ethylene. Modulating the concentration of the plant hormone ethylene has been proposed as a possible mechanism of plant growth promotion (Glick et al., 1998).

Osmotolerance

As soil bacteria and colonizers of the rhizosphere, azospirilla are subjected to manifold potential water stress situations. In saline soil, they have to cope with low osmotic potentials, and in drying soil, there are low matrix potentials—especially in the water-deleted rhizosphere—which may limit their activity and viability. From saline soil environments, bacteria with improved osmotolerance have been isolated, e.g., *Azospirillum halopraeferens* (Reinhold et al., 1987). To balance osmotic stress from the environment and to protect cellular functions, *Azospirillum* spp. can accumulate organic compounds, so-called "compatible solutes." In *Azospirillum* spp., trehalose, glycine betaine glutamate, and proline were characterized as compatible solutes (Hartmann, 1988a; Hartmann, 1988b; Hartmann et al., 1991). In general, the osmotolerance of *Azospirillum* spp. increases in the series *A. amazonense*, *A. lipoferum*, *A. brasilense* and *A. halopraeferens* (Hartmann, 1988a); the osmotolerance of *A. irakense* lies in between *A. brasilense* and *A. halopraeferens* (Khammas et al., 1989). *Azospirillum halopraeferens* is able to take up choline in a high affinity transport and to convert it into the potent osmoprotectant glycine betaine. It is known from some halotolerant plants that, e.g., choline is present in root exudates. In *A. brasilense* Sp7, a binding-protein-dependent, high affinity uptake of glycine betaine itself (K_m: 10 μM) was demonstrated. In *A. halopraeferens* and *A. brasilense*, glycine betaine stimulates growth and nitrogen fixation under salt stress (Hartmann, 1988b; Riou and Rudulier, 1990). In osmotolerant *Azospirillum* spp. like *A. brasilense*, *A. irakense* and *A. halopraeferens*, choline, glycine betaine, as well as, e.g., glutamate, aspartate or proline, are not or only weak sources of nitrogen or nitrogen and carbon (Hartmann et al., 1988b). Obviously, retarded utilization and degradation of osmoregulatory compounds is a prerequisite for using such compounds as osmolytes. Dehydroproline (DHP)-resistant bacteria with improved osmoregulatory properties could be isolated from *A. brasilense* Sp7 (Hartmann et al., 1992), as they could from enteric bacteria (Csonka and Hanson, 1991). The fact that DHP-resistant bacteria appear in some strains spontaneously at relatively high rates under selective conditions suggests an ecophysiological relevance of this trait (improved osmoregulation). Possibly, an insertion element associated transposition processes leading to chromosome rearrangements could be involved in this phenomenon, similar to the chromosome rearrangements demonstrated following the duplication of IS*50* associated with Tn*5* transposition in *A. brasilense* (Tripathi et al., 1998). Therefore, screening for improved osmoregulatory properties might be one way to improve the environmental fitness of inoculant strains for certain biotechnological applications (Hartmann, 1994a).

Siderophores

In aerobic environments, the availability of the essential micronutrient iron is limited by the extremely low solubility of ferric oxyhydroxy complexes, which have a dissociation constant as low as 10^{-38}. Most microorganisms excrete low molecular weight molecules (e.g., phenols and hydroxamates) with a very high binding affinity for ferric iron to sequester iron traces from environmental sources. A diversity of high affinity uptake systems for iron-loaded siderophores in microorganisms, plants and animals are well known to assimilate and store this solubilized iron (Römheld and Marschner, 1986; Braun et al., 1998). The presence of siderophores in soil and rhizosphere environments and the importance of siderophores for competitive growth of microorganisms (e.g., in the biocontrol of plant pathogens) are well known (Bossier et al., 1988).

Under iron-limiting conditions, *A. lipoferum* D-2 excretes the phenolate siderophores 2,3- and 3,5-dihydroxybenzoic acid conjugated with lysine and leucine as well as salicylic acid (Saxena et al., 1986). In *A. brasilense* RG, 2,3-dihydroxybenzoic acid conjugated with ornithine and serine, the so-called "spirillobactins," were characterized (Bachhawat and Ghosh, 1987b). Fe(III)-spirillobactin is taken up via a high affinity uptake process (K_M: 0.23 μM), which involves a specific outer membrane receptor (Bachhawat and Ghosh, 1987a). Since four outer membrane proteins with molecular weights between 72 and 87 kDa are induced under iron-limiting conditions, several structurally different siderophores could be recognized. Indeed, it has been shown for *A. brasilense* Sp245 that the fungal siderophores ferrichrome, ferrichrysine, and coprogen as well as the main siderophore of *Streptomyces* spp., ferrioxamine B, can be utilized as iron uptake carriers (Hartmann, 1988b). This property varies much among different environmental isolates and species: in *A. amazonense* Y1 and *A. lipoferum* SpBr17, no iron acquisition from external siderophores was observed, and in *A. brasilense* Sp7, iron acquisition was only slight. The siderophore iron uptake of *A. brasilense* Sp6 was studied more recently in molecular genetic terms and the *lon* gene was found to be involved in the iron uptake of *A. brasilense* (Mori et al., 1992; Mori et al., 1996).

Under severe iron-limiting conditions, spontaneous mutants were isolated, which could readily use coprogen and ferrichrysin as siderophores (Hartmann et al., 1992). For *A. halopraeferens* Au4, isolated from sodic alkaline soils which exert most severe iron-limiting conditions, the artificial iron scavenger dipyridyl was not inhibitory at all, while many *A. brasilense*, *A. lipoferum* and *A. amazonense* strains were inhibited to various degrees (Hartmann, 1988b). This could be explained by the medium range iron affinity of phenolate siderophores of the spirillobactin-type. Interestingly, it was recently demonstrated that *A. irakense* strains can hydrolyze and grow on ferrioxamine (Winkelmann et al., 1999). Moreover, the isolate *A. irakense* ASP-1 has been obtained from a sweet water pond through the ability to utilize ferrioxamines as carbon source.

Applications

All species of the *Azospirillum* cluster except *A. largimobile*, *Skermanella parooensis* and *Rhodocista centenaria* were shown to associate with a diversity of plants. Several authors using different methodological approaches proved that the colonization of plant roots by *Azospirillum* provokes structural and physiological changes in the root system. In some combinations of *Azospirillum* strains and plant cultivars, also endophytic colonization of the root was observed, although this seems to occur infrequently (Schloter and Hartmann, 1998). Efficient N_2-fixation and liberation and transfer of part of the nitrogen fixed by the bacterium to the plant have so far not been unequivocally shown for azospirilla colonizing the roots of healthy plants. Some increase of nitrogen fixation (acetylene reduction) was observed in so-called "para-nodules" induced by 2,4-dichlorophenoxyacetic acid (2,4-D) in wheat or when *A. brasilense* mutants with enhanced nitrogenase activity or ammonium release were used (Christiansen-Weniger et al., 1991; Pereg-Gerk et al., 2000). Mutants with improved environmental fitness, e.g., towards improved osmotolerance or iron acquisition, could be selected for some *A. brasilense* strains (Hartmann et al., 1992; Hartmann, 1994a). *Azospirillum* can contribute some nitrogen in cellulose-decomposing mixed cultures with *Cellulomonas gelida* (Halsall and Gibson, 1985a) or through the ability of straw decomposition by some specific N_2-fixing *Azospirillum* sp. isolates (Halsall et al., 1985b). Strains with improved competitiveness and sustained performance should be obtained through screening and selection procedures for superior rhizosphere fitness when agronomic application is envisaged. It has been shown that considerable differences exist in these properties in different strains of, e.g., *A. brasilense* (Hartmann, 1988b). Among *A. brasilense* isolates from Mexico, strains with elevated production of siderophore- and bacteriocin-like substances have been characterized by Tapia-Hernandez et al. (1990) as candidates for field inoculation trials. Furthermore, genetic improvement of *Azospirillum* inoculants tailored on the basis of the indepth knowledge of the physiology and regulation of key processes in *Azospirillum*, especially *A. brasilense*, seems to be a quite reasonable approach (Steenhoudt and Vanderleyden, 2000).

Under field conditions, detailed observations with various quantification methods clearly show that certain cultivars of sugar cane (Urquiaga et al., 1992), forage grass (Boddey and Victoria, 1986a), and *Pennisetum* (V. M. Reis, personal communication) derive as much as 30–60% of the plant N from atmospheric N_2, and thus nitrogen fixation by the associated diazotrophic population must occur. Because of the natural diversity of endophytic or epiphytic diazotrophs known to exist in these plants, the bacteria and sites in which nitrogen fixation and transfer of fixed nitrogen occur are not yet clear. The contribution of *Azospirillum* spp. within this plant-

associated bacterial community to this symbiotic process is still unknown. Responses of different plants, such as *Sorghum* and *Pennisetum*, to the inoculation by *Azospirillum brasilense* have been shown (Smith et al., 1984). However, the results of thorough field experiments with *Azospirillum* inoculants demonstrated an increase of uptake and assimilation of soil nitrogen and other plant nutrients rather than nitrogen fixation (Kapulnik et al., 1982; Lin et al., 1983; Boddey et al., 1986b). This was confirmed with a nitrate reductase-negative mutant of strain Sp245, which had no effect (Baldani et al., 1987). Wheat inoculated with two *A. brasilense* strains Sp245 and Sp107 repeatedly showed increased plant growth and N-uptake. Additionally, these strains were both isolated from surface-sterilized roots and shown to be established within roots of field-grown wheat upon inoculation (Baldani et al., 1986; Baldani et al., 1987). Statistically significant increases in plant dry-matter and/or N-yield have been shown in various cereals inoculated with *Azospirillum* strains (mostly *A. brasilense* Sp7 or Cd; Kapulnik et al., 1982; Baldani et al., 1986; Okon and Labandera-Gonzalez, 1994b; Dobbelaere et al., 2001).

An explanation for the observed increased nutrient uptake (of nitrate, potassium, phosphate and microelements) after *Azospirillum* inoculation in greenhouse and field experiments is the stimulation of root development (Fages, 1994). The proliferation of root hairs is enhanced and the cell arrangement in the outer layers of the root cortex, as observed in cross sections of inoculated plants, is changed (Fallik et al., 1994). The hydraulic conductivity of inoculated roots was increased (Sarig et al., 1992), and in plants subjected to osmotic stress, leaf senescence was delayed in inoculated plants, indicating an improved water uptake (Sarig et al., 1990). In field experiments, inoculated sorghum plants repeatedly showed higher leaf water potential, lower canopy temperature, and greater stomatal conductance and transpiration (Sarig et al., 1988). Moreover, inoculated compared to non-inoculated sorghum extracted more soil moisture (on the order of 15%) and from deeper soil layers (Fallik et al., 1994). In wheat, inoculation with *Azospirillum* improved coleoptile growth in seedlings grown in darkness under osmotic and salt stress (Alvarez et al., 1996). Effects on cell wall elasticity and/or apoplastic water status were evident (Creus et al., 1997). The production of plant growth-promoting substances such as auxins, gibberellins and cytokinins by the bacteria seems to be at least partially responsible for these effects (Dobbelaere et al., 1999). Therefore, it is very important to apply the optimum inoculum dose. For example in maize, seeds are coated with a peat-based inoculum at a final concentration of 10^7–10^8 colony forming units (CFU) per seed.

Field experiments involving *Azospirillum* inoculation during the 1990s were carried out in many countries including Belgium, France, Israel, Mexico, Uruguay, Argentina and South Africa (Dobbelaere et al., 2001). In Belgium, a combination of field experimentation and commercial application was carried out by Lipha-Lyon (France) and the Department of Applied Plant Sciences, Katholieke Universiteit, Leuven. Winter wheat as well as grain maize was inoculated with either *A. brasilense* Sp245 or *A. irakense* KBC1 in Tiegem, Belgium. Since Belgian soils are often very well or even overfertilized, leading to an excess of N in soil and groundwater, the increased N-uptake by cereals would help to reduce the amount of fertilizer used or even lost. An early effect of inoculation on root development and plant growth was observed. For winter wheat, a significant increase in plant dry weight of 62 and 46% above the control was evident upon inoculation with *A. brasilense* Sp245 and *A. irakense* KBC1, respectively. This corresponded to a 34% and 33% increase in the number of shoots and an early stimulation of root development (31% and 12%) after inoculation with Sp245 and KBC1, respectively (Dobbelaere et al., 2001). These effects occurred on plots without any additional nitrogen fertilizer at early and intermediate sampling dates, and in fertilized plots ($60\ kg \cdot N \cdot ha^{-1}$), they almost disappeared without producing significantly higher final yields. Similar effects especially on the nitric N-uptake by the plants were observed when the commercial inoculum AZOGREEN-m (Liphatech, France), which is a peat-based inoculum containing the strain *A. lipoferum* CRT1, was used on grain maize *Zea mays*; the inoculated plants took up more N than the control plants owing to the larger root system. In Mexico, extensive field inoculation trials of maize, wheat, barley, and sorghum with *Azospirillum* were conducted with significant yield increases (ranging from 20–70%; Caballero-Mellado et al., 1993). In a recent collaboration of the Ministry of Agriculture Research Institute (INIFAP) and the University of Cuernavaca, Mexico, an area of 450.000 ha of maize and 150.000 ha of sorghum, wheat and barley was inoculated with a mixture of local, prescreened *A. brasilense* strains, using sterilized peat as carrier containing a minimum of 5×10^8 viable cells $\cdot\ g^{-1}$ (Dobbelaere et al., 2001). By evaluating various cultivars of the crops and 171 sites all over Mexico with diverse soil and climatic conditions, the robustness of inoculation results was tested. The results showed consistent increases of on average 26%. Stimulation in fields with low N-levels was as much as 20–95%, while that in fields with high

N-level was only 0–19%, irrespective of the location and the inoculated crop (Dobbelaere et al., 2001).

An interesting area of application is the co-inoculation of legumes with *Rhizobium* and *Azospirillum*, which reportedly increases biomass parameters, nitrogen-content and yield (Burdman et al., 1998). These positive effects may be attributed to early and increased nodulation, enhanced N_2-fixation rates, and a general improvement of root development. Nodulation may be enhanced because a greater number of epidermal cells differentiate under the influence of *Azospirillum* into root hairs susceptible to rhizobium infection (Burdman et al., 1998). Nodulation may also be stimulated by an increased secretion of flavonoids that are involved in the activation of the nodulation genes in *Rhizobium* under the influence of co-inoculated *Azospirillum* (Burdman et al., 1996; Volpin et al., 1996).

Acknowledgment. We dedicate this chapter to Dr. Johanna Döbereiner, who died in October 2000 in Rio de Janeiro, in honor of her outstanding contributions and discoveries relating to *Azospirillum* and other rhizosphere diazotrophic bacteria.

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Prokaryotes (2006) 5:141–150
DOI: 10.1007/0-387-30745-1_7

CHAPTER 3.1.6

The Genus *Herbaspirillum*

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Historical Aspects

Owing to its cell form, growth behavior and habitat within grass roots, the first isolates of the later defined genus *Herbaspirillum* were initially thought to be a new *Azospirillum* species (Baldani et al., 1984). However, RNA-RNA hybridization experiments showed no close relatedness with *Azospirillum* spp. or *Aquaspirillum itersonii* (Falk et al., 1986). The first species of the newly defined genus *Herbaspirillum*, *Herbaspirillum seropedicae* (Baldani et al., 1986), was named after the location of the EMBRAPA National Center for Agrobiology (CNPAB) in Seropedica, Rio de Janeiro, Brazil. The genus *Herbaspirillum* was extended with [*Pseudomonas*] *rubrisubalbicans*, causative agent of "mottled strip disease" in some susceptible sugar-cane varieties, because DNA-rDNA and DNA-DNA reassociation hybridization studies showed a high degree of DNA similarity (Gillis et al., 1990; Baldani et al., 1992). Additional physiological and biochemical features, including the ability to fix nitrogen, confirmed the reclassification as *Herbaspirillum rubrisubalbicans* (Baldani et al., 1996). A group of clinical isolates (EF group 1) had to be included in the genus *Herbaspirillum* as "species 3" because of its molecular and overall physiological relatedness. However, members of *Herbaspirillum* species 3 do not exhibit nitrogen-fixing ability. More recently, several new species of *Herbaspirillum* were isolated from diverse plants like *Miscanthus sinensis* and *Pennisetum purpureum* (*H. frisingense*; Kirchhof et al., 2001) and nodules of *Phaseolus* (*H. lusitanum*; Valverde et al., 2003). On the basis of molecular relatedness, a group of bacteria having the ability to efficiently degrade chlorophenols was also included in the genus *Herbaspirillum* as *Herbaspirillum chlorophenolicum* (Im et al., 2004). Although most of the bacteria in the genus *Herbaspirillum* are N_2-fixing bacteria colonizing diverse plants endophytically (Döbereiner, 1992; Döbereiner et al., 1993), clinical and environmental isolates belong to this genus, too. This resembles the situation in other species of the Betaproteobacteria, where plant-associated or even symbiotic diazotrophs, opportunistic pathogens, and potent degraders of pollutants belong to the same genera like *Burkholderia* (Coenye and Vandamme, 2003), *Ralstonia* (Chen et al., 2001) and *Azoarcus* (Reinhold-Hurek and Hurek, 2000).

Taxonomy Aspects

Herbaspirillum spp. are members of the Betaproteobacteria which include many plant-associated bacteria such as the above-mentioned genera—*Azoarcus*, *Burkholderia* or *Ralstonia*. According to results based on DNA or RNA analyses, the genus *Herbaspirillum* belongs to the RNA superfamiliy III (De Smedt et al., 1980). DNA and RNA similarity studies clearly separate *Herbaspirillum* spp. from other betaproteobacterial genera and demonstrate a very high genomic DNA similarity in each of the *Herbaspirillum* spp.

Using the 16S-rDNA-based molecular phylogenetic approach the now known five species of *Herbaspirillum* form a close cluster within the Betaproteobacteria. The phylogenetic tree (Fig. 1) illustrates the position of the *Herbaspirillum* spp. and its closest relatives in the Betaproteobacteria. The tree was constructed by a maximum likelihood analysis, and the topology was confirmed by using a distance and maximum parsimony analysis. The 16S rDNA sequence similarity values within the genus *Herbaspirillum* are 98.5–99.4% and were clearly distinct from those of the next nearest relatives, i.e., the *Ultramicrobacterium* strains D-6 and ND5 (Iizuka et al., 1998) with 95.8–97.3% sequence similarity as well as *Janthinobacterium lividum* and *Oxalobacter formigens* with 95.4–96.2% and 94.6–95.4% (Sievers et al., 1998) sequence similarity, respectively. Within the different *Herbaspirillum* species, the 16S rDNA sequence similarities are very high. For example, *Herbaspirillum frisingense*, comprising isolates from different fiber plant tissues from Germany and Brazil, forms a tight cluster with 16S rRNA similarities of 98.9–99.4% (Kirchhof et al., 2001). Compared to the

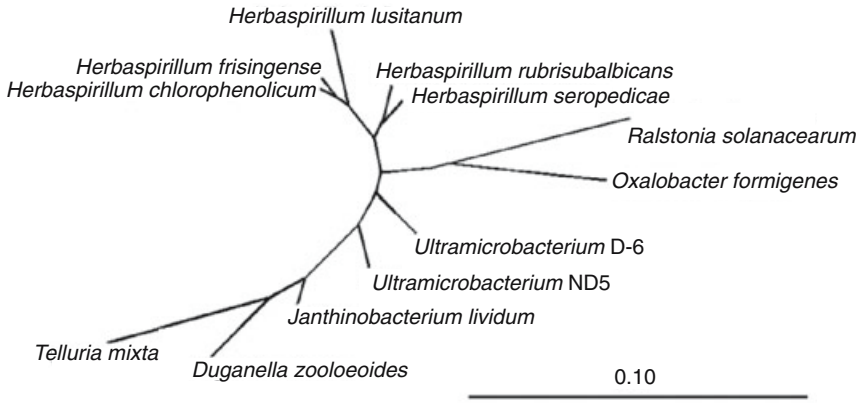

Fig. 1. 16S rDNA phylogenetic tree of *Herbaspirillum* (consensus tree). For the calculation of the phylogenetic tree, almost complete 16S rDNA sequences of the validly named *Herbaspirillum* spp. and most closely related members of the Betaproteobacteria were used. Only sequence positions which are represented in more than 50% of the members of the shown Betaproteobacteria were used for the calculation. The phylogenetic tree is based on "maximum likelihood" analysis and the topology of the tree was checked with "maximum parsimony" and "distance matrix" analyses.

Table 1. 16S rRNA-targeted oligonucleotide probes for fluorescence in situ hybridization (FISH-analysis) of *Herbaspirillum* spp.

Probe	Sequence 5′-3 position	Target	Specificity	Formamide (%)
HERB 68	AGCAAGCTCCTATGCTGC	68–85	Genus *Herbaspirillum*	35
HERB 1432	CGGTTAGGCTACCCACTT	1432–1449	Genus *Herbaspirillum*	35
Hsero 445	GCCAAAACCGTTTCTTCC	445–462	*H. seropedicae*	35
Hrubri 445	GCTACCACCGTTTCTTCG	445–462	*H. rubrisubalbicans*	60
Hfris 445	TCCAGAACCGTTTCTTCC	445–462	*H. frisingense*	50

From Kirchhof et al. 2001.

type strain of *Herbaspirillum seropedicae* (LMG 6513^T), *H. frisingense* strains have 16S rDNA sequence similarities of 98.7–99.1%. The high 16S rDNA similarity of 98.5–99.4% within the genus *Herbaspirillum* does not conclusively imply the differentiation of distinct species (Stackebrandt and Goebel, 1994). However, genomic DNA-DNA hybridization clearly allowed the differentiation, because the percentage of chromosomal DNA reassociation was 11% and 34% between *H. seropedicae* LMG 6513^T, *H. frisingense* [DSM13128]^T and *H. rubrisubalbicans* [LMG 2286]^T, respectively. Within the different species, the DNA-DNA hybridization values are 60–100% and the overall DNA G+C content (mol%) is 61–65% in all *Herbaspirillum* spp.

On the basis of the complete 16S rDNA sequences and the use of ARB-software for sequence analysis (Ludwig et al., 2004), it was possible to create a set of phylogenetic oligonucleotide probes on the genus and the species level (Hartmann et al., 2000; Kirchhof et al., 2001; M. Schmid and M. Rothballer, unpublished observation; Table 1). Using these probes, *H. seropedicae*, *H. rubrisubalbicans*, *H. frisingense* and *H. lusitanum* cells can easily be identified using the fluorescence in situ hybridization (FISH) technique (Amann et al., 1990; Wagner et al., 2003). In addition, 23S rDNA-directed oligonucleotide probes HS and HR complementary to a highly variable stretch of helix (position 55 to 59) of the 23S rRNA of *H. serpedicae* and *H. rubrisubalbicans* were developed (Kirchhof et al., 1997b). These probes were used for radioactive or nonradioactive filter hybridization in the identification of newly obtained isolates (Kirchhof et al., 1997a) but are not suitable for FISH analysis. They cannot be used, e.g., for specific differentiation between *H. rubrisubalbicans* and *H. frisingense*.

Polymerase chain reaction (PCR)-fingerprinting can be applied for the differentiation of DNA at the level of strains (Rademaker and De Bruijn, 1997). The clonal diversity of a variety of *Herbaspirillum* isolates was analyzed with different randomly amplified polymorphic DNA (RAPD) primers (Soares-Ramos et al., 2003) and primers directed to sequences derived from eukaryotic LINEs (long interspersed nuclear elements) conserved in all cells (Smida et al., 1996; Kirchhof et al., 2001; Valverde et al., 2003). The separation power was higher than the one obtained or achievable by amplified rDNA

restriction analysis (ARDRA) using four endonucleases *Alu*I, *Hae*III, *Hinf*I and *Rsa*I (Cruz et al., 2001). When different *Herbaspirillum* species were compared, RAPD- and LINE-analysis-derived banding patterns confirmed the different species, and it became additionally apparent that isolates of the same species (e.g., *H. frisingense*), originating from different plants, exhibit a different, although related, genomic fingerprint (Kirchhof et al., 2001). Isolates from roots, stems and leaves of banana formed a separate group (Soares-Ramos et al., 2003) which may even represent a new species. These findings indicate that the genetic diversity of plant-associated bacterial strains can be correlated with their plant origin (McArthur et al., 1988), reflecting a possible coevolution of plant endophytic bacteria with their hosts.

Habitats and Ecology

The origin of bacteria of the genus *Herbaspirillum* was mostly plant material, and the isolated strains showed the ability to fix nitrogen. In many cases, plant-associated *Herbaspirillum* spp. were found in apoplastic (Olivares et al., 1997; Elbeltagy et al., 2001) or intracellular locations (James et al., 1997; Olivares et al., 1997). When associated with plants, either as an asymptomatic bacterium or as a causal agent of mild disease, *Herbaspirillum* species have been found in species of the family Gramineae, like rice, wild rice (*Oryza officinalis*), *Sorghum bicolor*, *Miscanthus sinensis*, and *Pennisetum purpureum* (Baldani et al., 1996; Elbeltagy et al., 2000; Kirchhof et al., 2001). They are also associated with dicotyledoneous plants and could be isolated from root nodules of the legume *Phaseolus vulgaris* (Valverde et al., 2003) and roots as well as stems of different cultivars of banana (*Musa* spp.) and pineapple (*Ananas comosus* (L.) Merril; Weber et al., 1999; Weber et al., 2001; Cruz et al., 2001).

Some strains of *H. rubrisubalbicans* are mild pathogens of some susceptible sugar-cane varieties causing "mottled stripe disease"; they occur mainly in crops highly fertilized with nitrogen. However, all commercially used sugar-cane varieties in Brazil are resistant to this disease and *H. rubrisubalbicans* and *H. seropedicae* did not produce any characteristic symptoms when artificially inoculated into leaves by injection (Olivares et al., 1997). In addition, strains of *H. seropedicae* and *H. rubrisubalbicans* cause "red stripe disease" in *Pennisetum purpureum* as well as in *Sorghum bicolor* although symptoms are very mild in *Sorghum* leaves inoculated artificially (Pimentel et al., 1991; Olivares et al., 1997). No symptoms were observed in maize plants inoculated with *H. seropedicae* and *H. rubrisubalbicans*. In addition, no visible pathologic symptoms were apparent when *H. frisingense* was inoculated to *Miscanthus sinensis* seedlings (Eckert, 2003). In a survey to characterize the rhizobial community in nodules of *Phaseolus vulgaris*, isolates of a novel *Herbaspirillum* species, *H. lusitanum* ([LMG21710])^{T}, were obtained recently (Valverde et al., 2003). These bacteria were demonstrated to be infectious to *P. vulgaris* roots under axenic conditions, confirming the endophytic character of *H. lusitanum*.

In contrast to these plant associated *Herbaspirillum* spp., bacterial isolates (EF-group 1; Falsen, 1996) from different clinical specimens were grouped as *Herbaspirillum* sp. 3 (Gillis et al., 1991). Finally, an isolate from a 4-chlorophenol contaminated soil sediment was validly named "*H. chlorophenolicum* ([CPW301])^{T}" ([KCTC12096]^{T}; Im et al., 2004). This isolate was originally named "*Comamonas testosteroni*", collected from a stream near an industrial region in Cheongju, Korea, and selectively enriched using 4-chlorophenol as the sole carbon and energy source (Bae et al., 1996).

The majority of *H. seropedicae* and *H. rubrisubalbicans* isolates has been found in plants of tropical areas in numbers varying from 102 to 107 cells per g of fresh plant tissue (Table 2). Strains of *H. seropedicae* were first isolated from washed and surface sterilized roots of maize, sorghum and rice grown in two different soils in Rio de Janeiro State as well as from maize plants grown in a Cerrado soil in Brasilia, DF, Brazil (Baldani et al., 1986). Only a few isolates were obtained from rhizosphere soil (Baldani et al., 1986). Since *H. seropedicae* could not survive well in soil (Olivares et al. 1996), small root pieces could have been present in the rhizosphere soil used by Baldani et al. (1986).

Herbaspirillum seropedicae is a plant-endophytic bacterium (James and Olivares, 1998; James et al., 2002) infecting and colonizing tissues of rice roots mostly in the intercellular space, the apoblast. Using electron microscope analysis, *H. rubrisubalbican*s was localized in the intercellular space of the xylem and in the substomatal cavities of a mottled stripe susceptible sugar-cane variety, where the bacteria are restricted to microcolonies encapsulated within membranes of plant cell origin (Olivares et al., 1997). *Herbaspirillum seropedicae* and *H. rubrisubalbicans* were localized in the xylem in sugar-cane roots (Olivares et al., 1997) and *H. frisingense* in intercellular spaces of the root cortex and the root vascular tissue of *Miscanthus sinensis* roots (Eckert, 2003).

Table 2. Habitats and sources of isolation of *Herbaspirillum* spp.

Species	Country	References[a]
Herbaspirillum seropedicae		
Roots, stems and leaves of maize, sorghum, rice and sugar cane	Brazil	Baldani et al., 1986 Olivares et al., 1996
Roots of *Echinola crusgalli*, *Pennisetum purpureum*, *Panicum maximum*, *Digitaria decumbens*, *Brachiaria decumbens*, *Melinis minutiflora*	Brazil	Olivares et al., 1996
Stems of cultivated (*Oryza sativa*) and wild rice (*O. officinalis*, *O. barthii*, *O. rufipogon*)	Japan	Elbeltagy et al., 2000
Roots, stems and leaves of banana (*Musa* spp.)	Brazil	Weber et al., 1999, 2001
Herbaspirillum rubrisubalbicans		
Roots, stems and leaves of sugar cane and roots of *Digitaria insularis*	Brazil	Olivares et al., 1996
Roots, stems and leaves of banana and pineapple	Brazil	Weber et al., 1999
Herbaspirillum frisingense		
Roots, stems and leaves of *Miscanthus sinensis, M. sacchariflorus, Spartina pectinata*	Germany	Kirchhof et al., 1997, 2001
Roots and stems of *Pennisetum purpureum*	Brazil	Kirchhof et al., 2001
Herbaspirillum lusitanum		
Root nodules of *Phaseolus vulgaris*	Portugal	Valverde et al., 2003
Herbaspirillum species 3		
Different clinical specimen and infections (EF-group 1a and 1b)	Sweden	Falsen, 1996 Gillis et al., 1991
Herbaspirillum chlorophenolicum		
Contaminated sediment of a stream in an Industrial region in Cheongju	Korea	Bae et al., 1996 Im et al., 2004

[a]These references are representative of the literature in this area.

Isolation Procedures

Isolations of the nitrogen-fixing species *Herbaspirillum seropedicae*, *H. rubrisubalbicans* and *H. frisingense* take advantage of their ability to fix nitrogen under microaerobic conditions, as in the case of other microaerobic nitrogen-fixing bacteria like *Azospirillum* and *Gluconacetobacter* (Döbereiner, 1990). Serial dilutions of macerated root, stem or leave samples are inoculated into serum vials with nitrogen-free semisolid (1.75 g of agar/liter) NFb or JNFb medium (Table 3) and incubated at 32°C for one week (Döbereiner, 1995). In vials which exhibit a fine white pellicle, cells are examined under the microscope for the presence of small curved rods (0.6–0.7 × 4–6 μm). Following a transfer to fresh JNFb semisolid medium and incubation for 24–48 h, cultures are streaked out on solid JNFb medium containing 20 mg of yeast extract per liter and three times the bromothymol blue concentration of the JNFb medium. *Herbaspirillum seropedicae* and *H. rubrisubalbicans* form small moist white colonies with a green or dark blue center, in contrast to white colonies of *Azospirillum lipoferum* and *A. brasilense*. In the case of *H. frisingense*, the colored center of the colonies is not as highly marked as in the typical colonies of *H. seropedicae* and *H. rubrisubalbicans*. For final purification, single colonies are transferred to JNFb semisolid medium and cells from the typical pellicle are streaked onto moist BMS agar plates. Moist, smooth and small brownish colonies develop in the case of *H. seropedicae* and *H. rubrisubalbicans* (Baldani et al., 2003).

The original isolation of *H. lusitanum* (Valverde et al., 2003) was performed according to Vincent (1970) using YMA agar (Bergersen, 1961), because it was intended to isolate *Rhizobia*. On these plates, the colonies of *H. lusitanum* were mucoid, circular convex, white, slightly translucent, and usually 1–2 mm in diameter after two days at 28°C.

Herbaspirillum chlorophenolicum (Im et al., 2004), formerly *Comamonas testosteroni*, was isolated from a contaminated soil sediment near a stream in an industrial region of Korea (Bae et al., 1996) using 4-chlorophenol as the sole carbon and energy source.

Preservation of Cultures

Strains can be preserved in glycerol at –20°C or –80°C by mixing equal volumes of sterilized glycerol and washed, resuspended cells from a 48-h old culture grown in liquid JNFb medium (containing 20 mg of yeast extract and 5 mM ammonium chloride or sodium glutamate). Strains can also be kept lyophilized for many years. Cells grown on slant JNFb medium with D-glucose instead of malic acid for 48–72 h at 30°C are suspended in 2 ml of a 10% sucrose solution and

Table 3. Media used for the isolation and cultivation of diazotrophic *Herbaspirillum* spp.

Ingredient (per liter)	Semisolid NFb medium[a]	Semisolid JNFb medium[a]	Potato agar[b]
DL-Malic acid	5.0 g	5.0 g	2.5 g
Sucrose	None	None	2.5 g
K_2HPO_4	0.5 g	0.13 g	None
KH_2PO_4	None	None	None
$MgSO_4 \cdot 7H_2O$	0.2 g	0.25 g	None
NaCl	0.1 g	1.20 g	None
$CaCl_2 \cdot 2H_2O$	0.02 g	0.25 g	None
$Na_2MoO_4 \cdot 2H_2O$	None	None	None
Na_2SO_4	None	2.40 g	None
$NaHCO_3$	None	0.22 g	None
Na_2CO_3	None	0.09 g	None
K_2SO_4	None	0.17 g	None
Minor element solution[c]	2 ml	2 ml	2 ml
Bromthymol blue solution, 0.5% in 0.2 N KOH	2 ml	None	None
Fe-EDTA, 1.64%	4 ml	4 ml	None
pH (adjusted with KOH)	6.8	5.8	6.8
Vitamin solution[d]	1 ml	1 ml	1 ml
Agar	1.75 g	1.75 g	15 g

[a]Ingredients should be added to the medium in the stated order. For the cultivation under non-N_2-fixing conditions on solid agar plates ($15\,g \cdot liter^{-1}$) under air, 20 mM NH_4Cl has to be added.
[b]Totally, 200 g fresh potatoes are peeled and cooked for 30 min and filtered through cotton before other ingredients are added.
[c]$CuSO_4 \cdot 5H_2O$, 0.4 g; $ZnSO_4 \cdot 7H_2O$, 0.12 g; H_2BO_3, 1.4 g; $Na_2MoO_4 \cdot 2H_2O$, 1.0 g; $MnSO_4 \cdot H_2O$, 1.5 g; and H_2O, 1000 ml.
[d]Biotin, 10 mg; Pyridoxol-HCl, 20 mg; and H_2O, 100 ml.

Table 4. Discriminative phenotypic characteristics of *Herbaspirillum* spp.

	H. seropedicae	*H. rubrisubalbicans*	*H. frisingense*	*H. lusitanum*	*H. chlorophenolicum*
Assimilation of					
N-Acetyl-D-glucosamine	+	–	+	+	+
meso-Inositol	+	–	–	–	–
L-Rhamnose	+	–	–	+	–
meso-Erythritol	–	+	–	–	nd
Arabinose	+	+	–	+	nd

Symbols: +, present; –, absent; and nd, not determined.

5% peptone in 100 ml water. Aliquots are distributed into lyophilization ampoules and lyophilized.

Stock cultures can also be maintained on JNFb or BMS agar under a layer of sterilized mineral oil in tubes tightly sealed with rubber caps. Under these conditions, *H. seropedicae* remains viable at room temperature for at least 12 years (Baldani et al., 2003).

Identification

Cells of *Herbaspirillum* spp. exhibit Gram-negative staining. As originally described by Baldani et al. (1986) and Baldani et al. (1996), they generally have a vibroid cell shape, but they are, depending on the growth conditions, spirillum-shaped with a diameter of approximately 0.6–0.7 μm. Cell length depends on the culture medium and varies between 1.5 μm and 5.0 μm. They are very motile, using one to three flagella at one or both poles (Baldani et al., 2003).

The organisms have a strictly respiratory type of metabolism and sugars are oxidized but not fermented. With the exception of *Herbaspirillum* sp. 3 and *H. chlorophenolicum*, herbaspirilla are able to fix atmospheric N_2 under microaerobic conditions. They are oxidase and urease positive, but the catalase is variable or weak. The favored carbon sources are salts of organic acids like malate, pyruvate, succinate and fumarate both for NH_4^+ or N_2-dependent growth. Other carbon sources like glycerol, mannitol, D-glucose and sorbitol are also catabolized. However, sucrose cannot be utilized. Phenotypic characteristics which separate the five validly named *Herbaspirillum* spp. are summarized in Table 4. As shown by Valverde et al. (2003), *Herbaspirillum* spp. also exhibit a unique antibiotic resistance pattern which may also be used for differentiation.

The optimal temperature is 30–34°C and optimal pH, 5.3–8.0. The colonies on JNFb agar plates containing bromothymol blue are smooth and white with blue or green centers after one week of incubation.

Herbaspirillum seropedicae, *H. rubrisubalbicans* and *H. frisingense* can rapidly be identified using 16S rRNA-directed oligonucleotide probes and the fluorescence *in situ* hybridization (FISH) technique (Kirchhof et al., 2001; Table 1; Fig. 1).

Physiology

Herbaspirillum spp. are microaerophilic nitrogen-fixing bacteria except the mostly clinical *Herbaspirillum* species 3 and the very recently renamed species *H. chlorophenolicum* (Im et al., 2004). The diazotrophic herbaspirilla form a pellicle below the surface in nitrogen-free semisolid agar because of their microaerobic characteristic. They cannot grow or fix nitrogen in liquid N-free medium under air. However, nitrogenase activity can be detected under air when grown in liquid JNFb medium supplemented with L-glutamate and L-glutamine but not with L-serine, L-alanine or ammonium chloride when the nitrogen source is exhausted from the medium (Klassen et al., 1997). This is in contrast to some species of the genus *Azospirillum*, which can grow and fix nitrogen simultaneously, e.g., on glutamate as sole carbon and nitrogen source (Hartmann et al., 1988). Other nitrogen sources such as L-histidine, L-lysine, L-arginine or the amines methylammonium chloride, tetramethylammonium chloride, and ethylenediamine chloride do not support growth or nitrogen fixation by *H. seropedicae* (Klassen et al., 1997). *Herbaspirillum seropedicae* can assimilate or dissimilate nitrate to nitrite under oxygen limitation, but no nitrate-dependent anaerobic growth or visible gas production from nitrate is observed. However, small amounts of nitrous oxide (N_2O) are detected in the presence of 10% acetylene. Most strains of *H. rubrisubalbicans* also reduce nitrate to nitrite, but denitrification has not been observed. *Herbaspirillum chlorophenolicum* is not able to reduce nitrate to nitrite.

Compounds that can serve diazotrophic *Herbaspirillum* spp. as sole carbon and energy sources for N_2-dependent growth include malate, succinate, citrate, α-ketoglutarate, fumarate, pyruvate, *trans*-aconitate as well as mannitol, glycerol, sorbitol, glucose, galactose, and L-arabinose. *N*-Acetylglucosamine is used as sole carbon source for N_2-dependent growth by *H. seropedicae*, *H. frisingense* and *H. lusitanum* but not by *H. rubrisubalbicans*. In contrast, *meso*-erythritol is only used by *H. rubrisubalbicans* when the mannitol component of YMA medium is replaced by this carbon source and a nitrogen source like ammonium chloride is present in the medium.

Herbaspirillum seropedicae is the most intensively studied *Herbaspirillum* species. Since *H. seropedicae* is a diazotrophic plant growth promoting bacterium with potential for application as "green fertilizer," the studies focus on the nitrogen metabolism, especially the molecular organization and regulation of nitrogen fixation and ammonium assimilation genes and activities. The structural organization and regulation of the nitrogen fixation genes are well known (Machado et al., 1996; Klassen et al., 1999; Pedrosa et al., 2001). Nitrogen fixation in this organism occurs under microaerobic conditions and is tightly regulated by nitrogen compounds both at the level of synthesis and activity. In addition, ammonium causes a rapid and reversible switch-off of nitrogenase activity in *H. seropedicae*, as it does in *Azospirillum brasilense* and *A. lipoferum* (Hartmann et al., 1986; Fu and Burris, 1989). The central regulator of nitrogen control is the NifA protein, the *nif*-specific transcriptional activator in response to the levels of fixed nitrogen and oxygen (Souza et al., 1999). In addition, the general nitrogen control of the cell is regulated by NtrC, which also controls the expression of the *glnA* gene coding for glutamine synthetase, the key enzyme of the high affinity ammonium assimilation pathway (Persuhn et al., 2000; Souza et al., 2000). In contrast to the gamma-proteobacteria *Klebsiella pneumoniae* and *Azotobacter vinelandii*, where the NifL protein forms an inactive complex with the NifA protein in the presence of high levels of ammonium and oxygen, the NifA-protein is directly inactivated in response to increased levels of nitrogen and oxygen in *H. seropedicae* and the alpha-proteobacterium *Azospirillum brasilense* (Souza et al., 1991; Arsène et al., 1996). Although the mechanism of NifA activity control differs in these two groups of bacteria, the signaling pathways leading to the ammonium response have similarities. In strains of *A. brasilense* and *H. seropedicae*, which do not contain NifL, the P_{II} protein—the product of the *glnB* gene—is necessary for the ammonium control of NifA activity (Benelli et al., 1997). The signaling pathway for control of NifA activity by oxygen in rhizobia (*A. brasilense* and *H. seropedicae*) is probably sensed directly by their type of NifA protein (Monteiro et al., 1999). It has been suggested that the oxygen sensitivity of these NifA proteins involves a cysteine motif located at the end of the central domain and a linker region for the C-terminal domain, which resembles an iron-sulfur cluster-binding motif (Fischer et al., 1988). It has recently been demontrated that an alternative iron containing signal transducer for oxygen sensitivity of the NifA activity in *H. seropedicae* involves the Fnr protein, a general transcriptional regulator for the switch from aerobic to anaerobic metabolism

responsive to molecular oxygen (Monteiro et al., 2003). NifA expression is controlled by the general nitrogen regulation Ntr system which, in turn, is controlled by the state of the *glnB* product, the P_{II} protein. In *H. seropedicae*, the *glnA*, *glnB* and *ntrBC* genes have been identified (Benelli et al., 1997), suggesting that an Ntr/P_{II}-dependent signal transducer cascade senses the nitrogen levels in this organism, as it does in *A. brasilense*. A second P_{II}-like protein, called "GlnK" like in enteric bacteria, has been characterized in *H. seropedicae* (Benelli et al., 2002); it is regulated by uridylylation (Benelli et al., 2001).

Using gfp-reporter constructs, the in situ expression of the *nifH*-gene was recently demonstrated in *H. seropedicae* Z67 during the endophytic colonization of different gramineous plants (Roncato-Maccari et al., 2002). Similar results of *in situ nifH*-activity were obtained with *Azoarcus* sp. BH72 colonizing rice roots endophytically (Reinhold-Hurek and Hurek, 1998).

Application

Owing to their ability to fix nitrogen and to produce phytohormones (Bastián et al., 1998; Lambrecht et al., 2000), the diazotrophic *Herbaspirillum* spp. have the potential of plant growth promotion and associative nitrogen fixation (Baldani et al., 1995; Boddey et al., 1995; James, 2000). *Herbaspirillum* spp. are aggressive colonizers of the root interior, establishing themselves not only in the cortex and vascular tissues of roots but also systemically in the whole plant. Using axenic systems of different plants, a significant stimulation of root development due to inoculation by *H. seropedicae* (Baldani et al., 1993) and *H. frisingense* (Eckert, 2003) was demonstrated. Up to now only *Herbaspirillum seropedicae* strains have been applied in field experiments. Pereira et al. (1988) and Baldani et al. (2000) showed significant yield increases of sorghum and rice when inoculated with *H. seropedicae*. Increases of dry weight and grain yield were also observed in rice plants inoculated with several strains of *H. seropedicae* (Döbereiner and Baldani, 1998). Certain aluminum (Al)-tolerant rice varieties were stimulated in growth and nitrogen accumulation because of inoculation with *Herbaspirillum seropedicae* (Gyaneshwar et al., 2002). *Herbaspirillum*-inoculated Al-tolerant varieties (e.g., cv. Moroberekan) showed significantly more $^{15}N_2$ incorporation and higher N-contents than did the Al-sensitive variety IR45. Al-tolerant varieties secrete larger amounts of C in their root exudates, and bacteria colonizing the roots of cv. Moroberekan strongly expressed gusA- and NifH-proteins. Since *Herbaspirillum* spp. are frequently occurring in agricultural soils in the tropics and subtropics, the inoculation effect is sometimes difficult to assess because of the lack of a clear negative control. It is also possible that *Herbaspirillum* spp. are distributed and introduced through the seeds or plant stocks in the field.

Acknowledgment. This chapter is dedicated to the late Dr. Johanna Döbereiner. Her enthusiasm for plant associated nitrogen-fixing bacteria led to the original discovery of *Herbaspirillum* and many other diazotrophs at the EMBRAPA Center for Agrobiological Research in Seropedica, Rio de Janeiro, Brazil.

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Prokaryotes (2006) 5:151–162
DOI: 10.1007/0-387-30745-1_8

CHAPTER 3.1.7

The Genus *Beijerinckia*

JAN HENDRIK BECKING

Analysis of rRNA cistrons of *Beijerinckia* species (De Smedt et al., 1980) and rRNA cistron similarities as observed in DNA-rRNA hybridization experiments (De Vos et al., 1985) has demonstrated that the genus *Beijerinckia* is not phylogenetically related to the family of Azotobacteraceae (genera *Azotobacter* and *Azomonas*), but that it belongs to the alpha subclass of the purple bacteria (Stackebrandt et al., 1988) or the fourth rRNA superfamily of De Ley (De Smedt et al., 1980). Its closest relatives appear to be *Xanthomonas autotrophicus*, *Mycobacterium flavum*, a number of *Pseudomonas* species (*P. azotocolligans* and *P. diminuta*), and some authentic rhodopseudomonads. Furthermore, within the same subclasses as *Beijerinckia* are also found the genera *Azospirillum*, *Agrobacterium*, *Rhizobium*, *Acetobacter*, *Gluconobacter*, and *Zymomonas*.

Representatives of the genus *Beijerinckia* are characterized as nonsymbiotic (i.e., free-living), aerobic, chemoheterorophic bacteria with the ability to fix atmospheric nitrogen. They can be distinguished from other nitrogen-fixing bacteria by cell morphology and some physiological characteristics. Members of this genus have typical rod-shaped cells with round ends containing polar lipoid bodies. Moreover, *Beijerinckia* species show great acid tolerance, being able to grow and fix dinitrogen at pH 3.0–4.0. On agar media (nitrogen-free, glucose mineral agar), they usually produce highly raised colonies of very tenacious and elastic slime, and on liquid media they turn the whole medium viscous (see Fig. 3). For more detailed characteristics see the section on "Identification" in this Chapter and Becking (1974a).

The genus is named for M.W. Beijerinck (1851–1931), a famous Dutch microbiologist.

Habitats

Beijerinckia was originally isolated from a Malaysian quartzite soil (pH 4.5) by Altson (1936), from a Dacca, Bangladesh, soil (pH 4.9), from a soil of Insein, Burma (pH 5.2), by Starkey and De (1939; type species description). Later it was found to be widely distributed in the more acidic soils of mainly tropical regions. Although it is found primarily in acid soils, it can also be isolated from neutral and slightly alkaline tropical soils using an acid (pH 5.0) enrichment medium. *Beijerinckia* is a relatively slow grower and, using neutral or alkaline enrichment media, it cannot compete against the faster growing *Azotobacter* species. In the tropics, it is mainly present in eluvial, lateritic soils and normally absent in illuvial soils (Becking, 1961a, 1961b, 1974b; Kluyver and Becking, 1955). It is, however, also found sporadically outside the tropics.

In a large soil survey covering 392 soils of worldwide distribution, *Beijerinckia* was found in some temperate and subtropical soils of Europe (Yugoslavia), South Africa, continental Asia (India), China (Hong Kong, Kwantung), and Japan (Tokyo, Nikko National Park, etc.) (Becking, 1959, 1961a). Other authors mention its isolation from other nontropical soils, i.e., from an acidic volcanic ash soil of Sendai, Tohoku District, Japan (lat. 38°N) (Suto, 1954, 1957); from some Indian nontropical soils (up to lat. 27–30°N) (Barooah and Sen, 1959); in some subtropical and nontropical soils in Australia (lat. 15–43°S) (Tchan, 1953; Thompson, 1968); and in two soils of Egypt (Kharga Oasis, lat. 25°N) (Vančura et al., 1965). In addition, it was isolated from some Pacific Northwest soils in North America (Snake River Plain, Idaho, USA; lat. 44°N), from soil of the Squamish Bay Area, British Columbia, Canada (lat. 50°N) by Anderson (1966), and from a Montalto stony loam (pH 6.0) of Rockton, not far from the Delaware River in New Jersey, USA (lat. 40°N), by R. L. Starkey (personal communication, 1974). Also, Jordan and McNicol (1978) reported the isolation of *Beijerinckia* from a permanently cold, high Arctic soil (Devon Island, Northwest Territories; lat. 75°N).

In addition to some South African soils (Becking, 1959, 1961a) *Beijerinckia* was observed to be widely distributed in the more tropical soils of the African continent. Evidence of its ubiquitous occurrence was obtained in soils of Zimbabwe (Meiklejohn, 1968), Ivory Coast (Kauffmann and Toussaint, 1951a, 1951b) Sierra Leone, Nigeria, Ethiopia, Uganda, Kenya, Tanzania, and Congo/Zaire (Becking, 1961a). *Beijerinckia* was also found in many soil samples of the South American continent and neighboring islands: Trinidad Islands, Surinam, Venezuela, Bolivia, and Brazil (Becking, 1961a). Large surveys that

This chapter was taken unchanged from the second edition.

include many soils of different types from various tropical and nontropical regions of worldwide distribution are given by Becking (1961a) and for the Australian continent by Thompson (1968). The latter author surveyed tropical, subtropical, and nontropical Australian soils for *Beijerinckia* species (Thompson and Sherman, 1979) and 46% of 155 soils of Queensland and northern New South Wales (26°40′–30°30′ S) were *Beijerinckia* positive but only 10% of 10 soils of southern New South Wales (34°70′ S) were positive, indicating again a tropical preference of *Beijerinckia*. Seven soils of South Australia (34°91′ S) and 12 soils of Tasmania (40°70′–43°40′ S) did not contain *Beijerinckia*, but of 12 soils examined from Victoria (37°60′–38°40′ S), 16.7% contained *Beijerinckia*. *Beijerinckia* occurred in the above-mentioned soil samples in the pH range 4.5–6.9, but most frequently in samples from pH 5.0–6.4. In a certain type of grumusol (Oakes and Thorp, 1950), which exhibits gilgai microrelief by topographic variation in mounds and shelfs, in 15 randomly selected samples at each site, only the shelf soils contained *Beijerinckia* (33% of the soils) and none of the mound soils contained it. The shelf soils had a pH of 6.3 and the mound soils were of pH 7.7.

Döbereiner and Alvahydo (1959) and Döbereiner (1961) noted increased numbers of *Beijerinckia* in rhizosphere soils of sugarcane cultivated under irrigated conditions compared to normal soil. Ruinen (1956, 1961) found *Beijerinckia* as a regular component of the phyllosphere of tropical plants.

Diem et al. (1978) have used the fluorescent antibody technique to study the behavior of a *Beijerinckia* isolate in the rhizosphere and seed region (spermosphere) of rice seedlings. Also in rice, Karkhanis and Tikhe (1980) and Karkhanis (1987) reported not only its presence in the rhizosphere, but also its intracortical occurrence within the rice root. The latter observation, however, needs confirmation.

Isolation

The most selective method for the isolation of *Beijerinckia* is the use of an acid nitrogen-free enrichment medium (Becking, 1961a; Derx, 1950a, 1950b). *Beijerinckia* is acid-resistant; therefore, the use of such a medium eliminates the growth of other microbes, especially the faster growing *Azotobacter* species.

General Enrichment Methods

Enrichment Medium for *Beijerinckia* (Becking, 1961a; Derx, 1950a, 1950b)

Distilled water	1 liter
Glucose	20.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g

Adjust to pH 5.0.

Trace elements such as iron are provided by the soil used as an inoculum. The medium is poured in thin layers (2–3 mm) into petri dishes to allow good access of oxygen and to inhibit the development of anaerobic, butyric acid bacteria (such as the nitrogen-fixing *Clostridium pasteurianum)* and facultative dinitrogen-fixing anaerobes. Approximately 0.5 g of soil per petri dish is used as inoculum. The enrichment cultures are incubated at 30°C. After 2 or more weeks, these enrichment cultures are examined microscopically for the presence of the characteristic *Beijerinckia* cells (i.e., blunt rods with two lipoid bodies, one at each end of the cell; Figs. 1 and 2). In an advanced stage of development, the entire enrichment medium changes into a viscous mass (Fig. 3). When positive on the basis of microscopic examination, the enrichment culture is

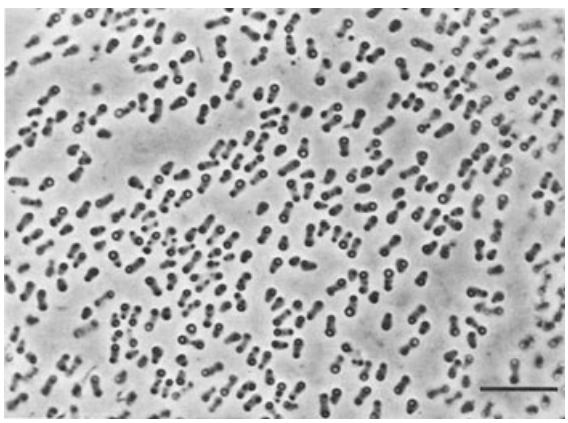

Fig. 1. *Beijerinckia indica* cells cultured in nitrogen-free glucose mineral agar (pH 5.0). The typical appearance of the cells and their intracellular polar lipoid bodies is illustrated. Living preparation, phase contrast microscopy. Bar = 10 µm.

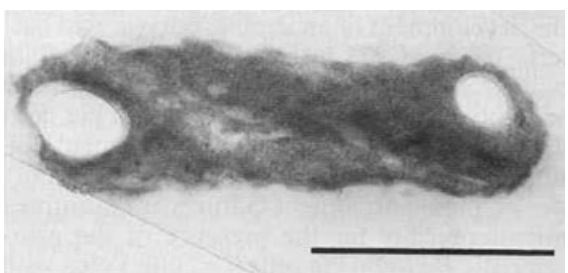

Fig. 2. *Beijerinckia indica* cell. Electron micrograph of a thin section showing the two polar lipoid bodies, which are surrounded by a membrane. Bar = 1 µm.

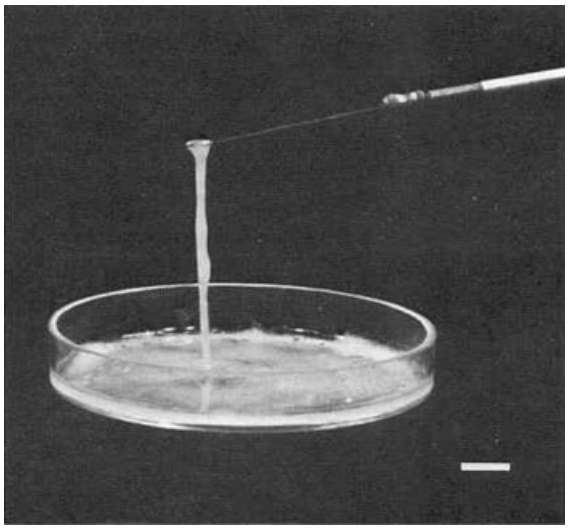

Fig. 3. Enrichment culture of *Beijerinckia*. The medium was inoculated with tropical soil. It demonstrates the highly viscous consistency of the medium after 3 weeks. Bar = 1 cm.

plated on the following nitrogen-free, mineral agar medium:

Isolation Medium for *Beijerinckia*

Distilled water	1 liter
Glucose	20.0 g
K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$FeCl_3 \cdot 6H_2O$	0.025 g (or 0.05 g)
$Na_2MoO_4 \cdot 2H_2O$	0.005 g
$CaCl_2$	0.05 g

Calcium may also be omitted; see "Cultivation." Adjust to pH 6.9.

Although an acid agar medium can be used for isolation, it is not recommended. After heat sterilization, such media tend to be soft or semisolid due to hydrolysis of the agar, and therefore the surface is difficult to inoculate with a needle.

Isolation from water sources (e.g., irrigation water of wet rice fields) is also possible, but the inoculum size should be large (10 ml or more/100 ml medium) because of the sparseness of this organism. More profitably, the sample is diluted with an equal volume of double-strength liquid enrichment medium (see above), and the pH of the medium is adjusted to 5.0 or lower. The medium is then dispensed into petri dishes in thin layers as described above.

For isolation from phyllosphere habitats, the same liquid enrichment medium (pH 5.0) can be used; the detached leaves are partially submerged in shallow layers of medium in petri dishes. On agar media, *Beijerinckia* develops characteristic, highly raised, glistening colonies of very elastic and tough slime (Fig. 4). For further purification, the same medium at neutral or alkaline pH is used with the addition of $CaCO_3$ (1 or 2% wt/vol) instead of $CaCl_2$. At alkaline pH, the slime is less tenacious and easier to dis-

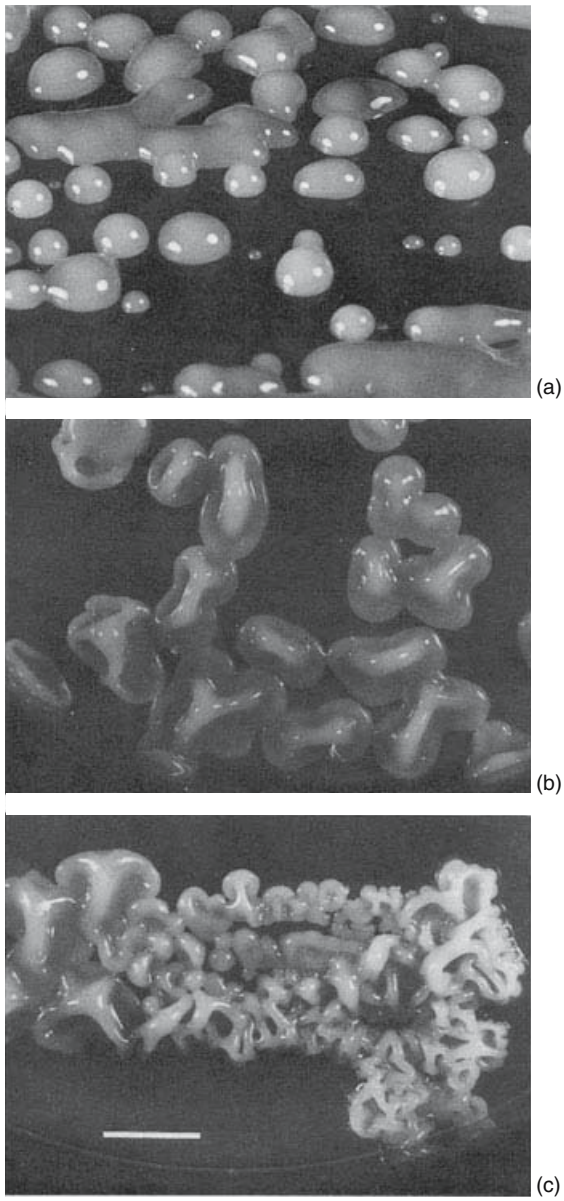

Fig. 4. (a) Typical colonies of *Beijerinckia indica* on nitrogen-free glucose mineral agar. The colonies are highly raised and have a very tough, elastic slime. In young cultures these colonies are colorless and transparent. (b) Typical colonies of an older culture of *Beijerinckia indica* on nitrogen-free glucose mineral agar. The colony turns opaque and its surface becomes plicated. (c) Typical colony of an aged culture of *Beijerinckia indica* on nitrogen-free glucose mineral agar. The colonies increase greatly in size due to copious slime production. The colonies become massive and opaque, with a plicated surface. In this stage they often become light reddish, pink, or cinnamon in color, especially on neutral or alkaline media. Bar = 1.0 cm.

solve in sterile tap water or in medium for the dilution series to obtain single-cell isolates.

When isolated, the *Beijerinckia* strains must be checked for purity by plating on plain peptone or broth agar. In contrast to *Azotobacter* and *Azomonas* species, *Beijerinckia* produces no growth on these substrates and contaminants are readily visible because they produce luxuriant development on these media.

Species-Specific Enrichment Procedures

So far, no species-specific isolation procedures are known for the five *Beijerinckia* species described. All strains obtained were random isolates from soil following the *Beijerinckia* general enrichment procedure, although the soil type may influence the occurrence of strains of certain species (see later). The isolated strains were generally only later assigned to the species level.

The use of various carbon compounds in the enrichment media offers no possibilities for species selection, because the utilization of these compounds is very variable within each species (see Tables 4.79 and 4.80, p. 318 in Becking, 1984). However, certain carbon sources might be used to increase the chance of obtaining certain species. For instance, citrate as sole source of carbon is used by 40% of the *Beijerinckia indica* strains and not by any of the other *Beijerinckia* species. Also, formate can be used for the specific enrichment of *Beijerinckia mobilis*, because it is weakly utilized by 70% of the *B. mobilis* strains, but not by the strains of any of the other species. In addition, propanol is utilized by all *B. mobilis* strains and only by 50% of the *B. indica* strains and not by *B. fluminensis* and *B. derxii*. Further, there is also a slight preference on the part of *B. mobilis* to utilize aromatic compounds (e.g., benzoate) compared to other *Beijerinckia* species. In this respect, it shows some resemblance to some representatives of the genus *Azobacter.*

The selection of the various species from plates obtained from the enrichment cultures is done primarily on the basis of colony morphology and chromogenesis (see "Identification" and "Species Differentiation," this chapter).

Cultivation

For routine maintenance or subculture, a nitrogen-free, glucose mineral agar medium of the following composition (g/liter of distilled water) can be used: Glucose, 20.0; K_2HPO_4, 0.8; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.5; $FeCl_3 \cdot 6H_2O$, 0.025 or 0.05; $NaMoO_4 \cdot 2H_2O$, 0.005; $CaCl_2$, 0.05; and agar, 15.0. The pH is adjusted to 6.9. The $CaCl_2$ may be omitted to obtain a calcium-free medium.

Also a medium of similar composition, but with $CaCl_2$ replaced by $CaCO_3$ (10–20 g/liter), can be employed. As noted elsewhere (see "Physiological and Biochemical Properties" in this Chapter) *Beijerinckia* species do not require calcium for growth and $CaCO_3$ sometimes prolonged the lag phase of growth. However, sometimes, a more alkaline medium (containing $CaCO_3$) is preferred, especially for the isolation of the organism in pure culture, since on this medium, the polysaccharide slime is usually less tenacious and sticky and therefore easier to transfer with an inoculation needle.

The preferential growth temperature for *Beijerinckia* species is 25°–30°C; the growth range is from 10°–35°C.

Preservation of Cultures

Beijerinckia strains are usually lyophilized in skim milk or dextran-sodium glutaminate solution on filter paper and stored in the dark at room temperature.

Storage has also been achieved on the usual agar media in tubes plugged with sterile rubber seals with storage in the dark at room temperature (Antheunisse, 1972, 1973); after 10 years, 33% of the cultures retained viability. In the author's (J. H. Becking) laboratory, *Beijerinckia* cultures are stored under a seal of sterile liquid paraffin or mineral oil. Usually a nitrogen-free glucose mineral agar (see "Cultivation" in this Chapter) is employed for this purpose. Such cultures generally survive for at least 3–5 years.

Strains may also be preserved indefinitely in liquid nitrogen. At the Type Culture Collection in Delft, The Netherlands, dimethyl sulfoxide (10% vol/vol) is added to the liquid cultures in or at the end of log phase, and the cultures are frozen as rapidly as possible in liquid nitrogen. For recovery, the vials are thawed rapidly in a water bath at 37°C.

For routine use, cultures can also be kept in a refrigerator at –4°C for several months. Experiments have shown that cells are resistant to freezing, and no reduction of viability occurs when stored for 3–4 months at –4°C (Becking, 1961a).

Identification

Morphological and physiological properties are both important for the identification of *Beijerinckia* from other aerobic N_2 fixers. First, they can be distinguished from these by their high acid tolerance, which allows them to grow and to fix nitrogen in nitrogen-free media of pH 4.0 or 5.0 (sometimes, even pH 3.0). They are further

characterized by the failure to form a pellicle on the surface of liquid media and by their ability to make a liquid medium viscous by polysaccharide slime production. Moreover, on solid nitrogen-free media (nitrogen-free, glucose mineral agar) they produce characteristic, large, glistening, slimy colonies with a tough, tenacious, sometimes elastic slime (Fig. 4a). Because of this slime, it is often difficult to subculture portions of a colony for purification. The slime is semi-transparent in liquid media but more opaque on solid media. Often the surface of the colony becomes strongly plicated (Fig. 4b and c). On aging, the colonies sometimes develop a brick-red (*B. indica*) or amber-brown to mahogany-brown (*B. mobilis*) color. One species, *B. derxii*, excretes into the medium a water-soluble, green, fluorescent pigment.

Beijerinckia cells can be distinguished from those of *Azotobacter* and Azomonas species by their generally smaller size; by their rather unique rod form, sometimes with a somewhat pear- or dumbbell-shaped appearance, with rounded ends; and by the characteristic presence of a highly refractile lipoid body at each pole (Figs. 1 and 2).

Unlike *Azotobacter* and *Azomonas* species and many other nitrogen-fixing bacteria, *Beijerinckia* strains do not grow on plain peptone agar. For this reason, plating on peptone agar can be used for a purity test of *Beijerinckia* isolates. Many strains of *Beijerinckia* utilize nitrate poorly or not at all. In this respect, they differ from strains of *Azotobacter* species.

Although both *Beijerinckia* and *Derxia* produce slimy colonies on agar and viscosity in liquid media, *Beijerinckia* strains can be distinguished by 1) a failure to produce dark brown or mahogany-brown pigmented colonies (except some strains of *B. mobilis*, in which the color tends to be more amber brown); 2) cells containing bipolar lipoid bodies rather than numerous lipoid bodies throughout the whole cell, as in *Derxia*; 3) failure to form a pellicle at the surface of liquid media; and 4) a positive catalase reaction of the cells (negative in *Derxia*).

In routine screening by microscopic examination of colonies on agar plates, the characteristic appearance of the *Beijerinckia* cell is conclusive for its identification. *Beijerinckia* cells are unicellular, but sometimes appear to be bicellular due to cross-wall formation in the middle of the longitudinal direction of the cell (Figs. 5 and 6). Under certain conditions, *B. mobilis* strains showed coccoid cells without terminal lipoid bodies, especially on aging (Fig. 7). In average, *Beijerinckia* cells measure 1.7–4.5 μm in length and about 0.5–1.5 μm in diameter. Rarely, misshapen cells occur, of dimensions 3.0 × 5.0–6.0 μm, which are, in addition, occasionally branched or forked. The large, very prominent lipoid bodies at each end of the cell consist of poly-β-hydroxybutyrate as determined by chemical analysis (Becking, 1974b) and X-ray diffraction analysis (Fig. 8). The lipoid bodies are

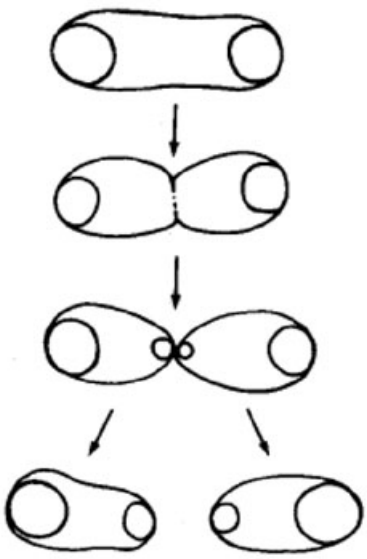

Fig. 5. Diagram of the life cycle of a *Beijerinckia* cell. A dividing cell forms a cross-wall in the middle of the longitudinal axis of the cell. In actively dividing cultures, intermediate stages can often be seen.

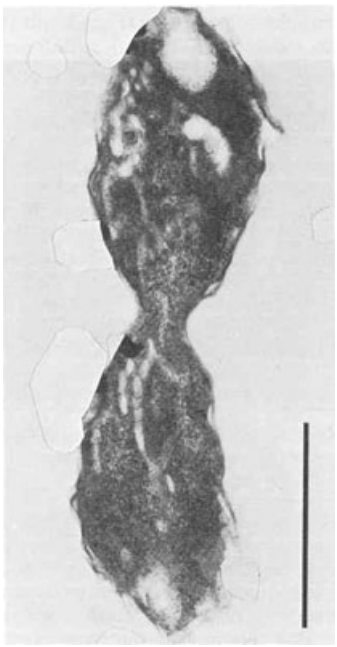

Fig. 6. Electron micrograph of a *Beijerinckia* cell in the process of division. The constriction in the middle of the cell is clearly visible and the two terminal lipoid bodies of the original cell can also be seen. Bar = 1 μm.

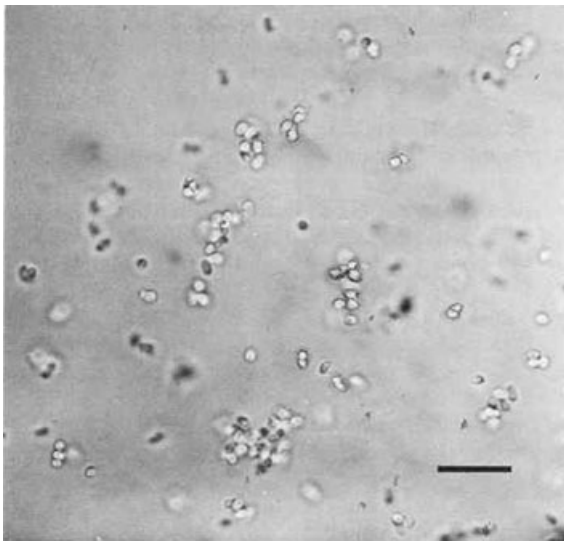

Fig. 7. *Beijerinckia mobilis* cells from an aged culture. The individual cells often lack the characteristic polar lipoid bodies and are more rounded in form, resembling certain *Azotobacter* species. Bar = 10 µm.

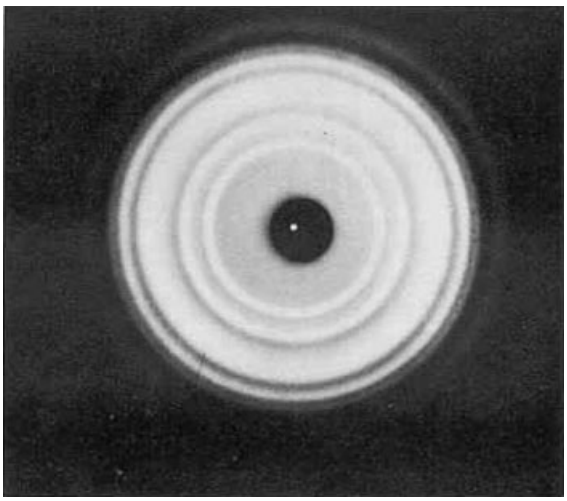

Fig. 8. X-ray diffraction diagram of crystalline poly-β-hydroxybutyrate powder obtained from *Beijerinckia*.

usually terminal in the cell, but sometimes, especially in *B. mobilis* strains, more than two lipoid globules per cell may occur (Fig. 9).

Cysts (enclosing one cell) and capsules (enclosing several cells) occur in some *Beijerinckia* species (*B. mobilis* and *B. fluminensis*, Figs. 10 and 11). The cells are Gram-negative. *Beijerinckia* species are motile by peritrichous flagella with the exception of some strains of *B. indica* (subsp. *lacticogenes*), but the flagellar arrangement has only been studied in a few strains (Hofer, 1944; Thompson and Skerman, 1979). Microscopic examination indicates that

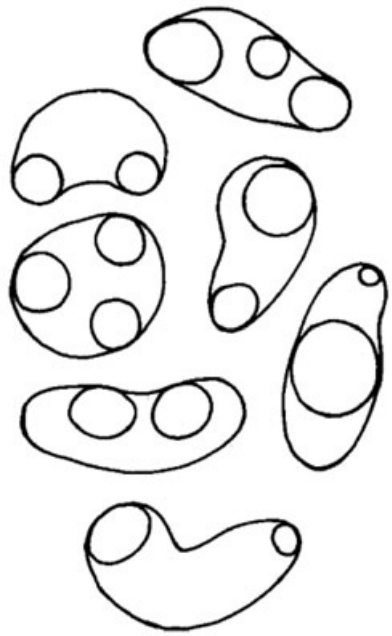

Fig. 9. Line drawing of *Beijerinckia mobilis* cells. Such misshapen cells may appear upon aging. The deformed cells show round, dumbbell, pear-shaped, or curved forms; sometimes even branched and forked forms may occur. Often, more than two lipoid bodies per cell may be present.

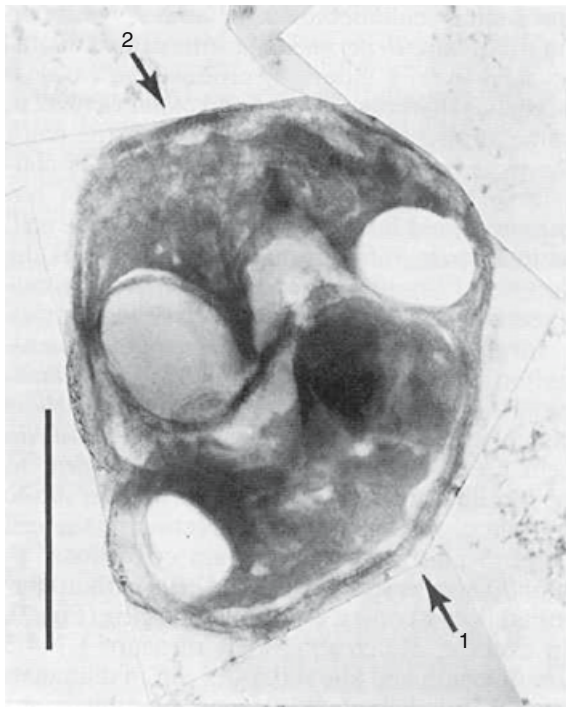

Fig. 10. Electron micrograph of a capsule of *Beijerinckia mobilis* containing two cells (see arrows 1 and 2). The terminal lipoid bodies of each cell and also the distinct capsular wall (see arrow 1) are visible. Bar = 1 µm.

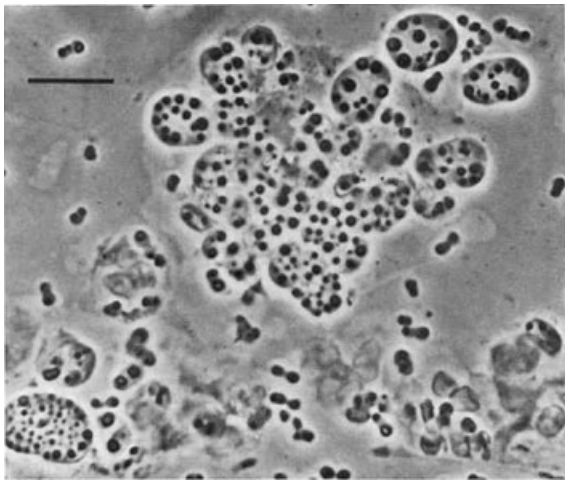

Fig. 11. *Beijerinckia fluminensis* cultured in nitrogen-free glucose mineral agar (pH 5.0), showing distinct capsule formation. The capsules enclose a large number of individual cells. Living preparation, phase contrast microscopy. Bar = 10 µm.

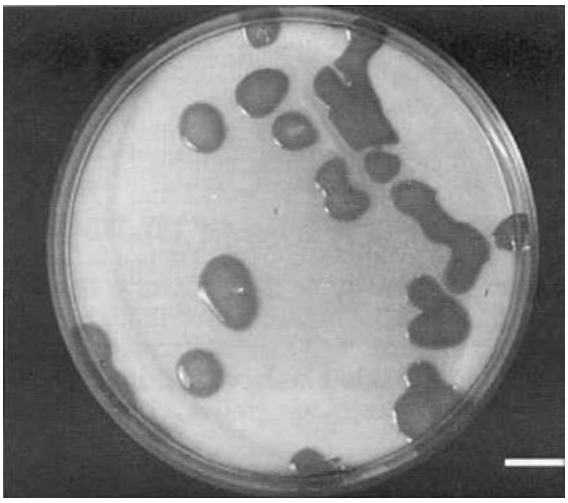

Fig. 12. Colonies of *Beijerinckia mobilis* on nitrogen-free glucose mineral agar containing $CaCO_3$. On alkaline media this species forms opaque, relatively flat *Azotobacter*-like colonies with a characteristic amber-brown pigment. The slime is less sticky than that of the other *Beijerinckia* species. Bar = 1 cm.

the flagella tend to originate in one-half of the often-dumbbell-shaped cells. The wave pattern is normal or curly, the wavelength has an average value of about 1.1–1.3 µm, and the amplitude is 0.26–0.35 µm. The amplitude of the waves in *B. fluminensis* and *B. derxii* strains is usually somewhat larger (up to 0.35 µm) than that in strains of *B. indica* (0.26 µm) (Thompson and Skerman, 1979). The GC content of the DNA of *Beijerinckia* species ranges from 54.7–60.7 mol% (T_m).

On nitrogen-free, glucose mineral agar (see "Cultivation" in this Chapter), colonies of *Beijerinckia* species exhibit various species-specific features in colony morphology and chromogenesis. For instance, *B. mobilis* colonies are flatter than those of *B. indica* and produce an uniform reddish brown or amber-brown colour on aging (Figs. 12 and 13). These colonies can readily be distinguished from those of *Azotobacter chroococcum* by their more reddish-brown color; *A. chroococcum* colonies are blackish brown. Moreover, in *B. indica* the slime is tenacious and difficult to remove, but in *B. mobilis* and *B. fluminensis* (Fig. 14) the slime is of more granular consistency, resembling that of *Azotobacter* or *Azomonas* species, and therefore is easier to remove. More details on colonial characteristics and pigmentation can be found under the individual species descriptions below. Thus colony characteristics can be used for rapid screening of isolates from plates obtained from enrichment cultures, prior to more precise identification by means of additional differential characteristics (see "Species Differentiation" in this Chapter).

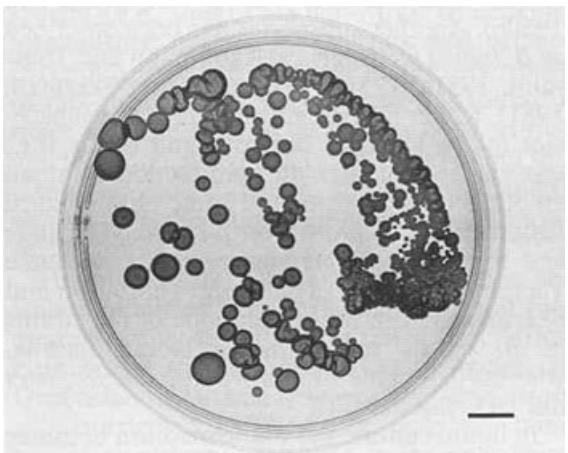

Fig. 13. Colonies of *Beijerinckia mobilis* on a nitrogen-free glucose mineral agar containing $CaCl_2$. On this transparent medium, the species forms only small raised colonies with a typical amber-brown color on aging. Bar = 1 cm.

Species Differentiation

Beijerinckia indica

Cells are straight or slightly curved rods, 1.6–3.0 µm in length and 0.5–1.2 µm wide. Lipoid bodies persist in aged cultures. No resting stages occur; cyst or ascococcus formation is never observed. Type description: (Starkey and De, 1939) Derx (1950a). The species *Azotobacter acida* Roy (1958), *Beijerinckia acida* (Roy)

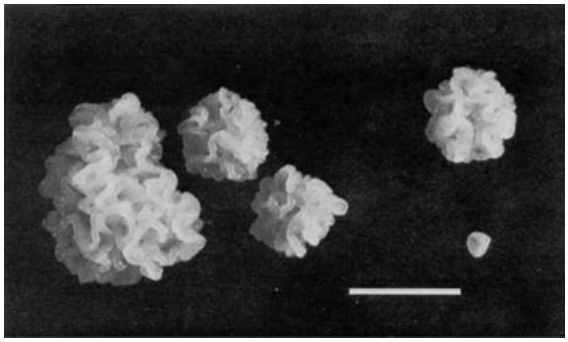

Fig. 14. Typical colonies of *Beijerinckia fluminensis* on nitrogen-free glucose mineral agar. This species forms rather small, raised colonies with a highly plicated surface. In this species the slime has a granular consistency. Bar = 0.5 cm.

Petersen (1959), and *Beijerinckia congensis* Hilger (1965) are regarded as synonymous with *Beijerinckia indica*.

This species produces highly raised colonies (Fig. 4), which on alkaline media on aging are colored brick red, cinnamon, or buff. In some strains, color production is very slow or even totally absent (i.e., *B. indica* var. *alba Derx*, 1950b). Another subspecies has been described as *B. indica lacticogenes* (Kauffmann and Tousaint, 1951a, 1951b; Thompson and Skerman, 1981), which is distinguished from the subspecies *indica* by more butyrous and brittle (i.e., less elastic) colonies, the production of small amounts of lactic acid, and some physiological features such as growth on *p*-hydroxybenzoate and the ability to metabolize protocatechuate via the *ortho*-cleavage pathway (Thompson and Skerman, 1979). Moreover, none of the strains of *B. indica* subsp. *indica* lacked flagella, whereas half of the strains of subsp. *lacticogenes* did lack flagella.

In liquid culture, the whole medium becomes viscous. On aging, color is produced but is less prominent than on agar.

Growth occurs between pH 3.0 and 10.0 (optimum rather broad, from pH 4.0–10.0; see Becking, 1961a, 1974b). Temperature range for growth is 10–35°C, with no growth at 37°C (Becking, 1961a).

Growth on and utilization of nitrate is poor, and N_2 is fixed in preference to utilization of nitrate in the medium (Becking, 1962). Weak growth occurs on malt agar, but no growth occurs in plain broth or on peptone agar.

The GC content is 54.7–58.5 mol% (T_m) (De Ley and Park, 1966; De Smedt et al., 1980).

It is the most common species, and it has been isolated from soils of all continents in tropical regions and sometimes also outside the tropics. It is particularly widely distributed in acid tropical soils.

Beijerinckia mobilis

Cells are straight, curved, or pear-shaped rods, 1.6–3.0 μm in length and 0.6–1.0 μm wide. Sometimes misshapen or forked cells occur. Ascococcus-like clusters of cells are often visible in older cultures. The typical polar lipoid bodies may disappear in aging cells, and the cells are then more rounded and resemble *Azotobacter* cells (Fig. 7). Motility is conspicuous. Type description: Derx (1950b).

This species has flatter colonies; on aging, they produce (also on relatively acid media) an amber-brown pigment (Figs. 12 and 13). Since its slime is neither elastic nor sticky, liquid media do not become viscous. There is a tendency to form a pellicle at the surface. Grows between pH 3.0 and 10.0. Optimum growth and N_2 fixation occur at pH 4.0–5.0 and decrease sharply at the more alkaline values (Becking, 1961a). Temperature range for growth is 10–35°C; no growth occurs at 37°C.

All strains tested grew well on nitrate or ammonium salts as the nitrogen source (in contrast to *B. indica*). Weak growth or no growth occurs on urea, glycine, glutamate, or tyrosine. All strains grow on leucine and casein agar. Moderate growth occurs on malt agar.

The GC content is 57.3 mol% (T_m) (De Smedt et al., 1980). This species was mainly isolated from very acid (pH mostly 4.0–4.5) tropical soils as well as in Southeast Asia, Africa, and South America. In agreement with these environmental conditions, physiological experiments showed optimum nitrogen fixation at pH 3.9; at pH 5.0, there is a sharp decline in growth and dinitrogen fixation (Becking, 1961a). Thus, soil pH is probably selective for its occurrence, and it might be possible to enrich this species selectively using more acid enrichment media (pH 4.0–4.5). It is particularly common in Indonesian (Javanese) soils.

Beijerinckia fluminensis

Cells are straight or slightly curved rods, 3.0–3.5 μm in length and 1.0–1.5 μm wide. Older cultures show characteristic large capsules enclosing 2–10 or more individual cells (Fig. 11). Division of the cells within the capsules has been observed. Motility is slow or absent, especially in older cells. Type description: Döbereiner and Ruschel 1958.

This species typically produces rather small, moderately raised colonies with irregular, rough surfaces (Fig. 14). The slime is not liquid, tenacious, or elastic, but more granular and stiff. Colonies are first opaque white, becoming, after 1–2 weeks on neutral and alkaline media, pinkish, reddish brown, or fulvous. Slime production

in liquid media is reduced as compared to that of *B. indica*. No pellicle or viscosity is produced, but a bluish-white turbidity of the whole medium is observed.

Growth occurs between pH 3.5 and 9.2. Temperature range for growth is 10–35°C (optimum is 26–33°C); no growth occurs at 37°C.

The GC content is 56.2 ± 1.8 mol% (T_m) (type strain) (De Ley and Park, 1966; De Smedt et al., 1980).

This species was originally isolated from a "Baixada Fluminensis" of pH 4.2–5.2 from the state of Rio de Janeiro, Brazil, and additionally from some other soils of Brazil (Döbereiner and Ruschel, 1958). It was also recovered from a large number of soils of Africa (including South Africa) and Southeast Asia (Indonesia) and from soils of India, Hong Kong, and China (Becking, 1961a).

Beijerinckia derxii

Cells are pear- or dumbbell-shaped, singly or occasionally in pairs, 1.3–2.1 µm in length and 1.8–2.9 µm wide. Sometimes larger or giant cells (3.3 µm–4.2 µm in length and 1.3–1.7 µm wide) may occur. Type description: Tchan (1957).

Agar colonies of this species are highly raised, slimy, and smooth. Colonies are at first semitransparent or opaque white, but after 2–3 weeks of incubation, a yellow-green fluorescent pigment is produced which diffuses from the colony into the medium. Pigment production is particularly prominent on iron-deficient media.

Two subspecies are recognized and also confirmed by the hierarchical classification of Thompson and Skerman (1979), i.e., a group containing the original type strain, *B. derxii* subsp. *derxii*, and a group of *B. derxii* subsp. *venezuelae*, originally described as *Beijerinckia venezuelae* (Materasssi et al., 1966).

The differences between the two groups are not markedly consistent. In general, strains of the subsp. *venezuelae* are characterized by the following: unable to utilize nitrate as sole source of N, nonmotile, not hydrolyzing glycogen, and growing over a slightly wider pH range (not more than 0.5 pH unit at each end of the range) and by differences in the utilization of several organic compounds as sole source of carbon (Thompson and Skerman, 1979).

In liquid culture, the whole medium turns uniformly turbid and viscous, but some strains produce less copious extracellular polysaccharides, resulting in only a turbidity, a flocculent bottom deposit, and show a slight tendency to pellicle formation.

The GC content is 59.1 mol% as examined in one strain (the type strain) (De Ley and Park, 1966).

B. derxii was originally isolated from Australian soil (Tchan, 1957); the soil pH was not specified. Later, the present author found it in several Asian and South American soils, mainly in the more alkaline ones (at least more alkaline than for the occurrence of the usual *Beijerinckia* species), e.g., soils of pH 6.5–7.0 (J. H. Becking, unpublished observations). Thus, soil pH apparently also has an influence on the occurrence of this species. This species also was found in more alkaline soils by Materassi et al. (1966) and by Florenzano et al. (1968), who found this green-pigment-producing species particularly abundant in the more alkaline Venezuelan soils (pH 6.8–8.3).

However, Meiklejohn (1954) mentioned one isolate obtained from an acid sandy soil (pH approximately 4.5) from Tanzania, East Africa, even before the type strain was described. In Queensland, Australia, where the type strain was isolated, J. P. Thompson (personal communication) mentioned that, in the undulating country of this region with alkaline soils of montmorillonite clay, *B. derxii* occurred preferentially in the soil of the shelves with a relatively more acidic reaction (pH 6.3–6.5), but rather alkaline for *Beijerinkia*, whereas in the more alkaline soil (pH 7.7) of the mounds (tops), *Beijerinckia* was entirely absent (see Thompson and Skerman, 1979, and "Habitats").

Physiological and Biochemical Properties

The efficiency of N_2 fixation in *Beijerinckia* strains is usually 10–13 mg N/g glucose consumed in a nitrogen-free medium containing 1 or 2% carbohydrate. In tests with 47 *B. indica* strains of various origins, it was observed that the efficiency of N_2 fixation was variable and related to the growth rate of the strains (J. H. Becking, unpublished observations). Of the strains tested, 21% were fast growers and poor N_2 fixers (6.0–9.9 mg N/g glucose consumed), 53% were moderate growers and moderate N_2 fixers (10.0–13.9 mg N/g glucose consumed), and 26% were slow growers and good N_2 fixers (14.0–16.9 mg N/g glucose consumed). The efficiency of N_2 fixation is also dependent on the age of the culture and on the carbohydrate concentration. Low carbohydrate levels tend to increase efficiency. In a study of some fast- and slow-growing strains of *B. indica*, the average amount of mg N fixed per g glucose consumed could be increased in the fast-growing strains from 10 to 20, and in the slow growing strains from 15 to 30, by decreasing the glucose level 10-fold (Becking, 1971, and J. H. Becking, unpublished observations).

Decreasing the partial pressure of O_2 below that of the normal air atmosphere also distinctly increases the N_2-fixing efficiency (Becking, 1971, 1978; Spiff and Odu, 1973).

The obligately aerobic character of their metabolism is also evident from the presence of cytochromes. Absorption peaks at 415–424 nm (Sorêt); 480 and 518 nm (β); 525–527, 551–556 nm (αc); and 604, 630 nm ($a_1aa_3a_2$) have been reported (Moss and Tchan, 1958). Therefore, the cells contain cytochrome *c* (γ_{max} 524 and 552 nm) and cytochrome *a* (γ_{max} 590 and 630 nm).

The mineral requirements of *Beijerinckia* species can generally be correlated with the mineral status of lateritic soils (Kluyver and Becking, 1955; Becking, 1961b, 1981). For example, such soils are rich in iron, and it is noteworthy that *Beijerinckia* species have a higher requirement for iron than do members of the Azobacteraceae, and also they are able to tolerate extremely high iron levels, even at low pH values (Becking, 1961a, 1961b). Thus, these organisms can be used as an indicator of the degree of "ferralitization" of these tropical soils (Dommergues, 1963). Ferralitization is the intense chemical weathering of soil, with the complete breakdown of all minerals except quartz, and the leaching of soluble salts and other exchangeable bases, usually producing an accumulation of iron and aluminum oxides.

Moreover, in agreement with the mineral status of lateritic soils and in contrast to *Azobacter* species, *Beijerinckia* does not require calcium for growth and dinitrogen fixation. Usually, $CaCO_3$ is even slightly inhibitory since it prolongs the lag phase of growth. The cause of this inhibition is unknown. It may be that a reduction of trace elements by precipitation and fixation by $CaCO_3$ during the heat-sterilization process are responsible. Media supplemented with 0.05% $CaCl_2{\cdot}2H_2O$ invariably showed good and relatively rapid growth.

Like most other N_2 fixers, *Beijerinckia* species require molybdenum for optimum growth and N_2 fixation. The molybdenum requirement in *Beijerinckia* species is variable, depending on the strain; for half-maximal growth under N_2-fixing conditions it ranges from 0.004–0.034 ppm Mo (0.4–3.5 μg Mo/100 ml). It is about 10–20 times higher than that in some *Azobacter vinelandii* strains and about two times higher than that in *Azomonas agilis,* whereas some strains of *A. chroococcum* have an equal to higher Mo requirement (Becking, 1962, 1974b). Moreover, unlike *Azobacter* species the molybdenum requirement cannot be replaced by vanadium (Becking, 1962, 1974b). Therefore, they probably do not possess an alternative vanadium-activated nitrogenase. Tests with nitrate-utilizing *Beijerinckia* strains showed that the Mo requirement of the nitrate system reductase is about 10 times lower than that of the nitrogenase system in the same strain (Becking, 1962, 1974b).

In alkaline nitrogen-free glucose mineral liquid media *Beijerinckia* strains decrease the pH of the medium to pH 4.0–5.0 by acid production. Analyses have shown that the acids produced are mainly acetic acid (Becking, 1961a) and a small amount of lactic acid (Kauffmann and Toussaint, 1951a, 1951b). In acid media (pH 4.0–5.0), no change of reaction occurred in spite of good growth, while in very acid media (pH 3.0) the pH of the medium is increased during growth by alkaline production.

In contrast to *Azotobacter,* many strains of *Beijerinckia* utilize nitrate poorly or not at all (Becking, 1962).

The polysaccharide slime of *Beijerinckia* species (Fig. 15) consists of glucose, galactose, mannose, glucuronic acid, and galacturonic acid, but no heptose. The main component (52% of the dry weight of the polysaccharide) is glucose (López and Becking, 1968).

The polar lipoid globules within the cell are poly-β-hydroxybutyrate (PHB), as shown biochemically, but also by elementary analysis (C, 52.5%; H, 7.5%; O, 37.8%) and by x-ray diffraction analysis (Becking, 1974b, and unpublished observations).

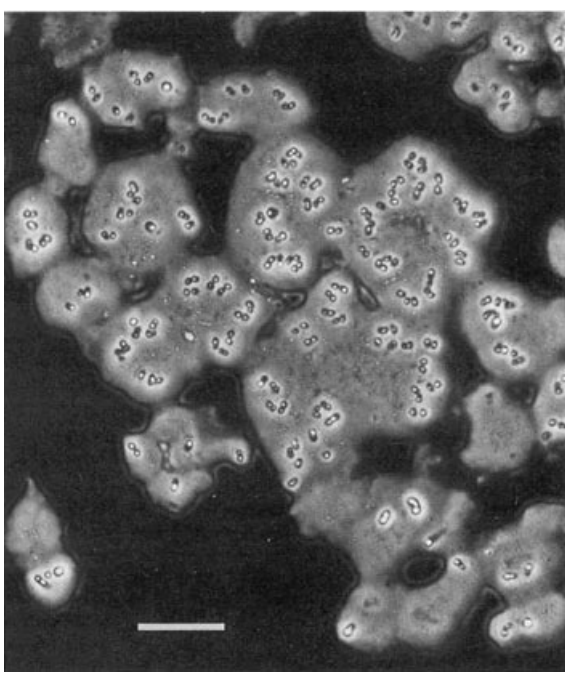

Fig. 15. Cells of *Beijerinckia indica* suspended in India ink, showing polysaccharide formation around the cells. Living preparation, phase contrast microscopy. Bar = 10 μm.

Applications

Although *Beijerinckia* was originally isolated from rice soils and tends to occur in larger numbers in the rhizosphere of rice plants, no evaluation has been made of the significance of this organism for rice culture. Its occurrence in these anaerobic water-logged rice soils is certainly not limited by oxygen supply, since rice plants have a continuous aerenchym system from the leaf and shoot to the roots, which transports air towards the root. In spite of the reduced status of the soil as evident by the black ferrous iron color of these iron-rich soils, along the rice roots a distinct layer of red ferric iron is observed (Becking, 1974b, 1978).

Increased densities of *Beijerinckia* have also been observed in sugar cane soils (Döbereiner and Alvahydo, 1959; Döbereiner, 1961) and a favorable effect of these populations on sugar cane growth was observed. Again, larger *Beijerinckia* numbers were found in the rhizosphere along the roots compared to plain soil (Döbereiner and Alvahydo, 1959). In greenhouse pot experiments, it was shown that sorghum (*Sorghum vulgare*) strongly stimulates the growth of *Beijerinckia* (Carneiro and Döbereiner, 1968).

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CHAPTER 3.1.8

The Family Acetobacteraceae: The Genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, and *Kozakia*

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Introduction and General Characteristics

Acetic acid bacteria comprise a widespread group of Gram-negative, obligately aerobic rods. They occur mainly in sugary, acidic and alcoholic habitats and have been studied extensively, since they can play a positive, neutral or detrimental role in foodstuffs and beverages. Some species of the Acetobacteraceae play a key role in the industrial manufacture of vinegar. The following genera belong to this family: *Acetobacter* (type genus), *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter* and *Kozakia*. The names *Acetobacter* and *Gluconobacter* are known in literature since 1898 and 1935, respectively, whereas the other genus names were published after 1989.

All members of the Acetobacteraceae are obligately aerobic and their metabolism is strictly respiratory with oxygen as the terminal electron acceptor. A common feature of the acetic acid bacteria (with the exception of *Asaia*) is the aerobic oxidation of ethanol to acetic acid, with accumulation of the latter in the medium. After the complete oxidation of ethanol, strains of *Acetobacter*, *Gluconacetobacter* and *Acidomonas* oxidize acetic acid further to CO_2 and H_2O (overoxidation of ethanol), whereas oxidation of acetate is absent in *Gluconobacter* and weak in *Asaia* and *Kozakia*. Growth occurs in the presence of 0.35% acetic acid (except for *Asaia* strains), and gluconic acid is usually produced from glucose. The characteristic ubiquinone of *Acetobacter* is the Q-9 type, whereas all other acetic acid bacteria possess Q-10. Most acetic acid bacteria possess the $C_{18:1\omega7}$ straight-chain unsaturated acid as predominant fatty acid in their cell envelopes.

Phylogeny

Phylogenetically the six genera of the acetic acid bacteria (*Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter* and *Kozakia*) belong to the Alphaproteobacteria. The simplified 16S rDNA dendrogram (Fig. 1) shows the phylogenetic position of the Acetobacteraceae among the major alphaproteobacterial families and groups. On the basis of DNA-rRNA hybridizations, Gillis and De Ley (1980) found that *Acetobacter* and *Gluconobacter* formed a separate branch in a rRNA superfamily which was later shown to belong to the alpha subclass of the Proteobacteria (Stackebrandt et al., 1988). The phylogenetic affiliations of *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconobacter*, *Gluconacetobacter* and *Kozakia* species and related acidophilic taxa were studied on the basis of 5S rRNA (Bulygina et al., 1992) and 16S rRNA/rDNA sequences (Sievers et al., 1994a; Kishimoto et al., 1995; Sievers et al., 1995b; Yamada et al., 1997; Sokollek et al., 1998b; Franke et al., 1999; Lisdiyanti et al., 2000; Lisdiyanti et al., 2001; Lisdiyanti et al., 2002; Schüller et al., 2000; Yamada et al., 2000b; Katsura et al., 2001; Katsura et al., 2002; Cleenwerck et al., 2002; Yamashita et al., 2004; Yukphan et al., 2004b). Within the Alphaproteobacteria the six genera of the family Acetobacteraceae form a distinct line of descent together with the phylogenetic neighbors *Acidiphilium* and *Acidocella*, as well as with the species *Acidisphaera rubrifaciens*, *Rhodopila globiformis*, *Roseococcus thiosulfatophilus*, *Craurococcus roseus* and *Paracraurococcus ruber* (Sievers et al., 1994a; Yurkov et al., 1994; Saitoh et al., 1998; Hiraishi et al., 2000; Figs. 1 and 2). All the above-mentioned organisms are characterized by an acidophilic phenotype, with the exception of *Roseococcus thiosulfatophilus*. Several of these taxa, such as *Acidiphilium*, *Acidisphaera rubrifaciens*, *Rhodopila globiformis*, *Roseococcus thiosulfatophilus*, *Craurococcus roseus* and *Paracraurococcus ruber*, contain bacteriochlorophyll *a* and carotenoids and belong metabolically to the group of aerobic bacteriochlorophyll-containing bacteria.

The detailed phylogenetic relationships among the actual species of the acetic acid bac-

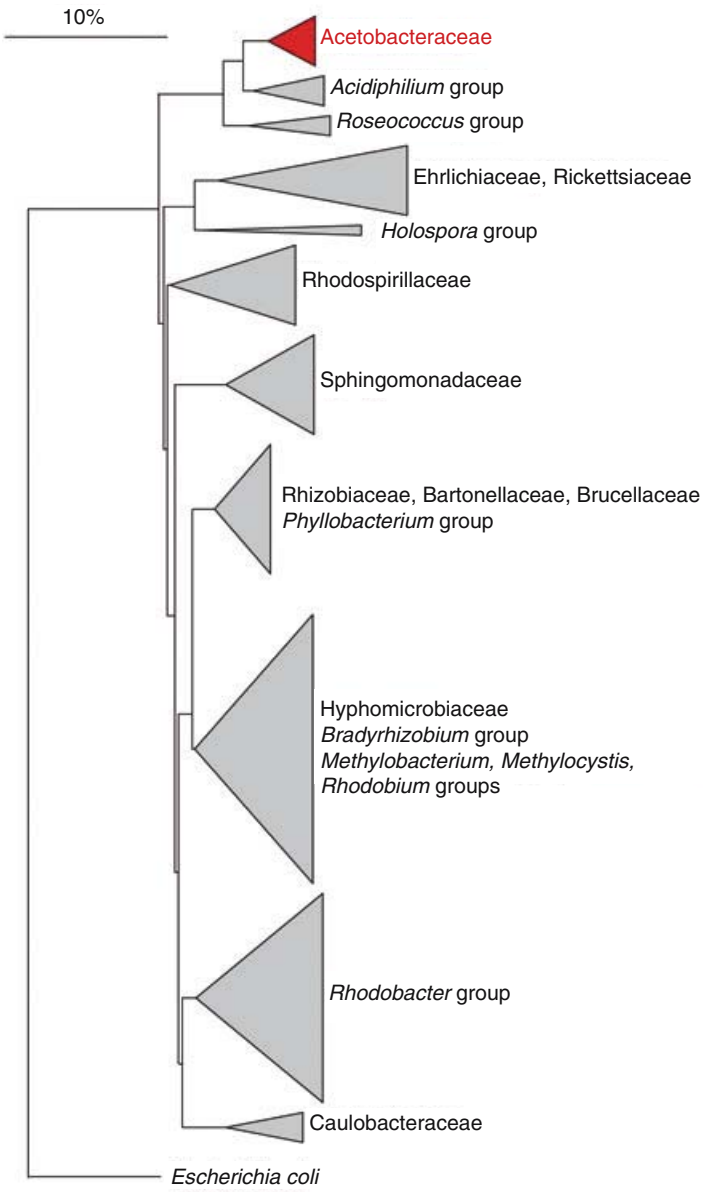

Fig. 1. Phylogenetic position of the Acetobacteraceae within the Alphaproteobacteria. The simplified neighbor-joining tree is based on the 16S rDNA sequences of the type strains of the type species of the alphaproteobacterial genera. The bar indicates 10% estimated sequence divergence. *Escherichia coli* was used as outgroup. The width of the triangles is proportional to the number of genera in the cluster. A more detailed phylogenetic tree of the Acetobacteraceae is represented in Fig. 2.

teria are shown in Fig. 2. Four major clusters can be recognized: one cluster containing all *Acetobacter* species; a second cluster with the *Gluconobacter* species; a third cluster grouping *Asaia* and *Kozakia*; and a fourth large cluster containing all *Gluconacetobacter* species, with a subcluster comprising *Ga. entanii*, *Ga. europaeus*, *Ga. hansenii*, *Ga. intermedius*, *Ga. oboediens*, and *Ga. xylinus* and a subcluster containing *Ga. azotocaptans*, *Ga. diazotrophicus*, *Ga. johannae*, *Ga. liquefaciens* (type species) and *Ga. sacchari*. *Acidomonas methanolica* (formerly *Acetobacter methanolicus*) forms a separate lineage among the acetic acid bacteria (Fig. 2): its 16S rDNA sequence similarity is less than 96.0% with all the other species of the Acetobacteraceae. Within the genus *Acetobacter*, the overall 16S rDNA sequence similarity values are above 95.5% and are less than 96.3% with those of other genera. Within the genera *Gluconobacter* and *Gluconacetobacter* the 16S rDNA sequence similarities are above 97.9% and 96.3%, respectively. *Gluconacetobacter europaeus*, *Ga. intermedius*, *Ga. oboediens* and *Ga. xylinus* are highly related as they share more than 99% sequence similarities (Fig. 2).

Taxonomy

Acetobacter and *Gluconobacter* have long constituted the core genera of the acetic acid bacteria (Asai, 1968; De Ley and Frateur, 1974a; De Ley and Frateur, 1974b; De Ley and Swings, 1984a; De Ley et al., 1984b; De Ley et al., 1984c; Swings,

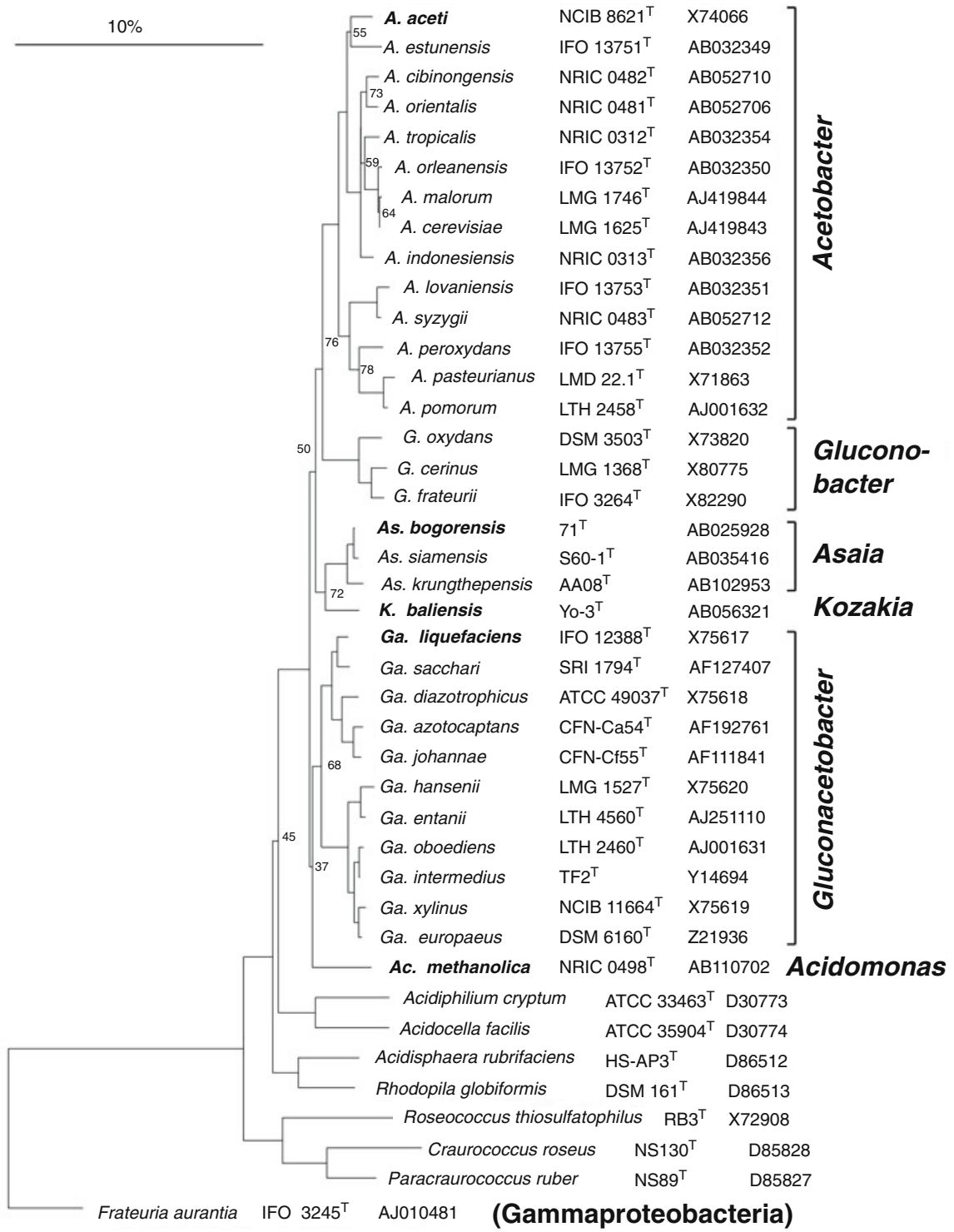

Fig. 2. Phylogenetic relationships of the Acetobacteraceae and some related taxa based on 16S rDNA sequences of the type strains (neighbor-joining method). Type species are in boldface; strain numbers and accession numbers are indicated. *Frateuria aurantia*, a member of the Gammaproteobacteria, shares phenotypic similarities with some acetic acid bacteria and was used as outgroup. The bar indicates 10% estimated sequence difference. Bootstrap values (derived from 1000 replications) greater than 80 are not shown. Abbreviations: *A.*, *Acetobacter*; *Ac.*, *Acidomonas*; *As.*, *Asaia*; *G.*, *Gluconobacter*; *Ga.*, *Gluconacetobacter*; and *K.*, *Kozakia*.

1992a). The generic designation "*Acetobacter*" was suggested in 1898 by Beijerinck (Beijerinck, 1898), whereas Asai (1935), studying for the first time oxidative bacteria living on various fruits, proposed the genus name *Gluconobacter* for those acetic acid bacteria displaying a limited ability to oxidize ethanol to acetic acid but a strong ability to oxidize glucose to gluconic acid. Bacteria belonging to *Gluconobacter* correspond to the "suboxydans" group of Frateur (1950). The status of the generic name *Gluconobacter* has been thoroughly discussed by De Ley and Frateur (1970). Various species and subspecies have been described in both genera, and taxonomic rearrangements have often been proposed. In his book on the acetic acid bacteria, Asai (1968) gives a detailed overview of the various classification systems for these bacteria. With the publication of the Approved Lists of Bacterial Names (Skerman et al., 1980), the genus *Acetobacter* was restricted to the following three species: *A. aceti* (with four subspecies), *A. pasteurianus* (five subspecies) and *A. peroxydans*, and the genus *Gluconobacter* contained one species (*G. oxydans*) with five subspecies.

The family name Acetobacteraceae was introduced in 1980 by Gillis and De Ley on the basis of results of DNA-rRNA hybridizations indicating that the peritrichously flagellated *Acetobacter* species were phylogenetically closely related to the polarly flagellated *Gluconobacter* species. A major change in the classification of the acetic acid bacteria was introduced when Yamada et al. proposed the genus *Gluconacetobacter* on the basis of partial sequence analysis of 16S rDNA and chemotaxonomic comparisons of ubiquinone systems (Yamada et al., 1997; Yamada et al., 1998). As a result, the following ubiquinone Q-10 containing *Acetobacter* species were transferred to the genus *Gluconacetobacter*: *A. diazotrophicus* (Gillis et al., 1989), *A. europaeus* (Sievers et al., 1992), *A. hansenii* (De Ley et al., 1984c), *A. liquefaciens* (Gosselé et al., 1983b) and *A. xylinus* (Yamada, 1983; Yamada et al., 1997; Yamada et al., 1998; Fig. 2).

Table 1 summarizes the current and part of the former classification (before 1998) of the acetic acid bacteria and includes the type strains and accession numbers of the 16S rDNA sequences of the validly published species names (situation June 2004). The facultative methylotrophic *Acetobacter methanolicus* (Uhlig et al., 1986) was transferred to the new genus *Acidomonas* (Urakami et al., 1989), a taxon occupying a separate position in the rDNA-dendrogram (Fig. 2). Yamada et al. (1999) and Lisdiyanti et al. studied a great number of acetic acid bacteria isolated from fruits, flowers and traditional fermented food collected in the tropical country Indonesia (Lisdiyanti et al., 2000; Lisdiyanti et al., 2001; Lisdiyanti et al., 2002; Lisdiyanti et al., 2003). These newly investigated biotopes led to the introduction of the two new genera *Asaia* (Yamada et al., 2000b; Katsura et al., 2001) and *Kozakia* (Lisdiyanti et al., 2002). In addition, since 1998, several novel species and new combinations were described in the genera *Acetobacter* and *Gluconacetobacter* (Table 1).

Table 2 summarizes the features which are useful for the differentiation of the six genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter* and *Kozakia*. Although identification at the genus level is relatively easy to make, differentiation and identification of the species are often more problematic. The identification methods, based on the phenotypic features of acetic acid bacteria (Swings et al., 1992b), are indeed time-consuming and not very accurate. In the last decade taxonomists increasingly used DNA-DNA hybridizations to reveal the genomic relationships and the species delineations among the acetic acid bacteria, particularly for the species of the genera *Acetobacter* (Sokollek et al., 1998b; Lisdiyanti et al., 2000; Lisdiyanti et al., 2001; Cleenwerck et al., 2002), *Asaia* (Yamada et al., 2000b; Katsura et al., 2001; Yukphan et al., 2004b), *Gluconacetobacter* (Franke et al., 1999; Schüller et al., 2000; Fuentes-Ramírez et al., 2001) and *Kozakia* (Lisdiyanti et al., 2002). The application of molecular methods based on the characterization of specific DNA segments (such as the 16S-23S rDNA internal transcribed spacer regions) will likely improve the speed and quality of the identification of acetic acid bacteria at the species level (Ruiz et al., 2000; Trček and Teuber, 2002; Yukphan et al., 2004a). Studies of new niches as well as the applications of rDNA-sequencing, DNA-DNA hybridizations and genomic fingerprinting techniques, together with phenotypic and chemotaxonomic characterizations, led in the past five years to an increase in the number of species and some nomenclatural rearrangements (Table 1).

Detailed descriptions of the various genera and species of the Acetobacteraceae can be found in the second edition of *Bergey's Manual of Systematic Bacteriology* (Sievers and Swings, 2005).

Habitats and Uses of Acetic Acid Bacteria

The following discussion will be focused not only on the classical niches of the acetic acid bacteria, i.e., beer and wine (where they occur as spoilers) or vinegar works (where they are the causative organisms), but also on lesser known niches. Since 1996, large numbers of acetic acid bacteria were isolated and characterized from a wide variety of habitats in Indonesia, Thailand and the Philippines (Yamada et al., 1999; Lisdiyanti

Table 1. Current and former classification of the Acetobacteraceae, together with the type strains and accession numbers of the 16S rDNA of the type strains.

Current classification (2003)[a]	References	Former classification (before 1998)[a]	References	Type strain[b]	Accession number 16S rDNA
Acetobacter aceti[T]	Beijerinck, 1898	*Acetobacter aceti*[T]	Beijerinck, 1898	NCIB 8621, JCM 7641	X74066, D30768
Acetobacter cerevisiae	Cleenwerck et al., 2002			LMG 1625	AJ419843
Acetobacter cibinongensis	Lisdiyanti et al., 2001			4H-1, NRIC 0482	AB052710
Acetobacter estunensis	Lisdiyanti et al., 2000	*"Acetobacter estunense"*[c]	Carr, 1958	IFO 13751	AB032349
Acetobacter indonesiensis	Lisdiyanti et al., 2000			5H-1, NRIC 0313	AB032356
Acetobacter lovaniensis	Lisdiyanti et al., 2000	*Acetobacter lovaniensis*[c]	Frateur, 1950	IFO 13753	AB032351
Acetobacter malorum	Cleenwerck et al., 2002			LMG 1746	AJ419844
Acetobacter orientalis	Lisdiyanti et al., 2001			21F-2, NRIC 0481	AB052706
Acetobacter orleanensis	Lisdiyanti et al., 2000	*Acetobacter orleanensis*[d]	Henneberg, 1906	IFO 13752	AB032350
Acetobacter pasteurianus	Beijerinck, 1916	*Acetobacter pasteurianus*	Beijerinck, 1916	LMD 22.1	X71863
Acetobacter peroxydans	Visser 't Hooft, 1925	*Acetobacter peroxydans*	Visser 't Hooft, 1925	IFO 13755	AB032352
Acetobacter pomorum	Sokollek et al., 1998a			LTH 2458	AJ001632
Acetobacter syzygii	Lisdiyanti et al., 2001			9H-2, NRIC 0483	AB052712
Acetobacter tropicalis	Lisdiyanti et al., 2000			Ni-6b, NRIC 0312	AB032354
Acidomonas methanolica[T]	Urakami et al., 1989	*Acetobacter methanolicus*	Uhlig et al., 1986	NRIC 0498	AB110702
Asaia bogorensis[T]	Yamada et al., 2000			71, NRIC 0311	AB025928
Asaia krungthepensis	Yukphan et al., 2004b			AA08, NRIC 0535	AB102953
Asaia siamensis	Katsura et al., 2001			S60-1, NRIC 0323	AB035416
Gluconacetobacter azotocaptans	Fuentes-Ramírez et al., 2001			CFN-Ca54	AF192761
Gluconacetobacter diazotrophicus	Yamada et al., 1997[e]	*Acetobacter diazotrophicus*	Gillis et al., 1989	ATCC 49037	X75618
Gluconacetobacter entanii	Schüller et al., 2000			LTH 4560	AJ251110
Gluconacetobacter europaeus	Yamada et al., 1997[e]	*Acetobacter europaeus*	Sievers et al., 1992	DES 11, DSM 6160	Z21936
Gluconacetobacter hansenii	Yamada et al., 1997[e]	*Acetobacter hansenii*	De Ley et al., 1984b	LMG 1527	X75620
Gluconacetobacter intermedius	Yamada, 2000	*Acetobacter intermedius*	Boesch et al., 1998	TF2, DSM 11804	Y14694
Gluconacetobacter johannae	Fuentes-Ramírez et al., 2001			CFN-Cf55	AF111841
Gluconacetobacter liquefaciens[T]	Yamada et al., 1997[e]	*"Gluconobacter liquifaciens"*	Asai, 1935	IFO 12388	X75617
Gluconacetobacter oboediens	Yamada, 2000	*Acetobacter oboediens*	Sokollek et al., 1998a	LTH 2460	AJ001631
Gluconacetobacter sacchari	Franke et al., 1999			SRI 1794, DSM 12717	AF127407
Gluconacetobacter xylinus[f]	Yamada et al., 1997[e]	*Acetobacter xylinus*[d,f]	Yamada, 1983	NCIB 11664	X75619
Gluconobacter cerinus[g]	Yamada and Akita, 1984	*Gluconobacter cerinus*	Yamada and Akita, 1984	LMG 1368, IFO 3267	X80775, AB063286
Gluconobacter frateurii	Mason and Claus, 1989	*Gluconobacter frateurii*	Mason and Claus, 1989	IFO 3264	X82290
Gluconobacter oxydans[T,h]	De Ley, 1961	*Gluconobacter oxydans*[T]	De Ley, 1961	DSM 3503	X73820
Kozakia baliensis[T]	Lisdiyanti et al., 2002			Yo-3, NRIC 0488	AB056321

Abbreviations: [T], indicates type species of each genus; ATCC, American Type Culture Collection, Manassas, Virginia, USA; CFN, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, Mexico; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; IFO, Institute for Fermentation, Osaka, Japan; IMET, National Collection of Microorganisms of the German Democratic Republic, Jena, Germany (see DSMZ); LMD, Laboratory of Microbiology, Technical University, Delft, the Netherlands; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; LTH, Institut für Lebensmitteltechnologie, Universität Hohenheim, Stuttgart, Germany; NCIB, National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland, United Kingdom; NRIC, NODAI Research Institute Culture Collection, Tokyo University of Agriculture, Tokyo, Japan; and SRI, Sugar Research Institute, Mackay, Australia.

[a]Quotation marks indicate that the species name did not occur on the Approved Lists of Bacterial Names (Skerman et al., 1980).

[b]Strain numbers of the type strains correspond to the strains used for sequence determination of their 16S rDNA (last column).

[c]On the Approved Lists of Bacterial Names (Skerman et al., 1980) "*Acetobacter estunense*" and *Acetobacter lovaniensis* were listed as subspecies of *Acetobacter pasteurianus*: *A. pasteurianus* subsp. *estunensis* and *A. pasteurianus* subsp. *lovaniensis*, respectively.

[d]On the Approved Lists of Bacterial Names (Skerman et al., 1980) *Acetobacter orleanensis*, *Acetobacter liquefaciens* and *Acetobacter xylinus* were listed as subspecies of *Acetobacter aceti*: *A. aceti* subsp. *orleanensis*, *A. aceti* subsp. *liquefaciens* and *A. aceti* subsp. *xylinum*, respectively.

[e]The original spelling of the genus name *Gluconoacetobacter* (Yamada et al., 1997) was corrected to *Gluconacetobacter* at the occasion of its validation (Validation List no. 64, Int. J. Syst. Bacteriol., 48, pp. 327–328 [1998]).

[f]*Acetobacter xylinus* contains the following subspecies: *A. xylinus* subsp. *sucrofermentans* and *A. xylinus* subsp. *xylinus* (Toyosaki et al., 1995).

[g]*Gluconobacter asaii* (Mason and Claus, 1989) is a junior subjective synonym of *Gluconobacter cerinus* (Katsura et al., 2002).

[h]*Gluconobacter oxydans* contains the following subspecies: *G. oxydans* subsp. *industrius*, *G. oxydans* subsp. *melanogenes*, *G. oxydans* subsp. *oxydans*, *G. oxydans* subsp. *sphaericus*, and *G. oxydans* subsp. *suboxydans*.

Table 2. Differential characteristics of the genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter* and *Kozakia*.[a]

Characteristics	Genera					
	1.	2.	3.	4.	5.	6.
Flagellation	Pe or nm	Po or nm	Pe or nm	Pe or nm	Po or nm	nm
Production of water soluble brown-pigment(s)	–	–	–	d	d	–
Production of cellulose	–	–	–	d	–	–
Production of mucous substance(s) from sucrose	d	–	–	–	–	+
Production of acetic acid from ethanol	+	+	–	+	+	+
Oxidation of						
Acetate to CO_2 and H_2O	+	+	w	+	–	w
Lactate to CO_2 and H_2O	+	– or w	w	+	–	w
Growth in the presence of 0.35% acetic acid	+	+	–	+	+	+
Growth on methanol	–[b]	+	–	–	–	–
Ketogenesis from glycerol	d	–	d	d	+	+
Production of γ-pyrones from						
D-Glucose	–	nd	–	d	d	–
D-Fructose	–	nd	d	–	+	d
Production of keto-D-gluconates from D-glucose						
2-Keto-D-gluconate	d	–	+	d	+	+
5-Keto-D-gluconate	d	–	+	d	+	+
2,5-Diketo-D-gluconate	–	–	–	d	d	–
Acid production from						
D-Arabinose	–	D	+	–	+	d
L-Rhamnose	–	–	d	–	–	–
D-Fructose	–	–	+	+	+	–
L-Sorbose	–	nd	+	d	+	–
Sucrose	–	–	+	–	+	d
Raffinose	–	–	–	–	–	+
D-Mannitol	–	–	d	d	+	–
D-Sorbitol	–	–	d	–	+	–
Dulcitol	–	–	d	–	–	–
Glycerol	–	+	+	+	+	+
Major ubiquinone	Q-9	Q-10	Q-10	Q-10	Q-10	Q-10
G+C content (mol%)	52–61	62–63	59–61	56–67	54–63	57

Symbols: +, 90% or more of the strains positive; w, weakly positive reaction; d, 11–89% of the strains positive; and –, 90% or more of the strains negative.

Abbreviations: Pe, peritrichous; Po, polar; nm, nonmotile; and Nd, not determined.

[a]1., *Acetobacter*; 2., *Acidomonas*; 3., *Asaia*; 4., *Gluconacetobacter*; 5., *Gluconobacter*; and 6., *Kozakia*.

[b]Contradictory reports were published for the growth on methanol by *A. pasteurianus*, *A. lovaniensis* and *A. pomorum* (Gosselé et al., 1983; Uhlig et al., 1986; Urakami et al., 1989; Sokollek et al., 1998b; Cleenwerck et al., 2002). (See text in the section *Acetobacter*.)

Data from Lisdiyanti et al. (2002) and Yamashita et al. (2004).

et al., 2000; Lisdiyanti et al., 2001; Lisdiyanti et al., 2002; Lisdiyanti et al., 2003; Seearunruangchai et al., 2004).

Grapes and Wine Starting in Pasteur's time, it was known that the acetification of wine was due to acetic acid bacteria (Vallery-Radot, 1924). The microbiologists at that time already knew that these bacteria could be isolated from the pellicle that forms on wine when it is exposed to air. They were also aware of the fact that the acetification requires oxygen (Wermischeff, 1893; Behrens, 1896; Fuhrmann, 1905). The high alcohol tolerance of acetic acid bacteria was already observed by Fuhrmann (1905), who isolated many acetic acid bacteria from wine must but was unable to isolate strains from either intact or injured grapes. Dupuy (1957) isolated *Acetobacter pasteurianus* (75% of the isolated strains) and *A. aceti* (19%) from wines in southern France. Acetic acid bacteria are present in about half of the samples of ripe grapes (Peynaud and Domercq, 1961), and they multiply on injured grapes. *Gluconobacter* occurs profusely on ripe grapes of the Bordeaux region (73% of all strains isolated), whereas *Acetobacter* and *Pseudomonas* constitute, respectively, only 12 and 15% of the isolates (Blackwood et al., 1969). *Gluconobacter* was present on intact and injured grapes but not during the fermentation. The ketogenesis by *Gluconobacter* strains on grapes leads to a higher requirement for SO_2 in the sulfitation process. These results apparently do not agree with the ones from Kahlon and Vyas (1972), who isolated *A. aceti*, *A. xylinum* and *A. pasteurianus* from grapes but no

Gluconobacter strains. The explanation is probably that they used an enrichment medium with ethanol, which favors the development of *Acetobacter*. Passmore and Carr (1975) isolated *Gluconobacter* but no *Acetobacter* strains from dried and mature grapes and vines. Ameyama (1975) isolated a strain of *G. oxydans* from grapes. In South Africa, Du Toit and Lambrechts (2002) reported *G. oxydans* as the dominating acetic acid bacteria in fresh grape must and *A. pasteurianus* and *Gluconacetobacter liquefaciens* during the fermentation process.

Sake The Japanese rice wine (sake) contains 14–24% ethanol (Prescott and Dunn, 1959). Rice starch is converted to fermentable sugar by the amylases of *Aspergillus oryzae*. Fermentation of this mixture yields sake. Takahashi (1907) studied several samples of altered "hyochi" sake (the term "hyochi" applies to sake altered after fermentation, clarification, and pasteurization). He isolated *Acetobacter pasteurianus* from samples that had the smell of acetic acid.

Palm Wine Palm wine is a typical tropical beverage, a product from the fermentation of sugary palm sap. Palm trees commonly used for this purpose are *Elaeis guineensis*, *Raphia vinifera*, *Cocos nucifera* and *Arenga pinnata*. Palm wines are popular beverages in Africa, South America, and the Far East.

To collect the sap, several tapping methods are practiced. One of them is to cut a hole at the base of the male flower bud, from which the juice oozes out. One tree may produce one to several liters of sap per day. Palm wine is a whitish, effervescent, acidic alcoholic beverage, the product of a mixed alcoholic, lactic acid, and acetic acid fermentation. As a first step, the sugar of the sap is fermented to ethanol within 8–12 h by *Zymomonas* and yeasts, thus creating a medium highly suitable for the development of acetic acid bacteria. A rather complex flora is present in palm wines (Swings and De Ley, 1977). During palm sap fermentation, the acetic acid bacteria appear after 2–3 days. Acetic acid bacteria utilizing glucose or sucrose or both may be present in earlier stages of the palm sap fermentation (Okafor, 1975). *Acetobacter* species were isolated from palm wines (Faparusi, 1973; Okafor, 1975) and from the immature spadix of the palm tree (Faparusi, 1973); *A. pasteurianus*, *A. lovaniensis*, *A. indonesiensis* and *A. tropicalis* from palm wine (Simonart and Laudelout, 1951; Lisdiyanti et al., 2003); and *Gluconacetobacter xylinus* from the leaflets of the palm tree and the surrounding air (Faparusi, 1973). *Gluconobacter oxydans* was found on the floret of the palm tree, in the tap hole, and in palm sap (Faparusi, 1973; Faparusi, 1974).

Tequila Greene and Breazeale (1952) described an *Acetobacter* infection in tequila that had developed cloudiness upon standing. The infection occurred during summer.

Fermentations of Cocoa Cocoa wine is made by fermentation of seeds of cocoa and is a popular drink in Nigeria (Bassir, 1968). The cocoa beans are ground and boiled in sugared water and allowed to cool to approximately 50°C before the inoculum is added. Its alcohol concentration (9.5–11.6% [w/v]) is significantly higher than that of palm wine (Bassir, 1968). *Acetobacter* and *Gluconobacter* strains have been isolated from cocoa wine (Bassir, 1968; Jones and Jones, 1984). Together with yeasts and lactic acid bacteria, the acetic acid bacteria contribute to the development of aroma and flavor as well as the necessary heat and acetic acid during the proper curing of the cocoa beans (Thompson et al., 1997). *Acetobacter* and *Gluconobacter* strains play a critical role during the aerobic phase of the natural fermentation of cocoa beans for the manufacture of chocolate (Schwan et al., 1995). To improve the reliability and quality of the cocoa fermentation process, Schwan (1998) successfully used a defined, mixed starter culture, including *A. aceti* and *G. oxydans*.

Cider In Europe, the word cider means apple wine. Acetic acid bacteria are associated with two bacterial cider disorders, i.e., the acetification of cider and "cider sickness." The acetification of cider (French: piqûre) by acetic acid bacteria, occurring previously in primitive cider manufacture, is almost completely ruled out in modern cider technology (Maugenet, 1962; Carr and Whiting, 1971). *Acetobacter* species are associated with another cider disorder known as "cider sickness" or as "framboisé" in France. This disorder of sweet ciders is characterized by: 1) an unpleasant odor and taste due to the formation of acetylaldehyde and 2) a milky colloidal precipitate that forms because the acetaldehyde combines with polyphenolic compounds (Guittonneau et al., 1939; Maugenet, 1962; Drilleau, 1977). Passmore and Carr (1975) extensively studied the acetic acid bacteria of the cider industry. The acetic acid bacteria involved in spoilage presumably hibernate in dried and injured apples, from which they spread to flowers in the spring. In September, they spread to the apples themselves. The apples for cider-making are shaken from the trees; most of them are bruised and become easily contaminated with a complex flora. As the pH range of the apple juice is 3.2–4.2 (Phillips et al., 1956), only acid-tolerant microorganisms such as *Zymomonas*, yeasts, lactic acid bacteria, and acetic acid bacteria can grow in the juice (Carr

and Whiting, 1971). The acetic acid bacteria presumably enter the cider factory with the damaged fruit: one week before the pressing season started, no acetic acid bacteria were found in apple silos, pressing equipment, or juice tanks, whereas two weeks later, several *Acetobacter* and *Gluconobacter* strains were isolated (Passmore and Carr, 1975). *Gluconobacter* was found mainly in the earlier stages of the cider-making process, which are rich in sugar, i.e., on the harvested apples, in the pressed pomace, and in the juice. Varieties of *Acetobacter aceti* and *A. pasteurianus* were found at every stage. Fernandez et al. (1994) observed a very similar composition of the bacterial flora during cider-making in the Basque country (Spain), and Maugenet (1962) isolated acetic acid bacteria from French cider samples and assigned them to the same two *Acetobacter* species.

Vinegar HISTORY OF VINEGAR. The word "vinegar" is derived from the French "vin" and "aigre" (in French: "vinaigre"), literally meaning "sour wine." Many interesting details about vinegar and its history can be found in the review article by Conner and Allgeier (1976). In the past, wine making and vinegar preparation were always linked. As early as 4000 B.C., vinegar was already mentioned in Babylonian writings, as a product of the date palm, the culture of which was highly developed (Nickol, 1979). Huber (1927) has given extensive details concerning the history of the role and the preparation of wines and vinegar in Babylon; "fruit honey," i.e., sugary syrups made from figs, apricots, grapes, and dates, was used in the kitchen and for the preparation of wine. After the process of wine-making was completed, the extracted dates together with their stones were used to make a drink composed of a sort of lemonade and vinegar. Vinegar was also a side product—mostly unwanted—of beer-brewing. Originally (4000–3000 B.C.), beer, wine and vinegar were homemade but later (3000–2000 B.C.) they were prepared in breweries. To obtain a higher alcohol content, the Babylonian and Assyrian breweries started mixing barley and dates. Raisin wine and raisin vinegar also became very popular in Babylon from approximately 1000 B.C. on.

In the Babylonian kitchen, it was quite common to add spices and herbs, such as tarragon, lavender, mint, celery, caper, and especially saffron, to vinegar, which was used as a seasoning agent together with salt, pepper, and sesame oil in salads of crude vegetables and as a dressing with lentils, beans, pears, or millet porridge. Because of the hot climate, the Babylonians also used vinegar as a pickling agent for fish, meat, vegetables (e.g., palm shoots, fennel, tarragon and anise), and fruits (e.g., melons, cucumbers, peaches, apricots, apples and pears). In antiquity, vinegar was the drink of the poor: the peasants, soldiers and travelers. Wine and beer were only drunk at home with meals and at festivities.

We are relatively well informed about the process of vinegar preparation in ancient Rome as well as vinegar's uses as a condiment and as a preservative and in medicine. Vinegar formation occurred spontaneously as a defect during wine manufacture. In the Greek settlements in Southern Italy, wine production and trade had become important from the eighth century B.C., and the Romans took over the Greek knowledge of vinegrowing and wine-making. In Rome, good cooking was an art for the rich, who eagerly accepted the Greek cooking heritage. One of the principal characteristics of the classical Latin taste was its preference for sweet-sour (honey-vinegar) combinations resembling those found in Chinese specialties (André 1961). Uncooked vegetables were eaten with a sort of French dressing ("vinaigrette"), the *acetaria* (Plinius, in André 1958). The following kinds of vinegar were known: *acetum piperatum*, vinegar containing pepper; *oxygarum*, made from vinegar, garum, and herbs; *oxymeli*, made by boiling vinegar, honey, water, and salt; and *oxysporium*, vinegar containing spices (André 1961). Plinius (in André 1958) recommended drinking vinegar to treat nausea, hiccupping, leprosy, freckles, ulcers, bites of dogs, and stings of scorpions, scolopendras, and other sting-possessing insects, but also by rectal injection to stop diarrhea.

Several natural sugar-containing juices or mashes that are first converted by fermentation to ethanol solutions can serve as raw materials for vinegar production. The vinegar is usually named after the original raw material, such as wine vinegar, cider vinegar or apple cider vinegar, malt vinegar, rice vinegar, etc. The latter type of vinegar is of course very common in Eastern countries.

The first commercial process for the production of vinegar used wooden casks in which, e.g., the wine was oxidized by the "mother of the vinegar," containing the acetic acid bacteria at the surface of the liquid. It was known as the "Orleans process" or "French method." In Japan, ancient techniques of vinegar making were probably introduced from China at the end of the fourth century, and the commercial production of vinegar started in the middle of the Edo period (1603–1867; Masai, 1980). The raw materials were rice, sake, and sake-cake, which is a by-product of the sake-production. Until the end of World War II, surface fermentation in wooden shallow tanks was the most common process used in Japan (Masai, 1980). The "German method" (or "quick process") was invented in Germany in the beginning of the nineteenth cen-

tury. Wine was added to the top of and allowed to trickle through a generator consisting of a tank packed with beechwood shavings. The acetic acid bacteria grow attached to the beechwood shavings and catalyze the oxidation of alcohol to acetic acid. Air is introduced through holes from the bottom of the tank. The latter generator method was only seldom used in Japan (Masai, 1980).

The development of scientific knowledge about vinegar and its formation is a reflection of the historical development of chemistry and microbiology. Milestones were: 1) the recognition of the alcoholic fermentation and vinegar formation as separate processes; 2) the role of oxygen in vinegar formation; 3) the determination of the chemical structure of acetic acid; and 4) the biological nature of vinegar production.

Kützing (1837) probably first suggested the biological nature of the acetification process. In France, Pasteur (1868) discovered that the cause of deterioration of wines was due to bacterial contamination during and after the yeast fermentation process, resulting in conversion of alcohol to acetic acid. He made also the observation that during vinegar manufacture similar bacteria, which he called "*Mycoderma aceti*," were responsible for production of the acetic acid by the oxidation of ethanol. Subsequently, Pasteur showed that partial sterilization or "Pasteurization" of wine at 55°C prevented the spoilage process.

VINEGAR PRODUCTION IN THE TWENTIETH CENTURY. In the twentieth century, the trickle vinegar generator was improved through mechanisms of forced aeration and temperature control, yielding 98% conversion of a 12% ethanol solution to acetic acid in 5 days. Hromatka and Ebner (1949) introduced the submerged reactor with an aeration system fixed at the bottom of the fermentor. This submerged process known as the "Frings acetator" has been used for many decades worldwide and allows a faster oxidation of ethanol. More details concerning vinegar production, including illustrations of the beechwood shavings generator and the Frings acetator, can be found in the chapter on Organic Acid and Solvent Production in Volume 1.

If one considers the interest that acetic acid bacteria inspired in many microbiologists, it is astonishing how little effect the available information about these bacteria has had on vinegar manufacture (Allgeier and Hildebrandt, 1960). This is particularly striking in regard to the application of the pure culture approach in the vinegar industry. Henneberg (1909) pointed out the enormous advantages of introducing pure cultures in vinegar manufacture: it would reduce the occurrence of vinegar eels, "Kahm" yeasts, and the unwanted contaminant *A. xylinum* (now named *Gluconacetobacter xylinus*). It would also permit the selection of suitable strains possessing the required technological and commercial qualities. Wiame and Lambion isolated several strains of acetic acid bacteria directly or after enrichment from acetators containing beechwood shavings (Wiame and Lambion, 1951a; Wiame and Lambion, 1951b). These organisms produced a satisfactory acetification under experimental conditions. Frateur and Simonart (1952) attempted to overcome the difficulties of isolating acetic acid bacteria from beechwood shavings by the use of "amphibian" cultures. They observed the appearance of extremely acid-resistant organisms, able to grow as dwarf colonies, which were identified as *A. pasteurianus* and *A. xylinum*. These species were also predominant in the normal flora. By examination of the plasmid profiles from the microfloras from both submerged and trickle vinegar fermentors it was concluded that only one strain was active in the former process, whereas a mixed-strain population was active in the latter (Sievers et al., 1989). The proof that the isolated strains were actually responsible for acetification could only be solved by the application of Koch's postulates. Shimwell (1954) isolated an *A. aceti* strain as a true working strain fulfilling Koch's third postulate. With this strain he produced up to 12% malt-, spirit-, or wine-vinegar of excellent quality at rates as fast as in the normal production process. Another strain, thought to be "the active one" in a quick vinegar process, was isolated from beechwood shavings by Wiame et al. (1959). It was characterized by a pronounced acidophily (its optimum pH was 3.1) and was appropriately named "*A. acidophilus*." Unfortunately, the original strains were lost (J. M. Wiame, personal communication).

Pure cultures are generally not used in the manufacture of vinegar. However, in the factory of the Finnish State Alcohol Monopoly, pure cultures of *A. pasteurianus* have been used to manufacture vinegar since 1945 (Suomalainen, 1961; Suomalainen et al., 1965), producing 10% acid in six days. No changes in the strain were observed over many years.

Turtura et al. (1973) isolated and identified more than 1000 strains of acetic acid bacteria from vinegars and soured pressed grapes; they found 56% of the isolates were strains of *A. pasteurianus*, 34% of *A. aceti*, and 10% of *Gluconacetobacter xylinus*. Passmore and Carr (1975) identified the following acetic acid bacteria in a cider-vinegar generator: *A. pasteurianus*, *A. aceti* and *Ga. xylinus*; they found no *Gluconobacter* strains. *Acetobacter pasteurianus* was also isolated from Japanese rice vinegar fermented by the traditional static method using

polished as well as unpolished rice (Nanda et al., 2001).

It may be surprising that even to the end of the twentieth century, the vinegar industry has mostly worked with acetic acid bacteria that were not properly characterized. The lack of defined and appropriate starter cultures is mainly due to problems associated with the isolation, cultivation and preservation of the acetic acid bacteria involved in high-acid vinegar production (Sievers and Teuber, 1995a). The availability of microbiologically defined starter preparations would, e.g., allow the accelerated start-up of the fermentation process or culture rotation as a method for controlling infections by bacteriophages (Sokollek and Hammes, 1997). Entani et al. (1985) developed a double layer agar technique using an acetic-acid-ethanol medium that allowed the cultivation of acetic acid bacteria capable of producing 10–15% acetic acid in commercial acetators. These improved techniques intensified studies on the bacterial flora in submerged high-acid vinegar fermentations, and the following new species isolated from high-acid vinegar plants have been described: *Acetobacter europaeus* (Sievers et al., 1992), *A. oboediens*, *A. pomorum* (Sokollek et al., 1988b) and *Gluconacetobacter entanii* (Schüller et al., 2000). The preservation methods for the handling of acetic acid bacteria outside the fermentation process were improved (Sokollek and Hammes, 1997) and applied for the cultivation and preservation of various isolates from industrial vinegar fermentations (Sokollek et al., 1998a).

Various aspects of the bacterial production of acetic acid are treated in detail in the chapter Organic Acid and Solvent Production in Volume 1.

Beer Acetic acid bacteria have been known as beer spoilers for many years. The acetification of beer was studied by the pioneers of microbiology, such as Pasteur, Hansen, Henneberg, and Beijerinck. In his book *Etudes sur la bière* Pasteur (1876) showed: 1) that beer sickness symptoms are always accompanied by the development of bacteria in wort or beer and 2) that the absence of "beer-sickness" symptoms coincides with the absence of bacteria. According to Pasteur (1876), "*Mycoderma aceti*" is involved in one of the seven forms of beer sickness, being characterized by an acetic smell and taste.

The infection of beer by acetic acid bacteria may lead to the formation of a film, cause turbidity, bring about a loss of alcohol accompanied by the formation of acetic acid, turn the beer ropy, and cause off-flavors and color changes (Vaughn, 1942; Shimwell, 1948). Infections due to acetic acid bacteria are reduced nowadays by modern sanitation practice, by the increased use of stainless steel, the use of single-strain yeast, and the controlled use of anaerobic conditions. The acetic acid bacteria can still cause problems in cask beer production (Ault, 1965; Rainbow, 1971). They are picked up at the end of the production process and will develop in the cask after air has entered to replace the beer drawn from it. The major beer-spoiling acetic acid bacteria are *Gluconacetobacter xylinus*, *Acetobacter pasteurianus*, and *Gluconobacter oxydans*. Acetobacters are part of the normal microbial flora in lambic beer (a Belgian beer obtained by spontaneous fermentation; Martens et al., 1991).

Fruits and Flowers Fruits, as sources of sugar and of ethanol, are excellent niches for acetic acid bacteria. Acetic acid bacteria have been found on apples, apricots, almonds, beets, bananas, coconuts, custard apples, figs, guavas, grapes, longans, loquats, mandarins, mangoes, oranges, papayas, passion fruit, pomegranates, pears, peaches, pineapples, plums, ponkans, persimmons, sapotas, star fruit, strawberries and tomatoes (Bhat and Rijsinghani, 1955; Asai, 1968; Kahlon and Vyas, 1972; Yamada et al., 1999; Lisdiyanti et al., 2000; Lisdiyanti et al., 2001; Lisdiyanti et al., 2003; Seearunruangchai et al., 2004). The presence of acetic acid bacteria on fruits can cause problems during the manufacture of cider, wine, etc. Most strains of the genus *Asaia* were isolated from tropical flowers collected in Thailand and Indonesia, such as the spider lily (*Crinum asiaticum*), orchid tree (*Bauhinia purpurea*), plumbago (*Plumbago auriculata*; Yamada et al., 2000b; Katsura et al., 2001) and *Heliconia* (Yukphan et al., 2004b). Some thermotolerant members of *Gluconobacter frateurii* were isolated from various kinds of fruits and flowers in Thailand and Japan (Moonmangmee et al., 2000).

Acetic acid bacteria can cause bacterial rot in pears and apples, which is characterized by different shades of browning and by tissue degradation. Allen and Ricker (1932) associated a bacterial rot of apples with infection by the apple maggot, *Rhagoletis pomonella*. They isolated *Acetobacter* strains both from decaying apple tissue and from the larvae and adults of the apple maggot. The authors also found that the occurrence of the rotting symptoms was correlated with an *Acetobacter* infection. Färber et al. (1957) isolated several *Acetobacter* and *Gluconobacter* strains and their typical endproducts of glucose and fructose metabolism (ketogluconic acids) from brown-rot on apples and identified them as a causal agents of bacterial rot. These publications remained almost unnoticed. Cole (1959) also studied bacterial brown rot of

apples and identified the causal agents as "*Pseudomonas melophthora*" and "*Pseudomonas pomi*." Dhanvantari et al. (1978) and Gillis and De Ley (1980) concluded that Cole's isolates belonged in *Acetobacter*. All pear and apple varieties were found to be susceptible to bacterial brown rot, but pears were more susceptible (Van Keer et al., 1981b). Bacterial brown rot could be induced by artificial inoculation, using an inoculum size as low as 100 cells. The optimal temperature for the development of rot symptoms was 25°C, but even at 4°C, rotting occurred (Vanden Abeele et al., 1980; Van Keer et al., 1981a; Van Keer et al., 1981b). *Gluconobacter* was also reported to be involved together with *Erwinia*, pseudomonads and fungi in the rapid colonization of apple wounds (Mercier and Wilson, 1994).

Soft Drinks Fruit juices and related beverages were introduced during the last two decades of the nineteenth century. A soft drink can be defined as a sugary solution at low pH, without free oxygen or growth factors, containing very little organic nitrogen. It is a very selective medium, allowing only the least exacting, acid-tolerant microorganisms to develop (Sand and Kolfschoten, 1970). In earlier microbiological examinations of fruit juices, the role of the acetic acid bacteria as spoiler-organisms was overlooked.

However, *Gluconobacter* is now considered to be a typical spoiler of soft drinks (Sand and Kolfschoten, 1970; Sand, 1971; Sand, 1976; Dittrich, 1972; Molitoris, 1973; Dachs, 1975; Dachs, 1976; Molitoris and Hubner, 1975; Fresenius et al., 1977–78; Cancalon and Parish, 1995). Lott and Carr (1964) identified *Gluconobacter* as the causative agent of a deleterious change in orange juice, characterized by a marked staleness of flavor. *Asaia* and *Gluconacetobacter sacchari* were reported as spoilage organisms in fruit-flavored bottled water (Moore et al., 2002a; Moore et al., 2002b).

Nata Nata is a white to creamy-yellow to pinkish, firm, gelatin-like substance formed through bacterial action on sugared fruit juices. "Nata de pina" and "nata de coco" are traditional dessert delicacies in the Philippines and other Southeast Asian countries (Villanueva, 1937; Alaban, 1962; Lapuz et al., 1967; Gallardo-De Jesus et al., 1973). Nata is considered as a healthy diet and became an important export product for the Philippines. Some confusion existed at first as to the chemical composition of nata and to the identity of the nata-producing organism. Its first students thought that nata was a dextran formed by a *Leuconostoc* species (Villanueva, 1937; Alaban, 1962). Today, it has been established that it is a form of cellulose produced by acetic acid bacteria belonging to *Gluconacetobacter* (Lapuz et al., 1967; Gallardo-De Jesus et al., 1973; Gosseléand Swings, 1985; Iguchi et al., 2000). More recently Bernardo et al. (1998) showed by 16S rDNA sequence analysis that the typical nata-producing strains are related to *Gluconacetobacter xylinus* and *Gluconacetobacter hansenii*. *Acetobacter pasteurianus*, *A. orleanensis*, *A. lovaniensis*, *G. oxydans* and *G. frateurii* were also found in the process of nata production (Lisdiyanti et al., 2003).

Pink Disease of Pineapple Fruit Acetic acid bacteria have been reported as causative agents of the pink disease of pineapple fruit (Buddenhagen and Dull, 1967; Rohrbach and Pfeiffer, 1976; Gosseléand Swings, 1986). However, various recent molecular studies indicate that the real causative organism is *Pantoea citrea*, a member of the Enterobacteriaceae (Cha et al., 1997; Pujol and Kado, 2000). The disease itself is recognizable by the appearance of a pinkish brown color after heating the fruit, although the fruit has an almost-normal appearance before the canning process.

Sugarcane and Coffee Plants Cavalcante and Döbereiner (1988) discovered a new nitrogen-fixing *Acetobacter* species in the stem and root of sugarcane in Brazil. Gillis et al. (1989) studied the isolates and proposed the new species *Acetobacter diazotrophicus*, which was later transferred to the genus *Gluconacetobacter* (Yamada et al., 1998; Table 1). This interesting endophytic bacterium represents a model system for the investigation of associations between monocots and diazotrophic bacteria (Muthukumarasamy et al., 2002b). *Gluconacetobacter sacchari* is closely related to *Ga. diazotrophicus* and was isolated in Australia from the leaf sheath of sugarcane as well as from the pink sugarcane mealy bug *Saccharicoccus sacchari*, living in the leaf sheath of sugarcane (Franke et al., 1999). *Gluconacetobacter sacchari* is not able to fix nitrogen. New species of nitrogen-fixing gluconacetobacters (*Ga. azotocaptans* and *Ga. johannae*) were isolated from the rhizosphere and the rhizoplane of coffee plants cultivated in Mexico (Fuentes-Ramírez et al., 2001; Table 1).

Bee Hives It is nearly impossible to determine the "normal" microflora of an insect because so many factors play a role in its composition. The honeybee, which visits many niches, undoubtedly comes into intimate contact with several pathogenic and nonpathogenic microorganisms. The digestive tracts of newborn worker honeybees are sterile. The microbial flora of the honeybee develops rapidly and appears largely to be the

result of chance. The bee first comes into contact with the microorganisms on their food and on the other bees of the hive. This means that the composition of the microbial flora will vary considerably depending on the food source of the bee colony. A considerable number of strains of bacteria, fungi, and actinomycetes have been isolated (Vecchi, 1959; Borchert, 1966; Batra et al., 1973). Nectar is transformed into honey by a complex process, beyond the scope of the present review; it is accompanied by a considerable loss of water. Ruiz-Argueso and Rodriguez-Navarro examined the microorganisms of ripening honey from an apiary in Madrid (Spain) surrounded by a flora of *Rosmarinus*, *Lavendula* and *Thymus* species (Ruiz-Argueso and Rodriguez-Navarro, 1973; Ruiz-Argueso and Rodriguez-Navarro, 1975). Two main groups of bacteria were identified: *Lactobacillus viridescens* and *Gluconobacter*. Occasionally, *Zymomonas mobilis* and several yeasts were also present. In natural honey, the number of bacteria decreases during the ripening process. An individual bee contains several hundred *Gluconobacter* cells, distributed equally between the intestine and the body surface. *Gluconobacter* was not isolated from larvae or from the sealed honey in the honeycomb. Lambert et al. (1981) isolated 56 *Gluconobacter* strains from honeybees in Belgium and characterized them by polyacrylamide gel electrophoresis of the soluble cell proteins. Not surprisingly, the sugar-loving and flower-associated gluconobacters occur on bees, which probably act as vectors in the dissemination of these and other bacteria.

Kombucha and "Tea Fungus" Kombucha is a slightly acidulous and effervescent beverage obtained by the fermentation of sugared black tea with a symbiotic culture of acetic acid bacteria and yeasts. The claimed health effects of kombucha have been discussed by Dufresne and Farnworth (2000) and Greenwalt et al. (2000). Several names have been given to the organisms responsible for the beverage obtained by acetic acid fermentation of tea infusions: kombucha, miracle fungus, fungus of charity, fungus of a long life, remedy for immortality, Ma Gu, Chinese or Japanese fungus, Hongo, Haipao, and Teekwasspilz (Dinslage and Ludorff, 1927; Kraft, 1959; Abadie, 1962). These names, often referring to a fungus, are confusing; in fact, what is meant is an association of a yeast with an acetic acid bacterium, forming a thick film on the surface of a tea infusion.

More than one thousand years ago the tea fungus was already used in Japan, China and India; and then in Russia, Poland and the Baltic States starting in about 1915; in the Balkans, Germany and Eastern Europe in about 1925; and in Spain, Italy, France and Switzerland in about 1955 (Kraft, 1959). The beverage is prepared from black tea to which sugar and the "tea fungus" are added. After approximately 3–5 days, the beverage is ready; it is slightly alcoholized, aromatic, acidic and refreshing. Hermann isolated *Gluconobacter oxydans* and *Acetobacter* (*Gluconacetobacter*) *xylinu*s from kombucha (Hermann, 1928a; Hermann, 1928b). *Acetobacter aceti*, *A. pasteurianus* and *A.* (*Ga.*) *intermedius* have also been identified in this beverage (Sievers et al., 1995c; Liu et al., 1996; Boesch et al., 1998), whereas the identified yeasts belong to the genera *Brettanomyces*, *Candida*, *Saccharomyces* and *Zygosaccharomyces* (Mayser et al., 1995; Liu et al., 1996). Abadie (1962) studied the relationship between the yeast *Candida mycoderma* and the *Acetobacter* cells as it occurs in the acetic fermentation of tea infusions. The bacteria are attached to the yeast cells and grow out to form a compact, granular, and gelatinous mass. The extracellular cellulose capsule is formed by the bacteria and incorporates the yeast cells. After about 20 days, the association is complete and well balanced. The author has also demonstrated that it was possible to constitute the association starting with pure cultures of yeast and acetic acid bacteria.

Flour At a moisture content of more than 13%, microbial growth can occur in flour, leading to acid fermentation, alcohol fermentation by yeasts, and acetic acid formation by acetic acid bacteria (Frazier, 1967). A strain of the species *Kozakia baliensis* (Lisdiyanti et al., 2002) was isolated from ragi, a starter used in Indonesia for the natural fermentation of rice flour.

Tanning Process The tanning process consists of the conversion of hides into leather by impregnation with either an infusion of tree bark, minerals, synthetic tannins, or other substitutes. Vegetable tanning liquors are characterized by an acetic acid "fermentation," which is preceded by a phase in which yeasts produce some ethanol from sugars. An extensive study of the acetic acid "fermentation" was made by Doelger (1936). *Acetobacter* may occasionally develop to a slimy pellicle at the surface of the liquor, and vinegar eels may thrive in it. This would suggest that the bacterium, besides its well-known acid tolerance, is also characterized by a pronounced tannin tolerance. Using mixtures of chestnut and oak bark extract (containing 25% tannin), the author noted the formation of acetic acid after 15 days of incubation.

Methanol Yeast Process Methylotrophic acetic acid bacteria were isolated from a septic methanol yeast process (Steudel et al., 1980) and from

sludge and described by Uhlig et al. (1986) as a new species, *Acetobacter methanolicus*, at present classified as *Acidomonas methanolica* (Urakami et al., 1989; Table 1).

The methanol-utilizing *Acidomonas methanolica* was reported in activated sludge from sewage treatment plants (Yamashita et al., 2004).

Fermented Meat Another unusual niche for acetic acid bacteria was described by Aries et al. (1982), who first isolated them from lactic-acid fermented meat used in the preparation of pet food. The isolates were further characterized as *Acetobacter pasteurianus* and *A. aceti* (Gosselé et al., 1984). The acetic acid bacteria alkalinize the meat by the dissimilation of lactic acid, thus allowing growth of pathogenic and toxigenic bacteria in a product where these bacteria had initially been inhibited by the low pH and by the lactic acid activity.

Isolation, Cultivation and Preservation A standard medium for the enrichment and isolation of acetic acid bacteria, with the exception of *Gluconacetobacter europaeus* and *Ga. entanii*, contains: yeast extract, 5.0 g; peptone, 3.0 g; D-glucose, 0.5 g; ethanol (99.8%), 15 ml; cycloheximide, 0.1 g; and distilled water, 1000 ml (Sievers and Swings, 2005). The following enrichment medium can also be recommended for most acetic acid bacteria except for isolates belonging to *Asaia*: yeast extract, 8.0 g; peptone, 15.0 g; D-glucose, 10.0 g; ethanol (99.8%), 5.0 ml; acetic acid, 3.0 ml; cycloheximide, 0.1 g; and distilled water, 1000 ml; final pH 3.5 (Lisdiyanti et al., 2003). Incubation is done at 30°C for 3–5 days and samples showing growth are then streaked on a $CaCO_3$-containing medium: yeast extract, 8.0 g; peptone, 5.0 g; D-glucose, 20.0 g; ethanol, 5.0 ml; $CaCO_3$, 3.0 g; agar, 15.0 g; and distilled water, 1000 ml. The acetic acid bacteria are selected as acid-producing strains that form clear zones around the colonies on the agar plates. Lisdiyanti et al. (2003) compared various media for the enrichment of acetic acid bacteria from Indonesian, Thai and Philippine sources. More specific methods for the isolation and cultivation of acetic acid bacteria are described in the sections of the individual genera.

Long-term preservation of strains of the Acetobacteraceae can be achieved by lyophilization or by storage in liquid nitrogen or by cryoconservation at –80°C using low-temperature refrigerators and appropriate cryoprotectants. Some of the acidophilic *Gluconacetobacter* strains require special precautions for their maintenance (see the section on Gluconacetobacter in this Chapter). Most acetic acid bacteria can be maintained at 4°C for several weeks on agar slants containing the appropriate medium (see sections on isolation and cultivation of the respective genera).

The Genus *Acetobacter* *Acetobacter* strains are easily differentiated from species of the genera *Gluconobacter*, *Gluconacetobacter*, *Acidomonas*, *Asaia* and *Kozakia* by their ability to oxidize acetate and lactate to CO_2 and H_2O and by having Q-9 as the major ubiquinone (Table 2). There are no exclusive phenotypic features to differentiate the 14 *Acetobacter* species. *Acetobacter aceti* (type species) and *A. pasteurianus* were mostly used in biochemical and genetic studies of *Acetobacter*.

Taxonomy In the eighth edition of *Bergey's Manual of Determinative Bacteriology* (De Ley and Frateur, 1974a), three species were recognized in the genus *Acetobacter*, basically referring to Frateur's classification system (Frateur, 1950): 1) *A. aceti* containing four subspecies (subsp. *aceti*, subsp. *orleanensis*, subsp. *xylinum*, and subsp. *liquefaciens*) and corresponding to Frateur's mesoxydans group; 2) *A. pasteurianus* encompassing five subspecies (subsp. *pasteurianus*, subsp. *lovaniensis*, subsp. *estunensis*, subsp. *ascendens* and subsp. *paradoxus*) and corresponding to Frateur's oxydans group with the exception of *A. paradoxus*; and 3) *A. peroxydans* corresponding to Frateur's peroxydans group. The names of these species and subspecies appeared on the Approved Lists of Bacterial Names (Skerman et al., 1980).

Gosselé et al. (1983), on the basis of numerical analyses of phenotypic features and protein profiles of more than 200 strains of acetic acid bacteria, rejected the subspecies concept in De Ley and Frateur's (1974a) classification and delineated four species in the genus *Acetobacter*: *A. aceti*, *A. hansenii*, *A. liquefaciens* and *A. pasteurianus*. These conclusive results appeared in *Bergey's Manual of Systematic Bacteriology* (De Ley et al., 1984c). However, Yamada (1983) revived *A. xylinus* as an independent species on the basis of its ubiquinone type. Subsequently, many new species were proposed in *Acetobacter*: "*Acetobacter polyoxogenes*" (Entani et al., 1985), *Acetobacter methanolicus* (Uhlig et al., 1986), *Acetobacter diazotrophicus* (Gillis et al., 1989), *Acetobacter europaeus* (Sievers et al., 1992), *Acetobacter pomorum* and *Acetobacter oboediens* (Sokollek et al., 1998b), and *Acetobacter intermedius* (Boesch et al., 1998). The establisment of the genus *Gluconacetobacter* (Yamada et al., 1997; Yamada et al., 1998) and the revival of the genus *Acidomonas* (Urakami et al., 1989) together with the transfer of *A. oboediens* and *A. intermedius* to the genus *Gluconacetobacter* (Yamada, 2000a) decreased the number of

Acetobacter species in 2000 to three: *A. aceti*, *A. pasteurianus* and *A. pomorum*.

On the basis of a polyphasic taxonomic study including DNA-DNA hybridizations, determination of ubiquinone composition and 16S rRNA gene analysis using strains freshly isolated from Indonesian sources together with strains from culture collections, Lisdiyanti et al. established five new species (*A. indonesiensis*, *A. tropicalis*, *A. orientalis*, *A. syzygii* and *A. cibinongensis*) and three new combinations (*A. estunensis*, *A. orleanensis* and *A. lovaniensis*) and revived *A. peroxydans* as a separate species in the genus (Lisdiyanti et al., 2000; Lisdiyanti et al., 2001). Cleenwerck et al. (2002) re-examined the classification of various *Acetobacter* strains, confirmed the entity of the new combinations and new species proposed by Lisdiyanti et al. (Lisdiyanti et al., 2000; Lisdiyanti et al., 2001), and additionally proposed two new species, namely *A. malorum* and *A. cereviseae*.

At present 14 species are recognized in the genus *Acetobacter* (Table 1; Fig. 2). *Acetobacter aceti* is the type species.

Habitats *Acetobacter* strains were isolated from vinegar, alcoholic beverages (such as sake, beer, wine, palm wine, cider, tequila, and cocoa wine), fermented foods (such as pickles, nata, and mash of alcoholic beverages), fruits (such as grapes, guava, sapodilla, starfruits, mangosteen, mango, banana, papaya, etc.), flowers (e.g., canna), and other materials (such as coconut juice, palm seed, and tofu; see the section Habitats and Uses of Acetic Acid Bacteria in this Chapter).

Isolation and Cultivation Basically, a medium containing glucose, ethanol and acetic acid is appropriate for the isolation of *Acetobacter* strains. Lisdiyanti et al. (2003) used an enrichment medium (glucose, 10.0 g; ethanol, 5.0 ml; acetic acid, 3.0 ml; peptone, 15.0 g; yeast extract, 8.0 g; cycloheximide, 0.1 g; and distilled water, 1000 ml) and described that more than 67% of the isolated acetic acid bacteria belonged to the genus *Acetobacter*.

Identification Features differentiating the genus *Acetobacter* from the other Acetobacteraceae are listed in Table 2. The sequence similarities of the 16S rDNA sequences of the *Acetobacter* type strains vary (97–99.5%). *Acetobacter aceti* is clearly distinguished from the other species by the formation of dihydroxyacetone from glycerol and the production of 2- and 5-ketogluconate from glucose, *A. pasteurianus* by its tolerance of ethanol, *A. peroxydans* by a negative catalase reaction and by its lack of acid formation from glucose, and *A. pomorum* by its requirement of acetic acid for growth. Differential characteristics of the 14 *Acetobacter* species are given in Table 3. At present DNA-DNA hybridization is recommended for the identification of *Acetobacter* strains at the species level.

Physiological and Biochemical Properties *Acetobacter* strains are motile by peritrichous flagella or nonmotile. They are obligately aerobic, oxidase negative, and oxidize ethanol to acetic acid. Acetate and lactate are oxidized to CO_2 and H_2O. Ethanol, glucose and glycerol are good carbon sources for growth. In liquid media *Acetobacter* strains develop usually rings or pellicles. They do not grow on Hoyer-Frateur medium (De Ley and Frateur, 1974a) or Frateur's modified Hoyer medium with glucose, ethanol or mannitol as a sole carbon source. They do not produce γ-pyrones from D-glucose or D-fructose and do not form 2, 5-diketogluconate from glucose (Table 2). Acids are formed from ethanol but not from sugar alcohols such as glycerol, sorbitol and mannitol.

Table 3. Differentiating features among the species of *Acetobacter*.[a]

Characteristics	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.
Catalase	+	+	+	+	+	+	+	+	+	d	–	+	+	+
Ketogenesis from glycerol	+	nd	–	–	–	–	nd	–	d	–	–	+	–	–
Acid formation from D-glucose	+	+	+	+	+	+	+	+	+	d	–	w	+	+
Formation from D-glucose of														
2-Keto-D-gluconate	+	+	+	+	+	–	+	+	+	–	–	–	–	+
5-Keto-D-gluconate	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Growth in the presence of 30% glucose	–	–	nd	–	–	–	+	nd	–	–	–	–	nd	–
Growth in the presence of 10% ethanol	–	nd	–	–	–	–	nd	–	–	+	–	–	–	–
Nitrate reduction[b]	–	nd	–	+	d	d	nd	–	d	+	–	nd	–	+

Symbols: +, 90% or more of the strains positive; w, weakly positive reaction; d, 11–89% of the strains positive; –, 90% or more of the strains negative; and nd, not determined.

[a]1., *A. aceti*; 2., *A. cerevisiae*; 3., *A. cibongensis*; 4., *A. estunensis*; 5., *A. indonesiensis*; 6., *A. lovaniensis*; 7., *A. malorum*; 8., *A. orientalis*; 9., *A. orleanensis*; 10., *A. pasteurianus*; 11., *A. peroxydans*; 12., *A. pomorum*; 13., *A. syzygii*; and 14., *A. tropicalis*.

[b]Nitrate reduction was tested in nitrate peptone according to Franke et al. (1999).

Data from Sokollek et al. (1998b), Lisdiyanti et al. (2000), Lisdiyanti et al. (2001), and Cleenwerck et al. (2002).

The oxidation of ethanol by *Acetobacter* strains is catalyzed by two cytoplasmic membrane-bound enzymes: ADH (alcohol dehydrogenase) and ALDH (aldehyde dehydrogenase). The ADH from *Acetobacter* was purified as a complex of several protein subunits; 72, 45 and 15 kDa in *A. aceti* (Inoue et al., 1989; Inoue et al., 1992; Matsushita et al., 1992b), and 78, 48 and 20 kDa in *A. pasteurianus* (Kondo et al., 1995). The larger subunit of the ADH possesses a heme *c* and a pyrroloquinoline quinone (PQQ) as cofactors and requires Ca^{+2} to be active, the second subunit is a cytochrome *c*, and the third and smallest subunit of the three-component ADH is considered to protect the catalytic subunit from proteolysis and to maintain the correct conformation of the ADH-complex for electron transport on the periplasmic surface (Kondo et al., 1995). For the breakdown of sugars, *Acetobacter* is equipped with the hexose monophosphate pathway and the tricarboxylic acid (TCA) cycle (Asai, 1968).

Contradictory data were reported concerning the growth on methanol or utilization of methanol by *A. pasteurianus*, *A. lovaniensis*, *A. pomorum* and *A. malorum* strains (Gosseléet al., 1983; Uhlig et al., 1986; Urakami et al., 1989; Sokollek et al., 1998b; Cleenwerck et al., 2002). Ambiguity about growth on methanol and utilization of methanol can be ascribed to unclear definitions of assimilation and utilization of methanol and growth on methanol. Differences in employed media may also contribute to contradictory reports concerning these features. Indeed, the yeast extract or peptone included in some media, together with impurities in the agar, can serve as carbon source for acetic acid bacteria, yielding false positive results for assimilation or utilization of methanol or both or false positive growth on methanol. We recommend that these characteristics should be tested on a defined medium, such as the yeast extract-omitted medium C of Yamashita et al. (2004), or by appropriate biochemical techniques.

Thermotolerant *Acetobacter* strains have been described and may find applications for the production of vinegar at higher temperatures (38–40°C; Lu et al., 1999; Adachi et al., 2003b). The DNA base composition of *Acetobacter* is 52–61 mol% G+C.

Genetics Genetic studies of *Acetobacter* are focused on the elucidation of the mechanism of acetic acid fermentation, resistance to acetic acid and ethanol, and other factors such as mutations associated with growth and environment (reviewed by Beppu, 1993). As a result of the development of host-vector systems (Fukaya et al., 1985a; Fukaya et al., 1990; Okumura et al., 1985; Okumura et al., 1988) and genetic transformation systems (Fukaya et al., 1985b; Fukaya et al., 1985c; Takemura et al., 1991), various genes of *Acetobacter* strains were cloned and characterized. In the context of the elucidation of the mechanism of acetic acid fermentation, the genes encoding the subunits of alcohol dehydrogenase (ADH) were cloned and characterized for *A. aceti* K6033 (Inoue et al., 1989; Inoue et al., 1990; Inoue et al., 1992), *A. aceti* 1023 (Fukaya et al., 1993b), *A. pasteurianus* NCI 1380 (Takemura et al., 1991; Takemura et al., 1993a) and *A. pasteurianus* NCI 1452 (Kondo et al., 1995). The ADH activity of *A. pasteurianus* is induced by ethanol (Takemura et al., 1993a).

For investigating the mechanisms of acetic acid and ethanol resistance, Fukaya et al. (1990) isolated acetic acid-sensitive mutants of *A. aceti* 1023 by chemical mutagenesis and cloned three genes (*aarA*, *aarB* and *aarC*) responsible for acetic acid resistance from the mutants. The *aarA* gene product is citrate synthase; the *aarC* gene product seems to be involved in acetate assimilation, and the function of *aarB* is still unknown (Fukaya et al., 1993a). Using the same mutagenesis method Takemura et al. (1993b) isolated an ethanol-resistant mutant strain of *A. pasteurianus* NCI 1380 and cloned a *his1* gene responsible for ethanol resistance. The *his1* gene encodes for a polypeptide displaying a significant homology with histidinol phosphate aminotransferase (HPAT) from *Escherichia coli* and other bacteria. However, the mutant still possesses almost the same HPAT activity as the parental strain. The mechanism of acetic acid and ethanol resistance remains still unclear and it was suggested that the energy generating system in the cell membrane may be linked to these mechanisms.

Quite often spontaneous mutations occur in *Acetobacter*. Ohmori et al. (1982) reported that a correlation exists between the loss of acetic acid resistance and ethanol oxidizing ability of *A. aceti* 1023. Kondo and Horinouchi (1997a) reported that insertion sequences (IS) integrated in the genome of *Acetobacter* are responsible for genetic instability leading to deficiencies in various physiological properties such as inactivation of ADH. *Acetobacter pasteurianus* strain NCI 1380 harbors five copies of IS*12528,* 10 copies of IS*1452* (Kondo and Horinouchi, 1997a), and 100 copies of IS*1380* (Takemura et al., 1991) with a total insertion element length of more than 6% of the nucleotide sequence of the genome (Kondo and Horinouchi, 1997a). Okamoto-Kainuma et al. (2002) cloned and characterized the *groESL* operon, believed to be associated with the resistance against various stress factors. The transcription of this operon in *A. aceti* IFO 3283 was induced by heat shock as well as by exposure to ethanol and acetic acid. Kashima et al. cloned and characterized the gene *est1*, coding

for the intracellular esterase which is involved in ethylacetate formation from ethanol and acetic acid (Kashima et al., 1999; Kashima et al., 2001). The transcription of this gene is induced by ethanol in *A. pasteurianus* and its regulation depends on the oxygen concentration.

Phosphatidylcholine, a major component of the membrane lipids, is found abundantly in cells of acetic acid bacteria. Hanada et al. (2001) cloned the *pmt* gene encoding phosphatidylethanolamine *N*-methyltransferase catalyzing the methylation of phosphatidylethanolamine to phosphatidylcholine in *A. aceti* and concluded that a correlation exists between phosphatidylcholine biosynthesis and acetic acid resistance.

Pathogenicity on Fruits *Acetobacter* strains can infect apples and pears and cause bacterial rot of these fruits, being characterized by browning and tissue degradation (see the section Habitats and Uses of Acetic Acid Bacteria in this Chapter). Bacterial rot of apples could be induced by artificial inoculation, using an inoculum size as low as 100 cells (Vanden Abeele et al., 1980; Van Keer et al., 1981a; Van Keer et al., 1981b).

Applications Several *Acetobacter* species (e.g., *A. aceti* and *A. pasteurianus*) were reported to be involved in the industrial production of vinegar (see the section Habitats and Uses of Acetic Acid Bacteria in this Chapter). More recently, various *Gluconacetobacter* species (e.g., *Ga. europaeus* and *Ga. entanii*) were isolated from vinegar production plants (see the section Gluconacetobacter in this Chapter). Vinegar has been used in the preservation of foods, in medicine as the first antibiotic known, as a common drink of slaves and soldiers in the Roman empire, and also as a cooking ingredient (see the section Habitats and Uses of Acetic Acid Bacteria in this Chapter and Organic Acid and Solvent Production in Volume 1).

Acetobacter peroxydans has been used as a biosensor for the determination of H_2O_2 by coupling immobilized living bacterial cells to an amperometric oxygen electrode (Rajasekar et al., 2000).

The Genus Acidomonas The facultatively methylotrophic *Acidomonas* strains share similar characteristics with other acetic acid bacteria such as growth at low pH and production of acetic acid and gluconic acid from ethanol and glucose, respectively. Utilization of methanol and a unique niche are characteristic for this genus.

Taxonomy The genus *Acidomonas* was validly established for Gram-negative, rod-shaped, acidophilic, facultatively methylotrophic bacteria by Urakami et al. (1989), incorporating *Acetobacter methanolicus* Uhlig et al. (1986). Sievers et al. (1994b) proposed a revival of *Acetobacter methanolicus*, but the separate phylogenetic position of the genus *Acidomonas* was justified by Yamada et al. (Yamada et al., 1997; Yamada et al., 2000b). Yamashita et al. (2004) emended the description of the genus *Acidomonas* on the basis of data using new strains isolated from activated sludge. The phylogenetic relationship of the genus *Acidomonas* as a member of the family Acetobacteraceae was reported by Bulygina et al. (1992) on the basis 5S rRNA sequencing and later confirmed by 16S rDNA sequencing (Sievers et al., 1994a; Sievers et al., 1994b; Yamada et al., 1997; Yamashita et al., 2004; Fig. 2). At present *Acidomonas methanolica* is the only species in this genus.

Habitats Currently 14 strains of *Ac. methanolica* are preserved in culture collections worldwide (Yamashita et al., 2004). Among the acetic acid bacteria, *Acidomonas* strains seem to occur in unique niches: sludge from sewage-treatment plants and a yeast fermentation process where methanol was the sole source of carbon and energy. They were not reported in fruits or plant material (Yamashita et al., 2004).

Isolation and Cultivation *Acidomonas* strains can be isolated by enrichment on medium C according to Yamashita et al. (2004). Medium C was described by Urakami et al. (1989) and its composition is as follows: methanol, 10 ml; yeast extract, 0.2 g; $(NH_4)_2SO_4$, 3.0 g; KH_2PO_4, 4.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 30 mg; $MnCl_2 \cdot 4H_2O$, 5.0 mg; $ZnSO_4 \cdot 7H_2O$, 5.0 mg; $CuSO_4 \cdot 5H_2O$, 0.5 mg; vitamin solution, 1.0 ml; and distilled water, 1000 ml. The vitamin solution contains: biotin, 2 mg; calcium pantothenate, 400 mg; pyridoxine·HCl, 400 mg; thiamine·HCl, 400 mg; *p*-aminobenzoic acid, 200 mg; folic acid, 2 mg; inositol, 2 mg; nicotinic acid, 400 mg; riboflavin, 200 mg; and distilled water, 1000 ml. Incubation is done at 30°C for 3–5 days in flasks with shaking. After enrichment, bacteria are streaked on agar plates containing medium C or PYMe medium. The composition of PYMe medium is: peptone, 5.0 g; yeast extract, 3.0 g; methanol, 10 ml, and distilled water, 1000 ml. The pH of both media is adjusted to 4.0 with HCl. Colonies are isolated, further purified on medium C, characterized and identified. *Acidomonas* strains can also be cultivated on PYM medium (peptone, 5.0 g; yeast extract, 3.0 g; malt extract, 3.0 g; glucose, 20.0 g; and distilled water, 1000 ml; pH 4.5) as described by Urakami et al. (1989).

Identification Features differentiating the genus *Acidomonas* from the other Acetobacteraceae are listed in Table 2. Phenotypically, *Acidomonas*

strains differ from other acetic acid bacteria in the utilization of methanol in medium C. Using Leifson's method *Acidomonas* strains oxidize acetate but do not (or weakly) oxidize lactate (Table 2). Further, *Acidomonas* strains are motile by a single polar flagellum (occasionally a polar tuft of flagella) or nonmotile, and their colonies on PYM agar medium are shiny, smooth, circular, convex, entire, beige to pink, and 1–3 mm in diameter after 5 days at 30°C. They grow at pH 3.0–8.0 and are therefore not acidophilic but acidotolerant. They grow at 30°C but not at 45°C. Acetic acid is produced from ethanol. Growth occurs in the presence of 0.35% acetic acid and 30% glucose. Yamashita et al. (2004) used 16S rDNA sequence analysis to assess the phylogenetic relationship between 14 strains of *Ac. methanolica* and reported that all strains formed a single cluster on the lineage of the Acetobacteraceae, clearly distant from the members of the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Asaia* and *Kozakia* (Fig. 2).

Correct identification of *Acidomonas* isolates may require sequencing of 16S rDNA as well as DNA-DNA hybridizations.

Physiological and Biochemical Properties *Acidomonas* strains were once regarded as promosing bacteria for the production of single cell protein from methanol. Therefore, their growth on and oxidation of several carbon sources have been studied in detail.

Acidomonas strains utilize methanol, glycerol or glucose as a sole carbon source for energy and growth via a periplasmic oxido-reductase system and a cytoplasmic ribulose monophosphate pathway (Steudel et al., 1980). The strains have a methanol oxidase respiratory chain (similar to most methylotrophs) in addition to an ethanol oxidase respiratory chain functioning at acidic pH. However, the methanol oxidase system of *Acidomonas* is totally different from its ethanol oxidase system. Under methylotrophic conditions, the methanol oxidase respiratory chain operates by linking methanol dehydrogenase to cytochrome *co* via soluble cytochromes *c* (Chan and Anthony, 1991; Matsushita et al., 1992a). The methanol dehydrogenase of *Acidomonas* strains exhibits two different forms: type I has an $\alpha_2\beta_2$ conformation consisting of 62- and 8.5-kDa peptides, and type II exhibits an $\alpha_2\beta_2\gamma$ conformation with a γ-subunit of 32 kDa showing a high homology to MoxJ of the methylotrophic *Methylobacterium extorquens* (Matsushita et al., 1993). In *Acidomonas* strains grown on glycerol, the ethanol oxidase respiratory chain consists of alcohol dehydrogenase, ubiquinone-10, and cytochrome *bo* ubiquinol oxidase (Matsushita et al., 1992a). The alcohol dehydrogenase of the strains consists of three subunits and is repressed under methylotrophic conditions (Frebortova et al., 1997).

The structures of the capsular polysaccharide and O-side-chain of the lipopolysaccharide of three *Acidomonas* strains have been determined with the aid of bacteriophages specific for *Ac. methanolica* (Grimmecke et al., 1991; Grimmecke et al., 1994a; Grimmecke et al., 1994b).

Acidomonas strains produce only gluconic acid from glucose. Pantothenic acid is required for growth. *Acidomonas* strains assimilate ammoniacal nitrogen with D-glucose and other carbon sources when pantothenic acid is supplied to the defined media (Yamashita et al., 2004). Acid is produced from L-arabinose, D-xylose, D-ribose, D-galactose, D-mannose, D-glucose, glycerol, *n*-propanol, *n*-butanol, isobutanol, ethanol and methanol but not from L-rhamnose, D-fructose, sucrose, maltose, lactose, raffinose, trehalose, D-mannitol, inositol, D-sorbitol, dulcitol or soluble starch. Methanol, ethanol, D-glucose, glycerol, D-mannose and succinic acid are assimilated as a sole carbon source on defined medium (Yamashita et al., 2004).

The major ubiquinone is Q-10 (Urakami et al., 1989; Yamashita et al., 2004), and the major cellular fatty acid is C18:1 and the major hydroxy acids are 3-OH C16:0 and 2-OH C16:0 (Urakami et al., 1989). DNA base composition is 62–63 mol% (Yamashita et al., 2004).

Genetics Bacteriophage Acm1, virulent for the type strain of *Ac. methanolica*, was identified by Kiesel and Wunsche (1993). Auxotrophic mutants for homoserine and threonine were obtained from the type strain of *Ac. methanolica* by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine treatment (Follner and Babel, 1992). Heterologous genes have been expressed in *A. methanolica* (Follner et al., 1994).

Applications *Acidomonas methanolica* seems to supercede *Gluconobacter* for the industrial production of gluconic acid. A two-phase process has been developed in which *Ac. methanolica* cells are grown on methanol in phase one, and subsequently high glucose concentrations can rapidly be converted to pure gluconic acid during the production phase (Babel et al., 1991; Poehland et al., 1993). More details can be found in the chapter Organic Acid and Solvent Production in Volume 1.

The Genus Asaia Compared to the classical acetic acid bacteria *Asaia* strains have some unusual characteristics because they produce no or only limited amounts of acetic acid from ethanol, and their growth is completely inhibited by 0.35% acetic acid. *Asaia* strains were mostly isolated

from tropical flowers and prefer sugars for growth.

Taxonomy The genus *Asaia* was first described by Yamada et al. (2000). At present the following three species are recognized: *Asaia bogorensis* (type species), *Asaia krungthepensis* and *Asaia siamensis* (Table 1). This classification is based on phenotypic and chemotaxonomic features, as well as on data obtained from DNA-DNA hybridizations and 16S rDNA sequencing (Yamada et al., 2000b; Katsura et al., 2001; Yukphan et al., 2004b). *Kozakia baliensis* is a phylogenetic neighbor of the genus *Asaia* (Fig. 2).

Habitats *Asaia* strains were mostly isolated from flowers from tropical plants, such as the orchid tree (*Bauhinia purpurea*), plumbago (*Plumbago auriculata*), crown flower (*Calotropis gigantea*), spider lily (*Crinum asiaticum*), ixora (*Ixora chinensis*), lantana, heliconia, roses, etc. One strain of *As. bogorensis* (NRIC 0320) was isolated from "tape ketan" (an Indonesian traditional fermented food made from glutinous rice). In a batch of fruit-flavored bottled water, Moore et al. (2002a) detected through partial 16S rRNA gene sequencing an *Asaia* strain as a spoilage organism.

Isolation and Cultivation *Asaia* strains can be enriched in a medium containing D-sorbitol or dulcitol at pH 3.5 without the addition of acetic acid. The composition of the isolation medium is as follows (in g per liter distilled water): dulcitol or D-sorbitol, 20.0; peptone, 5.0; yeast extract, 3.0; and cycloheximide, 0.1; adjusted to pH 3.5 with HCl. Incubation is done at 30°C for 3–5 days in flasks with shaking (Lisdiyanti et al., 2003). Cultures showing growth are streaked on $CaCO_3$ agar plates containing (g per liter distilled water): D-glucose, 20.0; ethanol, 5.0; yeast extract, 8.0; $CaCO_3$, 7.0; and agar, 15.0. Colonies of *Asaia* strains are pinkish or yellowish, do no produce water-soluble brown pigments, and will dissolve the $CaCO_3$ around the colonies weakly. Such colonies are isolated and further purified.

Identification Features differentiating the genus *Asaia* from the other genera of the Acetobacteraceae are listed in Table 2. The three *Asaia* species form a very closely related cluster in the phylogenetic tree based on 16S rDNA sequences (16S rDNA sequence similarities between the three species are as high as 99.5–99.9%; Fig. 2). *Asaia* species can best be differentiated by DNA-DNA hybridizations.

Characteristic features of *Asaia* strains are as follows: good growth at pH 3.0 but no growth in a medium containing 0.35% acetic acid. They do not (or weakly) oxidize ethanol to acetic acid, they oxidize acetate or lactate weakly to CO_2 and H_2O, and they possess Q-10 as the major ubiquinone. These characteristics are useful for differentiation of this genus from members of the genera *Acetobacter*, *Acidomonas*, *Gluconacetobacter*, *Gluconobacter* and *Kozakia* (Table 2).

The three species of *Asaia* can be phenotypically differentiated by the acid production from dulcitol (Table 4). *Asaia krungthepensis* strains display weak growth on maltose, whereas the type strains of the two other *Asaia* species fail to grow on this substrate (Yukphan et al., 2004b; Table 4).

Physiological and Biochemical Properties *Asaia* strains produce gluconic acid, 2-ketogluconate and 5-ketogluconate but no 2,5-diketogluconate from D-glucose. They show vigorous growth on a vitamin-free Hoyer-Frateur medium containing D-glucose as sole carbon source but not when ethanol is supplied as a sole C-source. Therefore, they do not require any growth factors for growth on D-glucose as sole carbon source. Acid is produced from D-glucose, D-mannose, D-galactose, D-fructose, L-sorbose, D-xylose, L-arabinose, *meso*-erythritol, glycerol, melibiose and sucrose but not from lactose. The DNA base composition is 59–61 mol% G+C.

The Genus Gluconacetobacter *Gluconacetobacter* strains are nonmotile or motile by peritrichous flagella. They oxidize ethanol to acetic acid, which is generally overoxidized to CO_2 and H_2O. They possess ubiquinone of the Q-10 type as major quinone. Strains of *Ga. diazotrophicus* are good nitrogen fixers and live in association with plants, such as sugarcane. *Gluconacetobacter xylinus* strains produce thick cellulose pellicles in liquid media, and other species, such as *Ga. europaeus*, are involved in high-acid vinegar fermentations.

Taxonomy The genus *Gluconacetobacter* was created by Yamada et al. to accommodate the ubiquinone Q-10 containing *Acetobacter* species, such as *A. liquefaciens*, *A. diazotrophicus*, *A. europaeus*, *A. hansenii* and *A. xylinus* (Yamada

Table 4. Differentiation of *Asaia* species.[a]

Characteristic	1.	2.	3.
Acid formation from dulcitol	+	+	–
Growth on maltose	–[b]	w	–[b]

Symbols: +, 90% or more of the strains positive; w, weakly positive reaction; and –, 90% or more of the strains negative.
[a]1., *As. bogorensis*; 2., *As. krungthepensis*; and 3., *As. siamensis*.
[b]Tested for the type strain only (Yukphan et al., 2004b).
Data from Yamada et al. (2000), Katsura et al. (2001) and Yukphan et al. (2004b).

et al., 1997; Yamada et al., 1998), which in 16S rRNA sequence analysis clustered remotely from the core of the Q-9 containing *Acetobacter* species. Note that already in 1984 it was proposed to accommodate the acetate-oxidizing acetic acid bacteria containing ubiquinone Q-10 (*A. liquefaciens* and *A. xylinus*) in a new subgenus *Gluconoacetobacter* of the genus *Acetobacter* (Yamada and Kondo, 1984b). The (sub)generic name *Gluconoacetobacter* has been corrected to *Gluconacetobacter* (Yamada et al., 1998) in accordance with rule 61 of the International Code of Nomenclature of Bacteria. Later the *Acetobacter* species *A. intermedius* and *A. oboediens* were also transferred to *Gluconacetobacter* on the basis of their 16S rRNA gene sequences (Yamada, 2000a), and at present the genus *Gluconacetobacter* contains 11 species (Table 1; Fig. 2), with *Ga. liquefaciens* as the type species. The high degree of DNA-DNA hybridization between the type strains of *Ga. intermedius* and *Ga. oboediens* indicated that these two species might be synonymous (Sievers and Swings, 2005). Phenotypic and genotypic heterogeneity were reported among strains of *Ga. hansenii* (Gosselé et al., 1983; Navarro et al., 1999b).

Habitats *Gluconacetobacter* strains were isolated from various sources such as flowers and fruits, sugarcane tissues, the pink sugarcane mealy bug, the rhizosphere and rhizoplane of coffee plants, tea fungus (kombucha), nata de coco, and vinegar production units. Some of the working strains in vinegar acetators belong indeed to *Gluconacetobacter* (e.g., *Ga. europaeus*; Sievers et al., 1992). Gluconacetobacters can also cause infections in beverages and spirituous liquors (more details can be found in the section Habitats and Uses of Acetic Acid Bacteria in this Chapter).

Isolation, Cultivation and Preservation Most *Gluconacetobacter* species, such as *Ga. liquefaciens*, *Ga. azotocaptans*, *Ga. diazotrophicus*, *Ga. hansenii* and *Ga. johannae*, can be grown on a medium containing mannitol, yeast extract and peptone (MYP medium). The recommended growth media for the various *Gluconacetobacter* species are listed in Table 5. A special N-free acidic LGI medium (pH 4.5) has been used for the isolation of the N_2-fixing *Ga. diazotrophicus*, *Ga. azotocaptans* and *Ga. johannae* strains from their respective host plants (Cavalcante and Döbereiner, 1988; Fuentes-Ramírez et al., 1993; Fuentes-Ramírez et al., 2001).

Until 1985 the working strains in high-acidic vinegar fermentors (up to 17% acetic acid) could not be grown on the media commonly used for acetic acid bacteria, and the bacteriological composition of the "seed vinegar" of such acetators remained largely unknown. The lack of defined pure starter cultures is due to problems in isolation, cultivation and conservation of acetic acid bacteria involved in high-acid vinegar production (Sievers and Teuber, 1995a). This drawback was partly overcome by the invention of a special double layer technique introduced by Entani et al. (1985) for the isolation of the microflora from vinegar fermenters in Japan. The double-layered modified AE-medium (Table 5) provides a constant supply of ethanol and high humidity from the bottom to the top layer of the medium and supports the growth of bacterial colonies at the agar surface. The incubators should contain a relative humidity of 92–97%. The application of these isolation and cultivation techniques led to the description of new species such as *Ga. europaeus*, *Ga. entanii*, *Ga. oboediens*, etc. (Sievers et al., 1992; Boesch et al., 1998; Sokollek et al., 1998b; Schüller et al., 2000).

Special precautions should also be taken for the preservation of *Gluconacetobacter* strains isolated from high-acid acetators, such as *Ga. entanii* and *Ga. europaeus*: 20% malt extract appears to be an effective cryoprotectant (Sokollek and Hammes, 1997; Sokollek et al., 1998a; Schüller et al., 2000). Freshly grown cells are harvested by centrifugation at 0°C and resuspended in 2 ml of ice-cold 20% malt extract solution. This suspension is poured dropwise in liquid nitrogen. The frozen culture is then lyophilized and stored at –20°C (Schüller et al., 2000).

Identification Features differentiating the genus *Gluconacetobacter* from the other Acetobacteraceae are listed in Table 2. The similarities of the 16S rDNA sequences of the *Gluconacetobacter* type strains vary between 96.6% and 99.6%. Characteristics differentiating the 11 *Gluconacetobacter* species are compiled in Table 5. Some species can be differentiated by their differences in growth behavior on media with high concentrations of acetate or glucose. Correct identification may require sequencing of the 16S rDNA as well as DNA-DNA hybridizations. An oligonucleotide probe based on the 23S rDNA of *Ga. europaeus* seems to be a useful tool for the differentiation of *Ga. intermedius* from *Ga. europaeus* and *Ga. xylinus* (Boesch et al., 1998).

Physiological and Biochemical Properties Gluconacetobacters grow best on ethanol, glucose or acetate as carbon source. Acids are formed from ethanol, glucose and usually from fructose and glycerol. The overoxidation of acetate to CO_2 and H_2O depends upon the acetate concentration in the medium. Depending upon the species, the pH optimum for growth varies between 2.5 and 6.0. Two species (*Ga. europaeus* and *Ga. entanii*) require acetic acid for growth (Table 5). The respiratory chain of *Gluconacetobacter*

Table 5. Differentiating features among species of *Gluconacetobacter*.[a]

Characteristics	1.	2.	3.	4.	5.	6.	7.	8.[b]	9.[b]	10.	11.
Growth on 3% (v/v) ethanol in the presence of 5–8% acetic acid	–	–	–	–	–	+	+	+	+	–	–
Requirement of acetic acid for growth	–	–	–	–	–	+	+	–	–	–	–
Growth only in the presence of acetic acid and ethanol and glucose[c]	–	–	–	–	–	+	–	–	–	–	–
Growth on medium of Carr and Passmore (1979)	+	+	nd	nd	+	–	–	w	nd	+	w
Formation from D-glucose of											
2-Ketogluconic acid	d	+	nd	nd	+	–	d	–	+	d	+
5-Ketogluconic acid	d	–	nd	nd	+	–	d	–	–	d	+
2,5-Diketogluconic acid	+	+	nd	nd	+	–	–	–	–	–	–
Growth in the presence of 30% D-glucose	–	+	+	+	+	–	–	+	+	–	–
Growth on 0.01% malachite-green agar	+	–	nd	nd	+	nd	–	nd	nd	nd	–
N_2 fixation	–	+	+	+	–	–	–	–	–	–	–
Cellulose formation	–	–	–	–	–	–	d	+	–	d	+
Recommended growth media[d]	GYC or MYP	GYC or MYP	MYP	MYP	GYC or MYP	AE	AE	RAE	RAE	GYC or MYP	GYC or MYP

Symbols: +, 90% or more of the strains positive; w, weakly positive reaction; d, 11–89% of the strains positive; –, 90% or more of the strains negative; and nd, not determined.
Abbreviations: GYC, glucose-yeast-$CaCO_3$; MYP, mannitol-yeast-peptone; AE, acetic acid and ethanol; and RAE, reinforced AE.
[a]1., *Ga. liquefaciens*; 2., *Ga. diazotrophicus*; 3., *Ga. azotocaptans*; 4., *Ga. johannae*; 5., *Ga. sacchari*; 6., *Ga. entanii*; 7., *Ga. europaeus*; 8., *Ga. intermedius*; 9., *Ga. oboediens*; 10., *Ga. hansenii*; and 11., *Ga. xylinus*.
[b]DNAs from the type strains of *Ga. intermedius* and *Ga. oboediens* showed species-level similarity of 76% among each other (Sievers and Swings, 2005).
[c]The sum of acetic acid and ethanol concentrations has to exceed 6% (Schüller et al., 2000).
[d]Composition of media (in g per liter, except when indicated):
GYC-medium: D-glucose, 100.0; yeast extract, 10.0; $CaCO_3$, 20; and agar, 15.0.
MYP-medium: D-mannitol, 25.0; yeast extract, 5.0; peptone, 3.0; and agar, 15.0.
AE-medium (acetic acid and ethanol medium): D-glucose, 5.0; yeast extract, 2.0; peptone, 3.0; acetic acid, 40.0ml (filter-sterilized); ethanol, 30.0ml (filter-sterilized); agar, 5.0 (bottom layer); agar, 10 (top layer); and 930ml distilled water. Add the filter-sterilized ethanol and acetic acid aseptically to the sterile basal medium. Pour the bottom layer AE-agar into a Petri dish and allow to solidify. Overlay the bottom agar surface with a thin coating (5–10ml) of top layer AE-agar and allow to solidify. Carefully streak the plates and incubate at 30°C for 1–2 weeks at 92–96% relative humidity (use, e.g., closed containers such as plastic boxes; Entani et al., 1985; Sievers et al., 1992).
RAE-medium (reinforced AE-medium): D-glucose, 40.0; yeast extract, 10.0; peptone, 10.0; $Na_2HPO_4 \cdot 2H_2O$, 3.38; citric acid, 1.5; acetic acid, 10.0ml (filter-sterilized); ethanol, 20.0ml (filter-sterilized); agar, 5.0 (bottom layer); agar, 10 (top layer); and 970ml distilled water. Use the double-layered system as described for the AE-medium (Sokollek and Hammes, 1997; Schüller et al., 2000).
Data from Sievers et al. (1992), Boesch et al. (1998), Sokollek et al. (1998b), Franke et al. (1999), Navarro and Komagata (1999a), Fuentes-Ramírez et al. (2001), Schüller et al. (2000), and Sievers and Swings (2005).

contains cytochrome *c*, ubiquinne, and a terminal ubiquinol oxidase (Matsushita et al., 1994). A complete TCA cycle is present. The citrate synthase from *Ga. europaeus* has been purified and its activity appears to be activated by acetate, inhibited by ATP, and not affected by reduced nicotinamide adenine dinucleotide (NADH; Sievers et al., 1997). *Gluconacetobacter oboediens* grows in the presence of 30% glucose and accumulates high concentrations of gluconic acid (at least 130 g per liter; Sokollek et al., 1998b). The DNA base composition of *Gluconacetobacter* is 56–67 mol% G+C.

OXIDATION OF ETHANOL AND VINEGAR PRODUCTION. The alcohol and acetaldehyde dehydrogenases of *Ga. europaeus* (a species isolated from high-acid vinegar fermentors) have been investigated in detail (Thurner et al., 1997). The membrane-bound alcohol and aldehyde dehydrogenase complexes consist of two and three subunits, respectively. The larger subunit of the alcohol dehydrogenase contains heme *c* and a pyrroloquinoline quinone (PQQ) as cofactor, whereas the second subunit is cytochrome *c*. The aldehyde dehydrogenase complex is organized as an operon containing three consecutive open reading frames (ORFs) corresponding to proteins with molecular masses of 84, 49 and 17 kDa. Its larger catalytic subunit contains heme *b* and a molybdopterin instead of PQQ; the middle subunit is a cytochrome *c* with three heme-*c* binding sites, and the smallest subunit contains two [2Fe-2S] clusters (Thurner et al., 1997).

Gluconacetobacter europaeus strains display an absolute requirement of acetic acid for growth on ethanol, whereas *Ga. entanii* strains require ethanol as well as acetate and glucose for growth (Sievers et al., 1992; Schüller et al., 2000; Table 5). The latter microorganism grows only when the total concentration of ethanol and acetic acid exceeds 6% (Schüller et al., 2000; Table 5).

FORMATION OF CELLULOSE AND ACETAN BY *GLUCONACETOBACTER XYLINUS*. Bacterial cellulose is characterized by its chemical purity, distinguishing it from plant cellulose, which is usually associated with hemicellulose and lignin. Because of its high mechanical strength and its ultrafine reticulated structure, bacterial cellulose has found many applications in paper, textile and food industries, as well as in medicine and cosmetics. Studies on the biogenesis of bacterial cellulose have been pioneered by Hestrin (1947), Hestrin (1962), Colvin (1977), Colvin (1980) and their coworkers (Hestrin et al., 1947; Hestrin and Schramm, 1954). *Gluconacetobacter xylinus* strains (previously named *Acetobacter xylinum* or *A. xylinus*) have been intensively used as model organisms for basic and applied studies on the biochemistry and genetics of cellulose formation (for reviews, see Cannon and Anderson [1991], Ross et al. [1991], Brown and Saxena [2000], Bielecki et al. [2002], Römling [2002], and Brown [2004]). This research on *Ga. xylinus* contributed also significantly to the elucidation of the mechanisms of biogenesis of cellulose in plants. Various studies focus on cellulose formation by "*Ga. xylinus* subsp. *sucrofermentans*" (Toyosaki et al., 1995; Watanabe et al., 1998; Chao et al., 2001). Most *Ga. intermedius* strains and some *Ga. hansenii* and *Ga. europaeus* strains form also bacterial cellulose (Table 5).

In static cultures, *Ga. xylinus* synthesizes a thick cellulose mat (called a "pellicle") covering the surface of the growth medium, whereas round balls of cellulose are formed in shaken cultures. Mutants which do not produce cellulose can be formed spontaneously in cultures aerated by shaking or stirring. Cells of cellulose producing bacteria are entrapped in the polymer matrix, often supporting the population at the liquid-air interface (Williams and Cannon, 1989). This facilitates oxygen and nutrient supply, since the concentration of nutrients in the cellulose matrix is enhanced by its absorptive properties, in contrast to the surrounding aqueous environment (Jonas and Farah, 1998).

Although bacterial cellulose is chemically identical to plant cellulose, its macromolecular structure and properties differ from the latter. Linearly ordered pores along the longitudinal axis of *Ga. xylinus* cells secrete long β-1,4-glucan chains that aggregate to form subfibrils with a diameter of 1.5 nm. These subfibrils aggregate to microfibrils by crystallization with subsequent assembly to ribbons (average thickness of 3–4 nm and a width of 70–130 nm) in the surrounding medium (Zaar, 1977; Zaar, 1979; Bielecki et al., 2002). The width of plant cellulose fibers is two orders of magnitude larger. The ultrafine ribbons of bacterial cellulose reach a length of 1–10 μm and form a very dense reticulated structure, stabilized by extensive hydrogen bonding (Jonas and Farah, 1998). The key enzyme in cellulose synthesis by *Ga. xylinus* is the membrane-bound cellulose synthase (UDP-glucose:1,4-β-D-glucan 4-β-D-glucosyl transferase), which uses UDP-glucose as substrate. The complete pathway from glucose to cellulose consists of the following four enzymatic steps (Ross et al., 1991):

Glucose → glucose-6-phosphate → glucose-1-phosphate → UDP-glucose → cellulose

The cellulose synthase is activated by cyclic diguanylic acid (c-di-GMP) acting as an allosteric effector and stimulating the enzymatic reaction rate up to 200-fold (Ross et al., 1986; Ross et al., 1987; Weinhouse et al., 1997). This unique

cyclic oligonucleotide is synthesized and degraded by the enzymes diguanylate cyclase and a Ca^{+2}-sensitive phosphodiesterase A, respectively, which have regulatory effects on cellulose biosynthesis. The biochemistry of the allosteric regulation mechanism of the cellulose synthase in *Ga. xylinus* has been thoroughly investigated and reviewed by Ross et al. (1991).

Most *Ga. xylinus* and *Ga. intermedius* strains produce besides the water-insoluble cellulose also a water-soluble polysaccharide called "acetan," a heteropolymer containing glucose, mannose, glucuronic acid and rhamnose in a molar ratio of 4 : 1 : 1 : 1 (Couso et al., 1987). Acetan formation seems to influence the degree of polymerization and crystallinity of the cellulose fibrils (Watanabe et al., 1998). Various genes of the acetan biosynthetic pathway (UDP-glucose dehydrogenase, GDP-mannosyl transferase, etc.) have been identified and sequenced (Griffin et al., 1994; Griffin et al., 1996; Petroni and Ielpi, 1996; Griffin et al., 1997).

NITROGEN FIXATION BY *GLUCONACETOBACTER DIAZOTROPHICUS*. The hitherto described N_2-fixing and plant-associated acetic acid bacteria (*Ga. diazotrophicus*, *Ga. azotocaptans* and *Ga. johannae*) are phylogenetically highly related (Fig. 2) and belong to *Gluconacetobacter*. *Gluconacetobacter diazotrophicus* is an endophytic organism of sugarcane and a few other plants (Caballero-Mellado et al., 1995), whereas the two other species were isolated from the rhizosphere and rhizoplane of coffee plants (Fuentes-Ramírez et al., 2001). Although associated with sugarcane, *Ga. sacchari* does not fix nitrogen. The interactions between *Ga. diazotrophicus* and sugarcane are an interesting model system for the study of the association between an endophytic nitrogen-fixing prokaryote and a monocot. *Gluconacetobacter diazotrophicus* plays a major role in the supply of nitrogen to the plant (Boddey et al., 1991; Sevilla et al., 2001). The biochemistry and genetics of the N_2-fixation by *Ga. diazotrophicus* have been thoroughly studied (Sevilla and Kennedy, 2000; Perlova et al., 2003a; see also the section Genetics and Genomics in this Volume). *Gluconacetobacter diazotrophicus* can fix nitrogen in the presence of nitrates and at low pH-values (Stephan et al., 1991; Burris, 1994; Muthukumarasamy et al., 2002a). Sucrose concentrations of 10% have a positive effect on the nitrogenase activity, protecting the activity of the enzyme complex against inhibition by oxygen (Reis and Döbereiner, 1998). James and Olivares (1997) described the mechanisms of infection and colonization of sugarcane by *Ga. diazotrophicus*. Ureta and Nordlund (2002) provided evidence that besides increased respiration, a putative FeSII protein plays a crucial role in the protection of nitrogenase against oxygen.

Genetics and Genomics A genome sequencing project has been initiated for the type strain of *Ga. diazotrophicus*, the endophytic diazotroph of sugarcane (see http://www.riogene.lncc.br/ and http://www.riogene.lncc.br/). Its genome size is 2.7 Mb. The cluster of *nif*, *fix* and associated genes of *Ga. diazotrophicus* was sequenced and analyzed (Lee et al., 2000): it is 30.5 kb in size, encodes for 32 proteins and represents the largest assembly of contiguous *nif-fix* and associated genes so far characterized in any diazotrophic prokaryote. The overall arrangement of the genes resembles that of the *nif-fix* cluster in *Azospirillum lipoferum*, whereas the individual gene products are more similar to those in *Rhodobacter capsulatus* or in species of the Rhizobiaceae (Lee et al., 2000). The regulatory features of three genes encoding P(II)-like signal proteins have been investigated in relation to the control of nitrogen fixation in *Ga. diazotrophicus* (Perlova et al., 2003b).

In *Ga. xylinus*, the genes for cellulose synthesis are localized in an operon. Differences in numbers of ORFs exist among the strains (Wong et al., 1990; Nakai et al., 1998). In *Ga. xylinus* ATCC 53582, the *acs* operon is functional for the final steps of cellulose biosynthesis and consists of three genes: the *acsAB* gene codes for the 168-kDa cellulose synthase, the *acsC* gene codes for a 138-kDa pore-protein, and the third gene (*acsD*) codes for a small protein involved in the crystallization of the microfibrils (Saxena et al., 1994; Brown and Saxena, 2000). The genes coding for diguanylate cyclase and phosphodiesterase A (involved in the synthesis and breakdown of the specific nucleotide regulator c-di-GMP) are organized on three unlinked homologous operons (Tal et al., 1998).

Insertion elements have been reported in several *Gluconacetobacter* strains and are responsible, just as in other members of the Acetobacteraceae, for genetic instability. In two different strains of *Ga. xylinus*, the following IS-elements have been reported, causing the loss of cellulose and acetan synthesis: IS*1031* (930 bp) and IS*1032* (916 bp). The former is inserted 500 bp upstream of the operon for cellulose biosynthesis (Coucheron, 1991; Coucheron, 1993; Standal et al., 1994) and the latter is inserted in the gene for acetan synthesis (Iversen et al., 1994).

Plasmids have been isolated and characterized from a variety of *Gluconacetobacter* strains and genetic transformation systems and plasmid vectors for gluconacetobacters have been established (Valla et al., 1985; Valla et al., 1987; Blatny et al., 1997). Plasmid p21R1 (a RK2-derivative

carrying the gene for levansucrase) was introduced in *Ga. diazotrophicus*, increasing the levansucrase production by this genetically modified acetic acid bacterium (Hernandez et al., 1999). Electroporation systems developed for *Ga. xylinus*, when used with heterologous plasmids, yielded transformation frequencies of up to 10^5 transformants per µg of DNA (Hall et al., 1992). Plasmid pAH4 was characterized from a cellulose-producing *Gluconacetobacter* strain, and a shuttle vector system of both this strain and *E. coli* was constructed by connecting pAH4 and pUC18 (Tonouchi et al., 1994). Fukaya et al. (1989) introduced the aldehyde dehydrogenase gene from "*Acetobacter polyoxogenes*" into *Ga. xylinus*, enhancing the production of acetic acid by overexpression of the cloned gene. Shuttle vectors for cloning and expression of genes in *Ga. europaeus*, *Ga. intermedius* and *E. coli* were constructed by ligation of plasmid pJK2-1 from *Ga. europaeus* and pUC18 (Trček et al., 2000).

Applications Some of the *Gluconacetobacter* species are involved in industrial vinegar fermentations, particularly in high acid acetators (e.g., *Ga. europaeus* and *Ga. entanii*). Most of such *Gluconacetobacter* strains were isolated and described from vinegar factories in Europe. The biotechnological and industrial aspects of vinegar production by acetic acid bacteria are treated in detail in the chapter Organic Acid and Solvent Production in Volume 1.

The cellulose produced by *Ga. xylinus* displays unique properties, such as high crystallinity, high mechanical strength, and a highly pure and ultrafine fiber network structure. Although cost differences between the bacterial and plant polymer are currently prohibitive to extensive commercial usage, bacterial cellulose from *Ga. xylinus* has found medical application (as a wound healing skin substitute), industrial use (in acoustic membranes for audio equipment), and application as a high-quality paper additive, thickener, insulator material, food stabilizer as well as a dietary fiber and probiotic. Cellulose produced by *Ga. xylinus* is also one of the basic components of the traditional food nata de coco, which is popular in Asian countries (Bernardo et al., 1998; see the section Habitats and Uses of Acetic Acid Bacteria in this Chapter). Attempts were and are being made to improve the production of bacterial cellulose, both in surface culture and in submerged culture via strain improvement and production process development (De Wulf et al., 1996; Jonas and Farah, 1998; Vandamme et al., 1998; Bielecki et al., 2002).

The plant-associated and nitrogen-fixing gluconacetobacters (e.g., *Ga. diazotrophicus*) may find applications to enhance the crop yield of other monocotyledonous plants besides sugarcane. *Gluconacetobacter diazotrophicus* is also of interest for bulk production of gluconic acid, because it grows well in a simple mineral salts medium and oxidizes glucose at high rates in acid conditions (pH 3.5) and (unlike *G. oxydans*) in the presence of high concentrations of gluconic acid (Attwood et al., 1991; Organic Acid and Solvent Production in Volume 1).

The Genus Gluconobacter *Gluconobacter* strains are motile by polar flagella or nonmotile and oxidize ethanol to acetic acid. In contrast to the other members of the family, they do not oxidize acetate or lactate to CO_2 and H_2O. All strains produce 2-ketogluconic acid from D-glucose, and most strains form also 5-ketogluconic acid. *Gluconobacter* strains prefer sugar-enriched environments in contrast to most *Acetobacter* and *Gluconacetobacter* strains, which prefer to grow on alcohol-containing media. Gluconobacters generally produce acid during growth on several carbohydrates (Table 2) and show a pronounced and efficient ketogenesis from polyhydroxyalcohols via a single-step oxidation. The latter property finds numerous applications in industry.

Taxonomy Acetic acid bacteria from sugary environments, such as flowers and fruits, were studied for the first time in 1935 in Japan by Asai. These strains exhibited characteristics quite different from those of the usual acetic acid bacteria, and mainly on the basis of their capacity to oxidize ethanol and glucose, Asai (1935) proposed to divide the acetic acid bacteria into two separate genera, *Acetobacter* and *Gluconobacter*. Asai's 1935 paper, being written in Japanese, remained virtually unknown in the West. Studies on flagellation and oxidation of acetate and lactate led Leifson (1954) to propose the division of the acetic acid bacteria into the genera *Acetobacter* and *Acetomonas*. De Ley and Frateur (1970) discussed the classification and nomenclatural problems related to the genus *Gluconobacter* and pointed out that *Gluconobacter* Asai 1935, the "suboxydans group" of Frateur (1950), and *Acetomonas* Leifson 1954 are all to be referred to the genus *Gluconobacter*. In the eighth edition of *Bergey's Manual* (De Ley and Frateur, 1974b), the genus *Gluconobacter* was considered as a member of the family Pseudomonadaceae and was composed of a single species *G. oxydans* with four subspecies, which appeared on the Approved Lists of Bacterial Names (Skerman et al., 1980). In the subsequent edition of *Bergey's Manual*, *Gluconobacter* belongs together with *Acetobacter* to the family Acetobacteraceae (De Ley et al., 1984b), and De Ley and Swings (1984a) proposed to discontinue the use of subspecies names and retained a single species *G. oxydans*.

At present the following four species are recognized in *Gluconobacter*: *G. oxydans* (type species), *G. asaii*, *G. cerinus* and *G. frateurii* (Table 1). This classification is based on phenotypic and chemotaxonomic features, as well as on data obtained from DNA-DNA hybridizations and rDNA-sequencing (Yamada and Akita, 1984a; Yamada et al., 1984c; Micales et al., 1985; Mason and Claus, 1989; Sievers et al., 1995b; Yamada et al., 1999; Tanaka et al., 1999). Notably, particularly in the biochemical literature, the name *Gluconobacter suboxydans* is still used instead of *G. oxydans* (e.g., Adachi et al., 2001b; Adachi et al., 2003c). Recently, a polyphasic study indicated that *G. asaii* should be considered as a junior subjective synonym of *G. cerinus* (Katsura et al., 2002).

Habitats *Gluconobacter* strains were isolated from sugar-rich biotopes, such as fruits and flowers, honey bees, grapes and wine, palm sap, cocoa wine, cider, beer and soft drinks, where they can cause off-flavors, e.g., in orange juice (see the section Habitats and Uses of Acetic Acid Bacteria in this Chapter).

Isolation and Cultivation Enrichment of *Gluconobacter* strains present in flowers, fruits, soft drinks, etc. can be done using the following medium: D-glucose, 100 g; yeast extract, 5.0 g; peptone, 3.0 g; acetic acid, 1.0 ml; cycloheximide, 0.1 g, and distilled water, 1000 ml. Incubation is done at 28–30°C for 3–5 days in flasks with shaking. Cultures showing growth are plated on a $CaCO_3$-containing medium, where the colonies dissolve the $CaCO_3$ (Carr and Passmore, 1979). The composition of the medium for the $CaCO_3$-ethanol test is as follows (Sievers and Swings, 2005): glucose, 0.5 g; yeast extract, 5.0 g; peptone, 3 g; $CaCO_3$, 15.0 g; agar, 12.0 g; ethanol (99.8%), 15 ml (filter-sterilized and added after after sterilization to the basal medium), and distilled water, 1000 ml. The oxidation of ethanol to acetic acid causes a clear zone around the colonies. The overoxidation of acetic acid by strains of the genera *Acetobacter*, *Gluconacetobacter* and *Acidomonas* results in a reprecipitation of the $CaCO_3$, whereas *Gluconobacter* strains will dissolve the $CaCO_3$ without the reprecipation of $CaCO_3$. Such colonies are isolated, further purified on GYC medium, characterized and identified.

Gluconobacter strains can be grown at 28–30°C on GYC- or MYP medium at pH 6.0–6.8 (see footnote [d] of Table 5 for the composition of the media).

Identification Features differentiating the genus *Gluconobacter* from the other Acetobacteraceae are listed in Table 2. The *Gluconobacter* species form a coherent closely related cluster in the phylogenetic tree (Sievers et al., 1995b; Fig. 2): 16S rDNA sequence similarities vary between 97.0% and 98.8%.

Phenotypic features differentiating the three species *G. oxydans*, *G. frateurii* and *G. cerinus* are summarized in Table 6. Note that the validly described species *G. asaii* (Mason and Claus, 1989) was found to be a junior synonym of *G. cerinus* (Katsura et al., 2002). Correct identification of *Gluconobacter* isolates may require sequencing of 16S rDNA as well as DNA-DNA hybridizations but can also be done on the basis of sequence and restriction analysis of the 16S-23S rDNA internal transcribed spacer regions (Yukphan et al., 2004a). On superficial examination, *Gluconobacter* strains may resemble *Frateuria*, a genus phylogenetically belonging to the *Xanthomonas*-group of the Gammaproteobacteria (Swings et al., 1980; Swings, 1992a; Fig. 2). The polarly flagellated *Frateuria* does not overoxidize acetate but oxidizes lactate to CO_2 and water and is characterized by Q-8 as major ubiquinone.

Physiological and Biochemical Properties *Gluconobacter* cells catabolize D-glucose via the hexose monophosphate pathway. In contrast to *Acetobacter* and other genera of the Acetobacteraceae, gluconobacters are not able to overoxidize acetic acid to CO_2 and H_2O, because they do not possess a complete TCA cycle, lacking succinate dehydrogenase (Greenfield and Claus, 1972). The respiratory chain in *Gluconobacter* consists of cytochrome *c*, ubiquinone, and cytochrome *o*

Table 6. Differentiation of *Gluconobacter* species.[a]

Characteristic	1.	2.	3.[b]
Growth on *meso*-ribitol[c]	–	+	–[e]
Growth on L-arabitol[c]	–	+	–[e]
Growth without nicotinic acid[c,d]	–	+	+
Acid formation from[f]			
meso-ribitol	–	+	–
D-Arabitol	–	+	+
L-Arabitol	–	+	–

Symbols: +, 90% or more of the strains positive; and –, 90% or more of the strains negative.

[a]1., *G. oxydans*; 2., *G. frateurii*; and 3., *G. cerinus*.

[b]The validly described species *G. asaii* (Mason and Claus, 1989) is now considered as a junior subjective synonym of *G. cerinus* (Katsura et al., 2002).

[c]Data from Mason and Claus (1989), Yamada and Akita (1984a) and Katsura et al. (2002).

[d]Three transfers into nicotinate-free media are necessary to demonstrate nicotinate-dependence (Mason and Claus, 1989).

[e]One strain was described as positive (Mason and Claus, 1989); the type strain and most other strains are negative (Katsura et al., 2002).

[f]Data from Tanaka et al. (1999).

as terminal ubiquinol oxidase. *Gluconobacter* cells contain also a cyanide-insensitive alternative ubiquinol oxidase (Takeda et al., 1992; Matsushita et al., 1994).

As in most acetic acid bacteria, the membrane-linked alcohol and aldehyde dehydrogenase complexes of *Gluconobacter* are composed of several subunits (Matsushita et al., 1994; Kondo and Horinouchi, 1997c). The larger subunit of the alcohol dehydrogenase complex contains heme *c* and pyrroloquinoline quinone (PQQ) as cofactors.

An outstanding biochemical feature of *Gluconobacter* is its ability to incompletely oxidize a great variety of carbohydrates, alcohols and related compounds. These rapid and incomplete oxidations of carbon sources are exploited in various biotechnological processes (Gupta et al., 2001; Deppenmeier et al., 2002).

Sugars, alcohols, and polyols are oxidized via two alternative pathways. The dehydrogenases of the so-called "direct oxidative pathway" are localized on the cytoplasmic membrane and their active sites are oriented towards the periplasm. Oxidizable substrates pass through the outer membrane and are almost quantitatively transformed to oxidation products, which are released into the medium via the porins of the outer membrane. These membrane-bound dehydrogenases are coupled to the respiratory chain and are responsible for the rapid oxidation of various biotechnologically important substrates. For the second type of enzyme system, uptake of the substrates into the cytoplasm is required, where oxidation takes place by soluble nicotinamide dinucleotide phosphate ($NAD[P]^+$)-dependent dehydrogenases and the resulting intermediates are phosphorylated and further metabolized via the pentose phosphate pathway. These $NAD(P)^+$-dependent enzymes participate in the biosynthesis of precursors and are involved in the maintenance of the cells (Matsushita et al., 1994). Some of these soluble NAD(P)-dependent pentitol- and hexitol-dehydrogenases have been crystallized and characterized (Adachi et al., 1999a; Adachi et al., 1999b; Adachi et al., 2001a).

Various membrane-bound dehydrogenases have been identified and characterized (Deppenmeier et al., 2002). Dehydrogenases for glucose, alcohol, aldehyde, glycerol, sorbitol, D-arabitol, fructose and gluconate contain PQQ as prosthetic group, and some of these dehydrogenases are also equipped with heme *c*-containing subunits (Matsushita et al., 1994; Matsushita et al., 1999; Matsushita et al., 2003; Choi et al., 1995; Shinagawa et al., 1999; Adachi et al., 2001a; Adachi et al., 2001b). In addition, membrane-bound flavin-dependent dehydrogenases have been described oxidizing D-gluconate, 2-keto-D-gluconate and D-sorbitol (Matsushita et al., 1994). Most likely, *Gluconobacter* strains contain many more dehydrogenases than those presently described (see, e.g., Adachi et al. [2003a], Adachi et al. [2003b] and Hoshino et al. [2003]). Several *Gluconobacter* strains display, e.g., a strong quinate dehydrogenase activity (PQQ-linked), producing 3-dehydroquinate (Adachi et al., 2003b; Adachi et al., 2003c). During these incomplete oxidations, coupling of electron transfer and proton translocation is not very efficient, resulting in low growth rates and loss of energy via heat production (Matsushita et al., 1989). The isolation and characterization of thermotolerant *Gluconobacter* strains might be advantageous for industrial applications to reduce the costs of cooling during fermentation (Moonmangmee et al., 2000; Adachi et al., 2003b).

Gluconobacter cells oxidize polyols with different carbon chain lengths ranging from glycerol to heptitols and octitols to the corresponding ketoses according to the so-called "rule of Bertrand-Hudson," whereby polyols with a *cis*-arrangement of two secondary hydroxyl groups in D-configuration to the adjacent primary alcohol group (a D-*erythro* configuration) are oxidized regioselectively to the corresponding ketoses (Kersters et al., 1965; Kulhanek, 1984; Kulhanek, 1989). Moreover, cyclitols and chemically modified pentitols and hexitols (such as deoxy-, deoxyamino-, deoxyhalogen polyols) can also be oxidized incompletely.

Cleton-Jansen et al. (1991) showed that a one-point mutation in the gene coding for the PQQ-linked glucose dehydrogenase of a *G. oxydans* strain resulted in the replacement of histidine at position 787 by asparagine. This single amino acid substitution changed the substrate specificity of the glucose dehydrogenase, resulting in the conversion of maltose to maltobionic acid in addition to the oxidation of glucose.

Gluconobacter strains generally produce acid during growth on D-glucose, D-fructose, D-xylose, D-mannitol and maltose and tolerate up to 10% glucose. The best carbon sources for growth are D-glucose, D-mannitol and D-sorbitol. The formation of water-soluble brown pigments on glucose-containing media is correlated with the production of 2,5-diketogluconate and γ-pyrones from D-glucose.

The major ubiquinone of *Gluconobacter* strains is Q-10 and their DNA base composition is 54–63 mol% G+C.

Genetics and Genomics Mutation studies and DNA-recombinant analyses focus on the genes of the various dehydrogenases involved in the direct oxidative metabolism operative in *Gluconobacter* (Gupta et al., 1997; Saito et al., 1997; Saito et al., 1998; Miyazaki et al., 2002; Sugiyama

et al., 2003). The microbiological production of 2-keto-L-gulonic acid (a precursor of vitamin C production) has been improved by cloning the genes of L-sorbose dehydrogenase and L-sorbosone dehydrogenase from *G. oxydans* T-100 and introducing them via a shuttle vector in *G. oxydans* G624, a strain accumulating L-sorbose (Saito et al., 1997; Saito et al., 1998). Similarly, Shibata et al. were able to introduce the *Gluconobacter* genes for L-sorbose dehydrogenase and L-sorbosone dehydrogenase into a strain of *Pseudomonas putida* (Shibata et al., 2000a; Shibata et al., 2000b), and replacement of the native L-sorbosone dehydrogenase promotor by the *Escherichia coli* tufB promotor improved the productivity of 2-keto-L-gulonic acid considerably. Cloning of the cytoplasmic xylitol dehydrogenase of *G. oxydans* resulted in an improved production of *meso*-xylitol from D-arabitol (Sugiyama et al., 2003). Gupta et al. (1999) described a *G. oxydans* mutant deficient in gluconic acid dehydrogenase unable to produce 2,5-diketogluconic acid. Kondo and Horinouchi (1997b) detected and characterized an insertion sequence IS*12528* in mutants of *G. oxydans* with reduced alcohol dehydrogenase activity. Such transposable elements are likely responsible for genetic instability of *Gluconobacter* strains.

The sequence of the *Gluconobacter oxydans* genome (±3 Mb) is being determined in Germany (http://www.genomik.uni-goettingen.de). Some 3000 ORFs were found and all the genes necessary to encode the enzymes of the pentose phosphate pathway and the Entner-Doudoroff pathway are present, whereas the genes encoding phosphofructokinase and succinate dehydrogenase have not been detected on the *G. oxydans* chromosome, confirming that the Emden-Meyerhof pathway is not functional and the citric acid cycle is incomplete and functions only for the synthesis of biosynthetic precursors (Deppenmeier et al., 2002). The genome sequencing revealed that more than 80 ORFs encode putative dehydrogenases and oxidoreductases with unknown function. Among them are more than 20 alcohol and 30 sugar dehydrogenases, indicating that *G. oxydans* harbors genes for novel dehydrogenases which can be of interest for future biotechnological applications.

Two *G. oxydans* plasmids pAG5 and pGO128 (5648 bp and 4340 bp, respectively) were described and sequenced (accession nrs. [genbank AB086443] and [genbank AJ428837], respectively; Tonouchi et al., 2003; Sievers and Swings, 2005).

At least three DNA-phages have been isolated from *Gluconobacter*: phage A-1 (Schocher et al., 1979; Jucker and Ettlinger, 1981) and phages GW6210 and JW2040 (Robakis et al., 1985). Phage A-1 caused abnormalities in the oxidation of D-sorbitol to L-sorbose by *G. oxydans* (Schocher et al., 1979).

Applications The biotechnological applications of *Gluconobacter* strains are mainly based on their outstanding capacity to incompletely and rapidly oxidize a wide range of carbohydrates, alcohols and polyols, whereby the oxidation products (acids and ketones) are excreted nearly quantitatively into the medium. Using *Gluconobacter* cells offers the great advantage of regio- and stereoselective oxidation of the substrates, a process that would otherwise require complex protection group chemistry. Some aspects of these industrial applications are summarized in this section, and we refer the reader for more detailed information to the following reviews: Gupta et al. (2001), Macauley et al. (2001), Deppenmeier et al. (2002), and Hancock and Viola (2002).

PRODUCTION OF GLUCONATE AND KETOGLUCONATES. Gluconic acid, its lactone, salts and keto-forms have a wide range of applications in the food, pharmaceutical and chemical industries. The production of gluconic acid by *Gluconobacter* (and other microorganisms) is treated in detail in the chapter Organic Acid and Solvent Production in Volume 1. Gluconobacters can oxidize glucose and gluconic acid to ketogluconic acids (2-ketogluconic acid, 5-ketogluconic acid and 2,5-diketogluconic acid). 2-Ketogluconate and 5-ketogluconate are interesting precursors for isoascorbic acid synthesis and tartaric acid production, respectively (Klasen et al., 1992). 2,5-Diketogluconate can be converted to 2-keto-L-gulonic acid as a penultimate intermediate in the industrial production of vitamin C.

PRODUCTION OF L-SORBOSE AND VITAMIN C. *Gluconobacter* strains are used for the oxidative conversion of D-sorbitol to L-sorbose, which is an important intermediate in the production of L-ascorbic acid (vitamin C; estimated world production: approximately 100,000 tons per year; Macauley et al., 2001). Vitamin C is important for human and animal nutrition and is used as an antioxidant in the food industry. It is currently produced by the Reichstein-Grüssner process, involving a series of chemically based unit operations and a single biological oxidative step, where D-sorbitol is regioselectively oxidized to L-sorbose by a membrane-bound D-sorbitol dehydrogenase of *G. oxydans* (Fig. 3). D-Sorbitol is obtained by electrolytic or catalytic reduction of D-glucose.

The overall practical yield of the Reichstein process, with the recent advances in chemical engineering, is thought to be around 50% (Boudrant, 1990). The effects of crosslinking

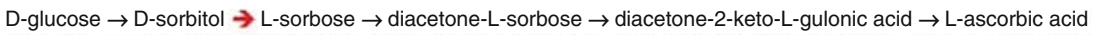

Fig. 3. The Reichstein-Grüssner process for the production of L-ascorbic acid. The biological oxidation of D-sorbitol to L-sorbose (red arrow) is catalyzed by *G. oxydans*. The black arrows indicate nonbiological steps.

Fig. 4. The conversion of D-sorbitol to 2-keto-L-gulonic acid by *Gluconobacter*. Red arrows indicate transformations performed by three different dehydrogenases of *G. oxydans*. The black arrow is a nonbiological step to yield vitamin C.

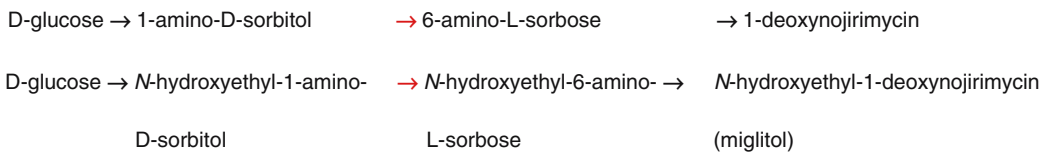

Fig. 5. Synthesis of 1-deoxynojirimycin and miglitol from D-glucose. Red arrows indicate the regioselective oxidation by a *G. oxydans* strain and the black arrows are nonbiological steps. The first step is a chemical reductive amination of D-glucose and the last step is a chemical stereoselective ring closure.

agents, such as glutaraldehyde and polyethyleneimine, on L-sorbose production by immobilized *G. oxydans* cells have been studied (Park et al., 1998). De Wulf et al. (2000) were able to obtain the theoretically maximal productivity of the biological oxidative step by using a *G. oxydans* mutant that was selected under conditions of substrate inhibition.

Several strategies including recombinant DNA-technologies are being developed to shift the synthesis of ascorbic acid from chemical procedures to purely bioconversion routes, where the synthesis of 2-keto-L-gulonic acid is a key intermediate (Deppenmeier et al., 2002). Some *Gluconobacter* strains convert D-sorbitol to 2-keto-L-gulonic acid via L-sorbosone. The 2-keto-L-gulonic acid can be converted nonbiologically to L-ascorbic acid (Hoshino et al., 1990; Saito et al., 1997; Fig. 4).

Overexpression of the L-sorbose dehydrogenase and the L-sorbosone dehydrogenase genes in *G. oxydans* results in improved yields of 2-keto-L-gulonic acid (Saito et al., 1997). In this respect the application of *Ketogulonicigenium* strains for the biological conversion of L-sorbose to 2-keto-L-gulonic acid may open new perspectives (Urbance et al., 2001). The latter genus belongs to the *Rhodobacter*-lineage of the Alphaproteobacteria (Fig. 1).

PRODUCTION OF DIHYDROXYACETONE AND ACETYLMETHYLCARBINOL. Dihydroxyacetone is used as a cosmetic tanning agent and as an intermediate for the synthesis of various organic chemicals and surfactants. The oxidation of glycerol to dihydroxyacetone by *G. oxydans* strains is catalyzed by a membrane-bound PQQ-dependent glycerol dehydrogenase (Claret et al., 1994) and can be optimized by using immobilized *G. oxydans* cells (Tkac et al., 2001). Similarly gluconobacters can be used to oxidize 2,3-butanediol to acetylmethylcarbinol.

SYNTHESIS OF 1-DEOXYNOJIRIMYCIN AND MIGLITOL. 1-Deoxynojirimycin and its N-substituted analog *N*-hydroxyethyl-1-deoxynojirimycin (miglitol) are strong inhibitors of α-glucosidases and are used for the treatment of non-insulin-dependent diabetes (Campbell et al., 2000). The industrial production of these compounds follows a combined biotechnological-chemical synthesis, whereby *G. oxydans* plays a key-role in the oxidation of 1-aminosorbitol derivatives (Deppenmeier et al., 2002; Fig. 5). As *Gluconobacter* cannot grow on aminopolyols, whole resting cells (grown on sorbitol) have to be used for this biotransformation process (Schedel, 2000).

GLUCONOBACTER AS BIOSENSOR. The cells and enzymes of *G. oxydans* find applications as biosensors for the estimation of the concentrations of various aldoses, polyalcohols, ethanol, glycerol, etc. (Lusta and Reshetilov, 1998; Tkac et al., 2000; Tkac et al., 2001; Macauley et al., 2001).

The Genus Kozakia During the isolation and identification of acetic acid bacteria from Indonesian sources, Lisdiyanti et al. (2002) detected a new cluster of acetic acid bacteria on the basis of 16S rRNA gene sequences. This new taxon, *Kozakia baliensis*, produces large amounts of mucous polysaccharides in a sucrose-containing medium. Genetic properties and applications of *Kozakia* strains have not yet been studied.

Taxonomy The genus *Kozakia* was first described by Lisdiyanti et al. (2002) as a new member of

the family Acetobacteraceae. At present it contains a single species, *Kozakia baliensis* (Table 1), and its classification is based on phenotypic and chemotaxonomic features, as well as data obtained from DNA-DNA hybridizations and 16S rDNA sequencing (Lisdiyanti et al., 2002). Its closest phylogenetic neighbor is the genus *Asaia* (Fig. 2).

Habitats Four strains of *Kozakia baliensis* were isolated in Indonesia and are presently available from culture collections: the type strain was isolated from palm brown sugar and three other strains originate from ragi (used as a starter for the preparation of fermented food).

Isolation and Cultivation *Kozakia baliensis* strains can be enriched on a medium containing: D-glucose, 10.0 g; ethanol, 5.0 ml; acetic acid, 3.0 ml; peptone, 15.0 g; yeast extract, 8.0 g; cycloheximide, 0.1 g; and distilled water, 1000 ml. The pH of the medium is adjusted to 3.5 with HCl. Incubation is done at 30°C for 3–5 days in flasks with shaking. Cultures showing growth are streaked on $CaCO_3$ agar plates containing: D-glucose, 20.0 g; ethanol, 5.0 ml; yeast extract, 8.0 g; $CaCO_3$, 7.0 g; agar, 15.0 g; and distilled water, 1000 ml. Colonies of *Kozakia baliensis* strains are slightly pinkish, mucous, do not produce water-soluble pigments, and dissolve the $CaCO_3$ around the colonies weakly.

Identification Phenotypically, the strains of *Kozakia baliensis* are similar to *Gluconacetobacter xylinus* because they oxidize acetate and lactate, have Q-10 as the major ubiquinone, and produce a large amount of levan-like mucous polysaccharides. The formation of levan by *Gluconacetobacter xylinus* has been reported by Tajima et al. (1997) and Kornmann et al. (2003). However, *Kozakia* strains are phylogenetically clearly distinct from the genus *Gluconacetobacter* (Fig. 2). The 16S rDNA sequence similarity between the type strain of *Kozakia baliensis* and the type strain of *Asaia bogorensis* is rather high (97.4%). *Kozakia baliensis* strains differ from *Asaia* strains in the production of acetic acid from ethanol, growth in the presence of 0.35% acetic acid, and absence of growth on 30% D-glucose (Table 2).

Physiological and Biochemical Properties *Kozakia* strains are nonmotile and produce levan-like mucous substance(s) on sucrose or D-fructose. They grow at pH 3.0 and 30°C, do not produce gelatinase, H_2S, indole or ammonia from L-arginine, and do not reduce nitrate. They oxidize acetate and lactate to carbon dioxide and water, but the activity is weak. They produce acetic acid from ethanol. Their growth is not inhibited by 0.35% acetic acid at pH 3.5. They grow on mannitol agar but not on glutamate agar and do not grow on 30% D-glucose. Ammoniacal nitrogen is not assimilated on glucose medium, mannitol medium, or ethanol medium without vitamins. The strains produce dihydroxyacetone from glycerol, produce γ-pyrones from D-fructose but not from D-glucose, and produce D-gluconate, 2-keto-D-gluconate and 5-keto-D-gluconate from D-glucose but not 2,5-diketo-D-gluconate. Acid is produced from L-arabinose, D-xylose, D-glucose, D-galactose, D-mannose, melibiose, raffinose, *meso*-erythritol, glycerol and ethanol but not from L-rhamnose, D-fructose, L-sorbose, lactose, D-mannitol, D-sorbitol or dulcitol. Acid production from D-arabinose and sucrose is variable depending on the strain. The major ubiquinone is Q-10 and the DNA base composition is 57 mol% G+C.

The Genera Saccharibacter *and* Swaminathania *Saccharibacter* (Jojima et al., 2004) and *Swaminathania* (Loganathan and Nair, 2004) are two new genera of acetic acid bacteria described and each containing one species. *Saccharibacter floricola* was isolated from pollen of Japanese flowers and is phylogenetically related to *Gluconobacter*. *Swaminathania salitolerans* strains were isolated from the rhizosphere, roots and stems of salt-tolerant wild rice and are able to fix nitrogen. *Swaminathania* is closely related to the genus *Asaia*.

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Prokaryotes (2006) 5:201–221
DOI: 10.1007/0-387-30745-1_10

CHAPTER 3.1.9

The Genus *Zymomonas*

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Habitat

Zymomonas mobilis has been reported mainly from tropical and subtropical habitats, e.g., sugar-rich, plant saps from agave (Mexico; Lindner, 1928), sugar cane (Brazil and Fiji Islands; reviewed in Falcao de Morais et al., 1993), and palm wine from central Africa (Swings and De Ley, 1977). Other sources of this organism include fermenting sugarcane juice (Goncalves de Lima et al., 1970), fermenting cocoa beans (Ostovar and Keeney, 1973), and bees and ripening honey (Ruiz-Argueso and Rodriguez-Navarro, 1975). In Europe, *Z. mobilis* also appeared in spoiled beer and cider. One of the first written descriptions of "cider sickness" was presented by Lloyd (1903), in which he noted the presence of "sulphuretted hydrogen" in spoiled ciders. Barker and Hillier (1912) were the first to study cider sickness extensively and gave a description of the responsible bacterium. Cider sickness is recognized by frothing and abundant gas formation, a typical change in the aroma and flavor, reduction of sweetness, and development of a marked turbidity forming a heavy deposit. From the complex microflora of sick cider, Barker and Hillier isolated and purified a bacterium that caused the typical strong aroma and flavor in sterile cider upon reinfection; unfortunately, they did not give a Latin taxon to the new organism. In 1951, Millis isolated several bacteria from ciders and perries that resembled Barker's strains (Millis, 1951). It was Millis who demonstrated quite clearly that the cider sickness organism was a *Zymomonas*. Various strains formed approximately 1.9 mole of ethanol per 1 mole of glucose and about the same amount of CO_2. Furthermore, some H_2S, acetaldehyde and lactic acid could be detected. The optimal pH range for growth was 4.5 to 6.5 and the optimal temperature range was 25 to 31°C.

Pulque and Palm Wine

During his stay in Mexico in 1923 to 1924, Lindner studied the fermentation of agave sap to pulque, an alcoholic beverage containing approximately 4 to 6% ethanol (Lindner, 1928). Lindner discovered the causal organism of the fermentation, a bacterium which he called *Termobacterium mobile* (Lindner, 1931). Over the years this organism received many names. This very motile bacterium was able to ferment sucrose, fructose and glucose to ethanol and CO_2. Lindner suggested that these bacteria occur strictly in tropical regions, where they account for the alcoholic fermentations in palm wines, chica beer, etc., and where they are used for bread manufacture. *Zymomonas* strains are very well adapted to a great variety of plant juices in tropical areas around the world because in such environments sucrose, glucose, fructose, amino acids and growth factors are present. The bacterium is resistant to ethanol and grows at low pH values under anaerobic conditions. Kluyver and Hoppenbrouwers (1931) studied Lindner's isolate. Neither Lindner nor Kluyver and Hoppenbrouwers related the pulque bacteria to the cider sickness organisms described earlier by Barker and Hillier (1912).

Beer

Shimwell (1937) isolated a *Zymomonas* strain from beer, from the surface of brewery yards, and from the brushes of cask-washing machines. The cells were Gram-negative plump rods, 2 to 3 by 1 to 1.5 μm in young cultures and longer (without endospores) in old cultures. The organism was indifferent to hop antiseptic and capable of growing in a wide range of beers. Later, Ault (1965) recognized that *Zymomonas* is a serious contaminant in keg beers. These infections can occur because of anaerobiosis and presence of priming sugars. The bacteria produce a heavy turbidity, and the unpleasant odor of rotten apples is due to traces of acetaldehyde and H_2S. *Zymomonas* has not been reported in lager beers; the low temperature of the process, 8 to 12°C, is unfavorable for its growth.

Isolation

Several procedures are described in the literature for the isolation of *Zymomonas* strains

(Swings and De Ley, 1977). Barker and Hillier (1912) isolated the cider sickness organisms on beer wort gelatin as small colonies after 11 days incubation at 22°C. Swings (1974) described the isolation of *Zymomonas* from fresh palm wines in WL differential medium, which is designed for brewing and fermentation processes but contains a yeast and mold inhibitor (Difco). When the samples were streaked on the medium in Petri dishes and incubated at 30°C in the Gas Pak anaerobic system, colonies of 1 to 4 mm in diameter were obtained after 4 to 5 days. However, it was very important to use young wine, about 24-h old, because isolation from palm wines more than 48-h old was impossible; the viability of *Zymomonas* rapidly decreases in aged fermentation broths. Swings and De Ley (1977) described a selection medium containing 0.3% malt extract, 0.3% yeast extract, 2% glucose, 0.5% peptone and 0.002% cycloheximide. The medium is adjusted to pH 4.0, autoclaved, and then supplemented with ethanol to a final concentration of 3%.

For *Zymomonas* cultivation, Fein et al. (1983) described a defined medium consisting of (g per liter of distilled water): glucose, 20; KH_2PO_4, 3.5; $(NH_4)_2SO_4$, 2.0; $MgSO_4 \cdot 7\ H_2O$, 1.0; $FeSO_4 \cdot 7\ H_2O$, 0.01; and 2-[N-morpholino]ethanesulfonate (MES), 19.52. The medium is adjusted to pH 5.5 and autoclaved. Then, a sterile solution of biotin and Ca-pantothenate is added to final concentrations of 0.001 g/liter each. A complete medium was introduced by Bringer-Meyer et al. (1985; g per liter of distilled water): D-glucose, 20; yeast extract, 10; KH_2PO_4, 1.0; $(NH_4)_2SO_4$, 1.0; and $MgSO_4 \cdot 7\ H_2O$, 0.5.

Growth and Conservation Conditions

Zymomonas grows best between 25 and 30°C; 74% of the strains grow at 38°C, but rarely at 40°C (Swings and De Ley, 1977). Growth is slow at 15°C (Millis, 1951) and absent at 4°C. Members of the subspecies *pomaceae* do not grow at temperatures above 36°C; therefore a growth temperature test is a good method for distinguishing between the two subspecies.

Most *Zymomonas* strains are able to grow between pH 3.8 and 7.5. At pH 3.5, 40% of the strains develop, illustrating a good acid tolerance. This is not surprising because the natural niche of this organism is in acid palm wines, cider and beers at pH 4. We observed that death rapidly occurs in batch fermentation after all the sugar is fermented. The bacterial cells are kept alive on complex media at room temperature and transferred weekly. When lyophilized, the organism can be kept alive for several years.

Identification

In the 1970s, Swings and De Ley applied modern molecular biological techniques to compare approximately 40 different strains of *Zymomonas*. They concluded that all the strains of *Zymomonas* by then described belong to a single species, *Zymomonas mobilis*, with two subspecies: *Z. mobilis* subsp. *mobilis* (the organism isolated from pulque or palm wine and currently envisioned for industrial ethanol production) and *Z. mobilis* subsp. *pomaceae* (the organism responsible for beer and cider spoilage; Swings and De Ley, 1977). Growth at 36°C is the best phenotypic test to differentiate the subspecies *mobilis* from *pomaceae*; only the former grows.

Once *Zymomonas* has been isolated on an appropriate medium, its identification is a relatively simple matter. A listing of the phenotypic characteristics of *Zymomonas* is given in Table 1 (Montenecourt, 1985).

Inoculation of *Zymomonas* into media containing sugar will lead to growth and gas production only when glucose, fructose or possibly

Table 1. Phenotypic description of the genus *Zymomonas*.

1. Gram-negative rods, 2- to 6-µm length, 1- to 5-µm width
2. Either motile or nonmotile; motility can be easily lost; one to four lophotrichous flagella
3. Pleomorphic cell arrangement, rosettes, chains, filaments
4. Spores, capsules, intracellular storage compounds (lipids, glycogen and poly-β-hydroxybutyrate) absent
5. Catalase positive, oxidase negative
6. Anaerobic and microaeroduric
7. Ferments glucose and fructose producing ≥1.5 moles of ethanol and CO_2
8. Sucrose utilization inducible, may be accompanied by levan production
9. No other monosaccharides, disaccharides, polysaccharides or fatty acids metabolized
10. Contains pentacyclic triterpenoids (hopanoids), vaccenic acid, and sphingolipids in its cell membranes
11. Forms sorbitol and gluconic acid from sucrose or glucose fructose (presence of a periplasmic enzyme, glucose-fructose oxidoreductase)
12. G+C content, 47.5 to 49.5 mol %; genome size approximately 2,085 kb

Adapted from Montenecourt, 1985.

sucrose is present. The ratio (moles of ethanol produced to moles of glucose fermented) is at least 1.5, and only small amounts of lactic acid and traces of acetylmethylcarbinol (acetoin) are formed; thus, a pH indicator will not register any acidity. This feature makes *Zymomonas* a unique ethanol-producing bacterium, and as far as is known, no other bacteria behave in this manner. If a finer distinction is to be made, tests listed by Swings and De Ley (1977) may be applied. Recently, it was demonstrated that in comparison to other Gram-negative bacteria, *Zymomonas* strains contained a high level of cis-vaccenic acid (>60% of the total fatty acids; Tornabene et al., 1982). Furthermore, the lipopolysaccharide fraction from this organism is different from that of other Gram-negative species inasmuch as deoxyhexoses, pentoses, hexoses, aminopentose, uronic acid, phosphate and myristic acid are the principal constituents, whereas ketodeoxyoctulonic acid (KDO), heptoses or hydroxy fatty acids are not evident.

Main Features

Zymomonas mobilis is an aerotolerant, fermentative bacterium with a number of exceptional characteristics: over 95% of the glucose utilized by this organism is converted to an equimolar mixture of ethanol and CO_2, and only a small percentage (<3%) is incorporated into cell mass (Swings and De Ley, 1977). Catabolism of the only carbon sources, glucose and fructose, proceeds via the Entner-Doudoroff pathway and with a net production of a single mole of ATP per mole of glucose catabolized. *Zymomonas mobilis* lacks other pathways for glucose catabolism, is incapable of gluconeogenesis, and has an incomplete tricarboxylic acid cycle (Gibbs and De Moss, 1954; Dawes et al., 1970; Swings and De Ley, 1977). The presence of pyruvate decarboxylase and alcohol dehydrogenase enables the organism to perform a pure ethanol fermentation, i.e., to produce almost two moles of ethanol per mole of glucose. Because of its rapid growth, sugar catabolism (about 1 μmole glucose · min^{-1} · mg^{-1} cell protein; Arfman et al., 1992), and tolerance towards high concentrations of substrate (up to 30% glucose; Kluyver and Hoppenbrouwers, 1931; Swings and De Ley, 1977; Loos et al., 1994) and product (up to 13% ethanol w/v; Rogers et al., 1982), *Z. mobilis* ferments ethanol very efficiently. *Zymomonas*, *Rhizomonas* and *Sphingomonas* belong to the same subclass (α-4) of the Proteobacteria (White et al., 1993). These genera contain sphinganine lipids in their membranes (Tahara and Kawazu, 1994). The physiological properties and biotechnological aspects of *Z. mobilis* have been reviewed (Swings and De Ley, 1977; Rogers et al., 1982; Baratti and Bu'Lock, 1986; Buchholz et al., 1987; Bringer-Meyer and Sahm, 1988; Viikari, 1988; Ingram et al., 1989; Sahm et al., 1992; Conway, 1992; Johns et al., 1992; Sprenger, 1993a; Sprenger et al., 1993b; Doelle et al., 1993; Yanase and Kato, 1994; Sprenger, 1996; Gunasekaran and Raj, 1999; Sprenger and Swings, 2000).

Metabolism: From Sugar Uptake to Ethanol Formation

Carbohydrate Transport

Zymomonas mobilis utilizes only three carbon sources: sucrose, glucose and fructose. Sucrose needs no uptake system because it is cleaved extracellularly and its moieties (glucose and fructose) are subsequently taken up into the cells by facilitated diffusion through a common transport protein (glucose facilitator GLF; DiMarco and Romano, 1985; Snoep et al., 1994; Weisser et al., 1996; Parker et al., 1997). *Zymomonas mobilis* appears to be the only known bacterium that relies solely on such an uniport type for sugar uptake, i.e., equilibration of external and internal sugar concentrations. No phosphoenolpyruvate (PEP)-dependent sugar uptake system has been detected yet (DiMarco and Romano, 1985). The uniporter needs no metabolic energy but cannot accumulate substances. Subsequent phosphorylation steps, however, distract free hexoses from the equilibrium so that effective sugar uptake and metabolism are warranted. Apart of glucose and fructose, various other hexoses and pentoses are GLF substrates (DiMarco and Romano, 1985; Schoberth and de Graaf, 1993; Parker et al., 1995; Parker et al., 1997; Weisser et al., 1995; Weisser et al., 1996), but D-mannose and D-xylose are no-growth substrates for wild-type *Z. mobilis* (but see Metabolic Engineering). Glucose is the preferred substrate (K_m ~4 mM) over fructose or xylose (K_m ~40 mM each). The GLF transports its substrates at high V_{max} (up to 1 μmole of substrate · mg^{-1} of cell protein · min^{-1}), with D-xylose being the best substrate, followed by glucose and fructose (Weisser et al., 1996; Parker et al., 1997). Growth on fructose leads to an increase in the transcription of *glf*, and the increased GLF may compensate for the lower affinity with fructose (Zembrzuski et al., 1992).

Owing to high glucose and fructose transport rates, equilibration between external and internal sugar concentrations can be rapid and may contribute to osmotic adjustment (Struch et al., 1991). Although not a growth substrate for *Z. mobilis*, the compatible solute sorbitol accumulates (up to 1 M, intracellular concentration) and further improves growth of *Z. mobilis* in high-

sugar environments (Loos et al., 1994). The sorbitol transporter is dependent on the proton motive force (Loos et al., 1994). Ethanol diffusion across the cell membrane has been determined using nuclear magnetic resonance (^{13}C-NMR) techniques (Schoberth et al., 1996).

Glycolytic Flux and Regulation of Metabolism

The glycolytic enzymes of the Entner-Doudoroff pathway and the fermentative enzymes pyruvate decarboxylase and alcohol dehydrogenase of *Z. mobilis* represent over half of the organism's soluble protein (Algar and Scopes, 1985; Osman et al., 1987; An et al., 1991). These enzymes (see Table 2 for details and references) are considered to operate at or near substrate saturation and do not seem to be subject to major regulations, e.g., by allosteric control (Algar and Scopes, 1985). The overall catabolic activity of *Z. mobilis* leads to a nearly unlimited flow of carbon through the glycolytic pathway. Thus each minute, *Z. mobilis* consumes an amount of glucose equal to one-third of its mass (Snoep et al., 1996; Parker et al., 1997). Regulation of enzyme activity has been shown for glucokinase through an absolute demand for inorganic phosphate, and to a lesser degree, by feedback inhibition through glucose-6-phosphate (Scopes and Bannon, 1995). Fructokinase is inhibited by glucose (K_i of 0.14 mM; Scopes et al., 1985b). Pyruvate kinase, which is allosterically regulated in most other organisms, appears not to be subject to any allosteric activator (Steiner et al., 1998). Growth on fructose induces about twofold the transcription of genes for peripheral sugar metabolism which lead to the introduction of fructose into glycolysis (i.e., by fructose uptake via GLF, fructose phosphorylation by fructokinase FRK, and

Table 2. Glycolytic enzymes and their genes.

Gene	Function/enzyme	Protein characterization	Sequence reference
glf	Glucose/fructose transporter	DiMarco and Romano, 1985 Parker et al., 1995, 1997 Weisser et al., 1995, 1996	Barnell et al., 1990
glk	Glucokinase	Scopes et al., 1985	Barnell et al., 1990
frk	Fructokinase	Doelle, 1982 Scopes et al., 1985 Weisser et al., 1996	Zembrzuski et al., 1992
pgi	Glucose 6-P isomerase	Hesman et al., 1991	Hesman et al., 1991
zwf	Glucose 6-P dehydrogenase	Scopes et al., 1985 Scopes, 1997	Barnell et al., 1990
pgl	6-Phosphogluconolactonase	Scopes, 1985	n.a.
edd	6-Phosphogluconate dehydratase	Scopes and Griffiths-Smith, 1984	Barnell et al., 1990
eda	KDPG aldolase	Scopes, 1984	Conway et al., 1991
gap	Ga 3-P dehydrogenase	Pawluk et al., 1986	Conway et al., 1987d
pgk	Phosphoglycerate kinase	Pawluk et al., 1986	Conway and Ingram, 1988
pgm	Phosphoglycerate mutase	Pawluk et al., 1986	Yomano et al., 1993
eno	Enolase	Pawluk et al., 1986	Burnett et al., 1992
pyk	Pyruvate kinase	Pawluk et al., 1986 Steiner et al., 1998	Steiner et al., 1998
pdc	Pyruvate decarboxylase	Bringer-Meyer et al., 1986 Neale et al., 1987	Brau and Sahm, 1986 Conway et al., 1987b Neale et al., 1987 Reynen and Sahm, 1988
adhA	Alcohol dehydrogenase I (zinc-dependent)	Wills et al., 1981 Neale et al., 1986	Keshav et al., 1990
adhB	Alcohol dehydrogenase II (iron-dependent)	Wills et al., 1981 Neale et al., 1986	Conway et al., 1987c
pdhA-D	Pyruvate dehydrogenase complex		Neveling et al., 1998
gfo	Glucose-fructose oxidoreductase	Zachariou and Scopes, 1986 Hardman and Scopes, 1988 Wiegert et al., 1997	Kanagasundaram and Scopes, 1992a Wiegert et al., 1997
gnl	Gluconolactonase		Kanagasundaram and Scopes, 1992b
sacA	Sucrase or invertase (intracellular)	Gunasekaran et al., 1990 Yanase et al., 1991	
sacB	Sucrase or invertase B (extracellular)	O'Mullan et al., 1992 Yanase et al., 1995	Kyono et al., 1995
levU	Levansucrase	Yanase et al., 1992 Yanase et al., 1991	Song et al., 1993 Kyono et al., 1995

isomerization to glucose-6-phosphate by phosphoglucose isomerase PGI; Scopes et al., 1985b; Hesman et al., 1991; Barnell et al., 1992; Zembrzuski et al., 1992).

Control on glycolytic flux is mainly exerted by glucose-6-phosphate dehydrogenase as shown by overexpression studies of several glycolytic enzymes of *Z. mobilis* (Snoep et al., 1995; Snoep et al., 1996). When overexpressing each of the first genes (*glf*, *zwf*, *edd*, *glk*) of the Entner-Doudoroff pathway (see Fig. 1), only recombinants with elevated glucose-6-phosphate dehydrogenase had a 10–13% higher glycolytic flux than that of the native organism, whereas increasing the expression of various other glycolytic operons caused a significant decrease in the glycolytic flux and growth rate. The latter was attributed to a protein burden effect (Snoep et al., 1995). Additional evidence for a major role of glucose-6-phosphate dehydrogenase in flux control was given by kinetic investigations which showed that the enzyme is allosterically inhibited by phosphoenolpyuvate (Scopes, 1997). Furthermore it was proposed that differences in transcript stability of the glycolytic enzymes of Z. mobilis represent a mechanism to balance the high levels of the ethanologenic enzymes (Meija et al., 1992). Hence, expression of glycolytic and

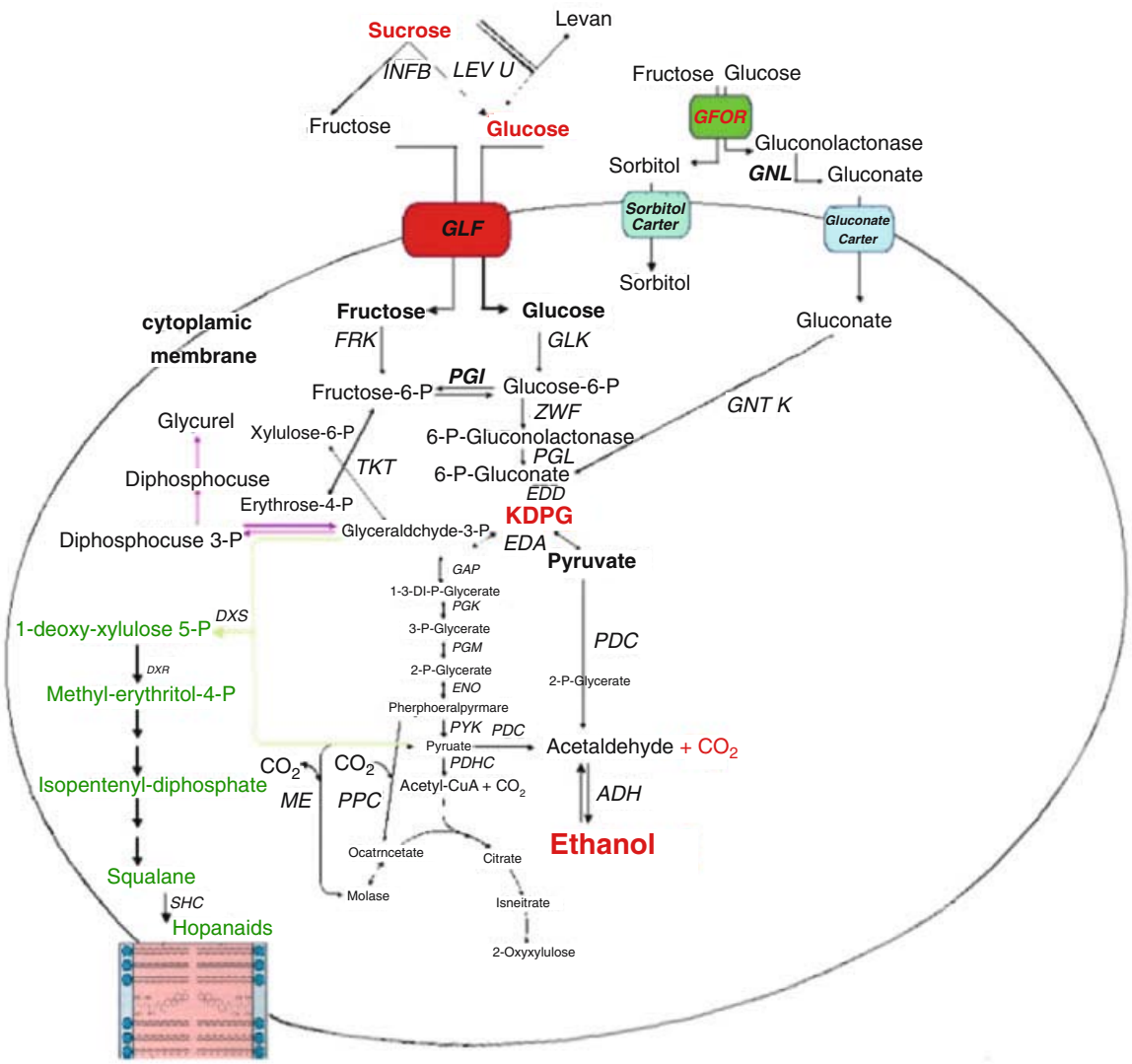

Fig. 1. Overview of main metabolic features in a schematic *Zymomonas mobilis* cell. Steps in carbohydrate metabolism (uptake and metabolism of sucrose, glucose, fructose, gluconate, sorbitol) are given together with anabolic reactions (incomplete tricarboxylic acid (TCA) pathway, formation of by-products, formation of hopanoids). Abbreviations: DXR = deoxyxylulose 5-phosphate reductoisomerase; DXS = 1-deoxy-xylulose 5-phosphate synthase; GNT K = gluconate kinase; ME = malic enzyme; PGL = phosphogluconolactonase; PPC = phosphoenol-pyruvate carboxylase; TKT = transketolase. For other abbreviations of enzymes see Table 2.

fermentative enzymes relies mainly on mRNA stability (the half-lives of transcripts for the glycolytic enzymes were in the range of 8–18 minutes; Meija et al., 1992). This relative mRNA stability may distinguish highly expressed glycolytic genes from biosynthetic genes. Differential expression of the *gap* and *pgk* genes, which form an operon, also has been attributed to differences in mRNA stability (Eddy et al., 1989).

When used in in vitro fermentations, the Entner-Doudoroff enzymes were remarkably resistant to ethanol inactivation and produced up to 16.5% (w/v) ethanol (Scopes and Griffiths-Smith, 1986). In living cells, 3-phosphoglycerate accumulated in the presence of 10% (w/v) ethanol, as detected by ^{31}P NMR spectroscopy. Enzyme assays confirmed that phosphoglycerate mutase and enolase were inhibited 31% and 40%, respectively, in the presence of 10% (w/v) ethanol in the test system (Strohhäcker et al., 1993).

A global regulatory protein (Grp) with high sequence similarity to the *Escherichia coli* global regulator Lrp has been discovered and can complement a glutamate-uptake mutant of *E. coli* (Peekhaus et al., 1995). The *grp* gene lies adjacent to an operon for a high-affinity glutamate carrier; its true function, however, remains to be elucidated (Peekhaus and Krämer, 1996).

Pyruvate Decarboxylase

Pyruvate decarboxylase (PDC, EC 4.1.1.1), a key enzyme in ethanol fermentation, catalyzes the conversion of pyruvate to acetaldehyde and carbon dioxide and depends on thiamine diphosphate and Mg(II) ions for its catalytic activity. Though PDC is widely distributed in fungi and higher plants, it is rare in prokaryotes and unknown in animals (Candy and Duggleby, 1998). In addition to being in *Z. mobilis* (Hoppner and Doelle, 1983; Bringer-Meyer et al., 1986; Neale et al., 1987; Diefenbach and Duggleby, 1991), bacterial PDCs have been found in *Sarcina ventriculi* (Stephenson and Dawes, 1971; Lowe and Zeikus, 1992), *Acetobacter* species (King and Cheldelin, 1954; De Ley and Schell, 1959), and *Erwinia amylovora* (Haq and Dawes, 1971; Haq, 1984). In *Z. mobilis*, PDC is one of the most abundant proteins, contributing 4–6% to the soluble cell protein content (Bringer-Meyer et al., 1986; An et al., 1991). The enzyme is a homotetramer with a subunit molecular mass of 60.79 kDa calculated from the DNA sequence (Neale et al., 1987; Conway et al., 1987b; Reynen et al., 1988). In contrast to genes in *Saccharomyces cerevisiae*, only one gene seems to code for PDC in *Z. mobilis* (Neale et al., 1987; König, 1998). *Zymomonas mobilis* PDC exhibits normal Michaelis-Menten kinetics with a K_m for pyruvate of 0.3–0.4 mM (Bringer-Meyer et al., 1986; Neale et al., 1987). This is exceptional because all other PDCs that have been characterized up to now are allosterically regulated by the substrate or other activator molecules such as pyruvamide (König, 1998). Recently, determination of the 3D crystal structure of the PDC from *Z. mobilis* revealed that the interface area between the dimers of the enzyme is much larger than in the yeast PDC. In addition, the dimers are more tightly packed in the PDC from *Z. mobilis*, thus preventing large rearrangements in the quarternary structure and locking the enzyme in an activated conformation (Dobritzsch et al., 1998). The critical amino acid residues involved in cofactor binding (Asp^{440}, Asn^{467}, Gly^{469}), substrate binding (Asp^{27}, His^{113}, His^{114}, Tyr^{290}, Thr^{388}, Glu^{473}) and catalysis (Glu^{473}) of the *Z. mobilis* PDC have been identified by site-directed mutagenesis (Sun et al., 1995; Pohl, 1997; Candy and Duggleby, 1998; Chang et al., 1999). A side reaction catalyzed by pyruvate decarboxylase is the carboligase activity, where the activated acetaldehyde bound to the thiamine diphosphate cofactor is condensed to a second aldehyde molecule (Bringer-Meyer and Sahm, 1988; Bornemann et al., 1995; Bruhn et al., 1995). This acyloin condensation reaction is used industrially for the production of phenylacetylcarbinol ([R]-1-hydroxy-1-phenylpropan-2-one), an intermediate in the synthesis of L-ephedrine. In *Z. mobilis* PDC, alanine replacement of the tryptophan (Trp^{392}) located near the active center enhanced the carboligase activity of the enzyme, increasing phenylacetylcarbinol formation by a factor of four (Bruhn et al., 1995).

Alcohol Dehydrogenase Isoenzymes

Zymomonas mobilis possesses two isoenzymes of fermentative alcohol dehydrogenase (ADH, EC 1.1.1.1): ADH I (a zinc-containing enzyme) and ADH II (an iron-containing enzyme; Wills et al., 1981; Hoppner and Doelle, 1983; Kinoshita et al., 1985; Neale et al., 1986). ADH activity is essential for the obligatory fermentative metabolism of *Z. mobilis*; therefore it is not surprising that the two ADH isoenzymes are abundant proteins in *Z. mobilis*, representing 2–5% of the soluble cell protein (Mackenzie et al., 1989). The ADH I, encoded by *adhA*, is a homotetramer with a subunit molecular mass of 36 kDa, and it contains one zinc atom per subunit (Wills et al., 1981; Neale et al., 1986; Keshav et al., 1990). The protein level of ADH I, which is more active at the early stages of growth, was found to decline in stationary-phase cells (Viikari, 1988; Keshav et al., 1990; An et al., 1991). The gene *adhB* encodes ADH II, also a homotetrameric enzyme with a subunit molecular mass of 40 kDa and one

iron atom per subunit. This enzyme catalyzes half the acetaldehyde reduction in *Z. mobilis* (Wills et al., 1981; Neale et al., 1986; Conway et al., 1987d). Under aerobic conditions, ADH I activity is fully conserved, but ADH II activity decays as the enzyme-bound Fe^{2+} atoms are oxidized (Tamarit et al., 1997). Whereas ADH I also oxidizes butanol, ADH II has almost no activity towards this substrate (Kinoshita et al., 1985; Neale et al., 1986). The enzyme has been found to transfer the pro-*R* hydrogen of NADH onto acetaldehyde (Glasfield and Brenner, 1989). Together with ADH IV of *Saccharomyces cerevisiae* and propanediol oxidoreductase of *E. coli*, ADH II of *Z. mobilis* forms a group of structurally related enzymes belonging to the iron-activated group III of dehydrogenases (Conway and Ingram, 1989; Reid and Fewson, 1994). These proteins share no homology with zinc-containing ADH enzymes (Cabriscol et al., 1994). The ADH II of *Z. mobilis*, identified as a major stress protein, was induced both by exposure to ethanol and by elevated temperature (Michel, 1993). The enzyme is expressed from tandem promoters which share partial sequence identity with the *E. coli* consensus sequence for heat shock proteins (An et al., 1991; Mackenzie et al., 1989). By a polymerase chain reaction (PCR)-mediated random mutagenesis, ADH II of *Z. mobilis* has been altered to produce more thermally stable variants. The same in vitro random mutagenesis technique enabled isolation of variant enzymes that had substrate specificities different from that of the wild-type enzyme, e.g., mutant enzymes active with butanol or with NADP (Rellos et al., 1997).

Ethanolic Fermentation

Zymomonas mobilis performs a highly productive ethanol fermentation and offers a number of advantages over the traditional yeast fermentation, i.e., higher sugar uptake and ethanol yield, lower biomass production, and oxygen independence (Rogers et al., 1982). Fermentations with *Z. mobilis* compared to those with yeast have a limited substrate range, which is restricted to glucose, fructose and sucrose (Swings and De Ley, 1977). Furthermore, the low salt tolerance of *Z. mobilis* poses problems for the fermentation of molasses, which usually have a high salt content (Montenecourt, 1985; Skotnicki et al., 1982). Nevertheless, it has been shown that glucose as well as unsterile hydrolyzed B-starch can be converted efficiently to ethanol in a continuous process employing a fluidized bed reactor (Bringer et al., 1984; Weuster-Botz, 1993a; Weuster-Botz et al., 1993b). In addition, processes for simultaneous saccharification and ethanol fermentation of starch or sugar cane have been developed (Kim and Rhee, 1993; Krishna et al., 1998). When glucose is fermented by nonaerated cultures, only insignificant amounts of by-products are formed. However, during growth of *Z. mobilis* in fructose-containing media the formation of acetoin, acetic acid, acetaldehyde, glycerol and dihydroxyacetone was more pronounced, and the cell yield was lower than when grown in glucose (Viikari, 1988; Johns et al., 1992; Horbach et al., 1994). Various genes encoding enzymes required for utilization of other carbon sources, e.g., starch, cellulose, raffinose, lactose, xylose or mannose, have been transferred into *Z. mobilis*; for details, the reader is referred to the chapter on metabolic engineering and to recent reviews (Doelle et al., 1993; Sprenger, 1993a; Gunasekaran and Raj, 1999). Another strategy for an efficient ethanol fermentation of such carbon sources is the transfer into *E. coli*, other enteric bacteria and also Gram-positive bacteria of the *Z. mobilis* genes *pdc* and *adhB* encoding pyruvate decarboxylase and alcohol dehydrogenase II, respectively (for a review see Ingram et al., 1998; Ingram et al., 1999).

During fermentation of sucrose by *Z. mobilis*, the formation of by-products such as levan and sorbitol decreases the ethanol yield (Viikari, 1988). Three types of transfructosylation occur in the presence of sucrose, resulting in the formation of free fructose, oligosaccharides and levan (Viikari, 1988). *Zymomonas mobilis* possesses three different sucrases: an intracellular sucrase (SacA or syn. InvA), an extracellular levansucrase (SacB or syn. LevU or syn. SucZE2), and an extracellular sucrase (SacC or syn. InvB or syn. SucZE3) (Kannan et al., 1997; Yanase et al., 1998; Song et al., 1999). Extracellular levansucrase and extracellular sucrase are involved in the sucrose metabolism of *Z. mobilis*, whereas the function of the intracellular sucrase is not understood (Kannan et al., 1997). The genes encoding for the extracellular levansucrase and sucrase are clustered on the chromosome (Gunasekaran et al., 1995), and the transcription of both genes was induced significantly when sucrose was added to the medium (Song et al., 1999). Extracellular levansucrase and sucrase do not carry an amino-terminal signal peptide usually found in proteins translocated across the cytoplasmic membrane (Gunasekaran et al., 1995; Kyono et al., 1995). In *E. coli* that carry the *Z. mobilis sucZE2* gene, a part of the expressed levansucrase was translocated across the inner membrane (Yanase et al., 1998), whereas the *Z. mobilis* gene for the extracellular sucrase expressed in *E. coli* did not lead to the enzyme's secretion (Kannan et al., 1995; Yanase et al., 1998). Levansucrase (EC 2.4.1.10) hydrolyses β-fructosides but not α-glucosides and catalyzes

levan formation from sucrose as well as from raffinose (Sangiliyandi et al., 1999). The optimum temperature for the polymerase activity (30°C) was lower than that for the hydrolase activity (50°C; Yanase et al., 1992; Sangiliyandi et al., 1999).

Sugar and Ethanol Tolerance

GLUCOSE-FRUCTOSE OXIDOREDUCTASE (GFOR) AND THE FORMATION OF SORBITOL The formation of sorbitol and gluconic acid from sucrose (or mixtures of glucose and fructose) by *Z. mobilis* has attracted much interest in the past, as yields of up to 90% for gluconic acid and up to 92% of sorbitol were reported for mixtures of glucose and fructose of up to 750 g/liter (Rehr et al., 1991; Silveira et al., 1999). A novel enzyme, glucose-fructose oxidoreductase (GFOR), with tightly bound NADP(H) as cofactor, has been detected (Zachariou and Scopes, 1986). The 3D crystal structure of the homotetramer GFOR enzyme has been elucidated recently (Kingston et al., 1996). So far, this enzyme is unique to *Zymomonas mobilis*, where it occurs in both subspecies, *mobilis* and *anaerobia* (Sprenger and Swings, 2000). The physiological function of this periplasmic enzyme (Loos et al., 1991; Aldrich et al., 1992) apparently lies in the formation of the compatible solute sorbitol. Sorbitol is accumulated in the cell (up to 1 M; Loos et al., 1994) and helps to counteract the detrimental effects of high sugar concentrations (Loos et al., 1994). A GFOR-deficient mutant, unable to form sorbitol (ACM3963; Kirk and Doelle, 1993), failed to grow in 1 M sucrose solutions, but after introduction of the wild-type *gfo* gene, it regained sorbitol forming ability and subsequently grew in high sucrose media (Wiegert et al., 1996). The periplasmic location of GFOR takes advantage of the concomitant presence of both substrates, glucose and fructose, at saturating concentrations (Sprenger, 1996), as the glucose facilitator prefers glucose over fructose (Weißer et al., 1995). The GFOR is formed as a preprotein with an unusually long N-terminal extension that serves as a signal sequence for the protein's export to the periplasm (Wiegert et al., 1996; Halbig et al., 1999). This signal sequence contains a so-called twin-arginine motif, which is a hallmark of a new class of Sec-independent protein export, the TAT or twin-arginine translocation in bacteria (Berks, 1996; Sargent et al., 1998; Berks et al., 2000). The TAT pathway transports folded proteins together with their redox cofactors to the bacterial periplasm.

HOPANOIDS IN *Z. MOBILIS*: MEDIATORS OF ETHANOL TOLERANCE AND FIRST INDICATORS OF THE EXISTENCE OF A NOVEL BIOSYNTHETIC PATHWAY FOR THE ISOPRENIC C_5 UNIT *Zymomonas mobilis* is capable of tolerating ethanol concentrations up to 13% (w/v). This ethanol tolerance is remarkably high and comparable to that of *Saccharomyces cerevisiae* (Rogers et al., 1982). The *Z. mobilis* cytoplasmic membrane contains a number of different hopanoids (Fig. 2): these membrane-stabilizing, pentacyclic

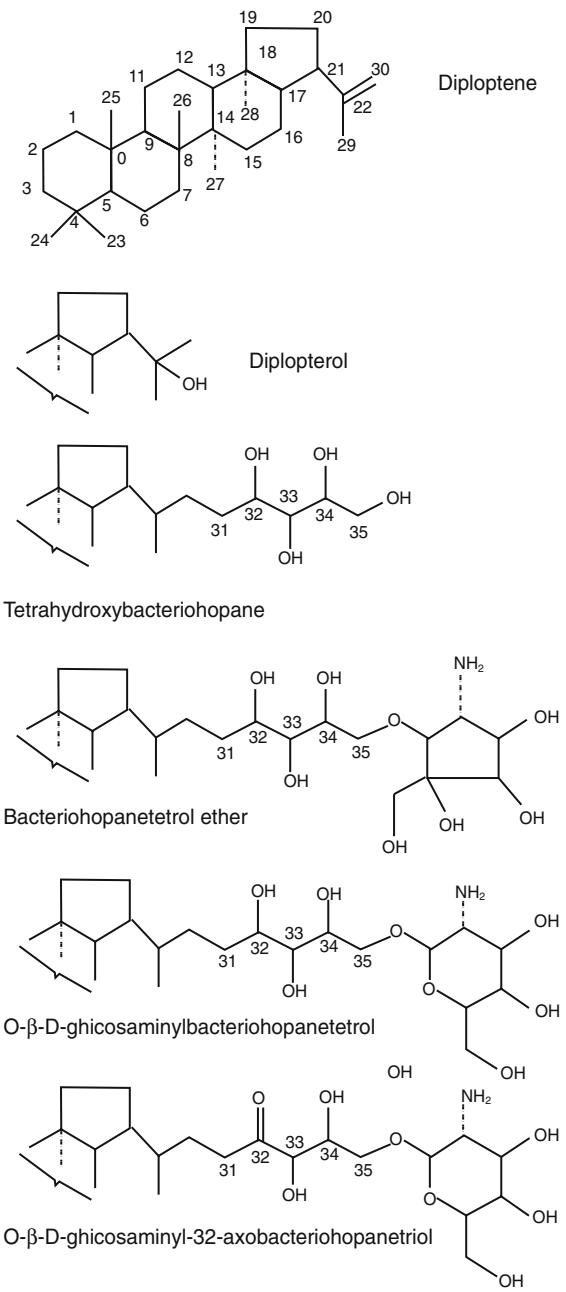

Fig. 2. Hopanoids from *Zymomonas mobilis*.

triterpenoids are present at concentrations of 30 mg/g cell dry weight (i.e., they contribute 40–50% to the organism's total lipid content; Hermans et al., 1991; Sahm et al., 1993; Moreau et al., 1997; Kannenberg and Poralla, 1999). Hopanoids play an important role in the ethanol tolerance of *Z. mobilis* by reinforcing the cytoplasmic membrane against the fluidizing effects of high concentrations of ethanol (Horbach et al., 1991; Sahm et al., 1993). Exposure to high levels of ethanol leads to an induction of several stress proteins (An et al., 1991) and their genes *adhB*, *dnaK*, and *groESL* (encoding for alcohol dehydrogenase II and the heat shock proteins DnaK, GroES and GroEL) have been cloned and characterized (Conway et al., 1987c; Michel, 1993; Barbosa et al., 1994). In continuous cultures at high ethanol concentrations and low dilution rates, sustained oscillations of biomass occur due to fluctuations of cell viability (Ghommidh et al., 1989; Vaija et al., 1993; Daugulis et al., 1997).

In vitro studies with cell-free extracts of *Z. mobilis* revealed that biosynthesis of the triterpenic moiety of the hopanoids proceeded from isopentenyl diphosphate via the intermediates farnesyl diphosphate and squalene (Shigeri et al., 1991); two enzymes involved in this part of isoprenoid biosynthesis in *Z. mobilis*, squalene synthase and squalene cyclase, were studied in detail and the corresponding genes were cloned and characterized (Reipen et al., 1995; Koukkou et al., 1996; Perzl et al., 1998).

However, the results of feeding studies with ^{13}C-labeled glucose and NMR spectroscopic analysis of the resulting labeling pattern of the triterpenoid moiety of the hopanoids clearly contradicted the classical mevalonate pathway for C_5 isoprenoid building-block isopentenyl diphosphate (IPP) biosynthesis (Flesch and Rohmer, 1989; Rohmer et al., 1989; Rohmer et al., 1993; Rohmer et al., 1996; Horbach et al., 1993; Sahm et al., 1993). Only recently was it evident that the unique characteristics of *Z. mobilis* could be of substantial help for the discovery of a novel, mevalonate-independent pathway for IPP biosynthesis, with glyceraldehyde-3-phosphate and pyruvate as precursors of isoprenic units (Rohmer et al., 1996). In this pathway, 1-deoxyxylulose 5-phosphate is formed as the first intermediate (Broers, 1994; Sprenger et al., 1997; Lois et al., 1998). Four further reactions of the pathway have been identified (Fig. 3; Takahashi et al., 1998; Rohdich et al., 1999; Lüttgen et al., 2000; Herz et al., 2000). The novel pathway is present in bacteria, green algae and the chloroplasts of higher plants (methylerythritol-4-phosphate pathway; Rohmer, 1999).

Special Features: Aerobic Metabolism, Pyruvate Dehydrogenase Multienzyme Complex

Aerobic Metabolism

Although strictly fermentative, *Z. mobilis* is an aerotolerant bacterium owing to the presence of a respiratory chain and the antioxidant enzymes catalase and superoxide dismutase (Belaich and Senez, 1965; Bringer et al., 1984; Pankova et al., 1985). However, at glucose concentrations >100 mM, oxygen inhibits growth due to an accumulation of acetaldehyde (Bringer et al., 1984; Pankova et al., 1985; Ishikawa et al., 1990). In the presence of oxygen, less NADH is available for the reduction of acetaldehyde to ethanol and therefore acetaldehyde accumulates to growth-inhibiting concentrations (Bringer et al., 1984; Pankova et al., 1985). Recently, continuous chemostat cultures of *Z. mobilis* growing aerobically with low glucose concentrations (<100 mM) exhibited a 2–2.5-fold increase in molar growth yields (Toh and Doelle, 1997; Zikmanis et al., 1997; Zikmanis et al., 1999). It was suggested that an inhibition of the proton pumping membrane ATPase by acetaldehyde in the presence of oxygen is the main cause for this increase. In this situation, membrane energization would be brought about by the respiratory activity (Toh and Doelle, 1997; Zikmanis et al., 1999).

The composition and function of the *Z. mobilis* electron transport chain is poorly understood, although the presence of a constitutive respiratory chain, containing cytochromes b, c and d, has been reported long ago (Belaich and Senez, 1965). Further studies have shown a structure with several membrane oxidoreductases and with branched electron pathways to oxygen. A hypothetical scheme is shown in Fig. 4. Cytoplasmic membrane vesicles oxidize NADH with a high specific rate, reaching 0.2-0.3 U · mg protein^{-1} (Bringer et al., 1984; Strohdeicher et al., 1990; Kalnenieks et al., 1995). Apart from the NADH oxidase, *Zymomonas* contains a membrane-linked NADPH oxidase with a similar activity (Bringer et al., 1984; Pankova et al., 1985). Two minor membrane oxidase activities have been reported: glucose oxidase, corresponding to a pyrroloquinoline quinone (PQQ)-containing glucose: ubiquinone oxidoreductase (Strohdeicher et al., 1988; Strohdeicher et al., 1990), and D-lactate oxidase (Kalnenieks et al., 1998). It has been demonstrated that electrons from both NADH and glucose are transported to oxygen via the quinone pool (Strohdeicher et al., 1990). Coenzyme Q_{10} (ubiquinone) has been found to

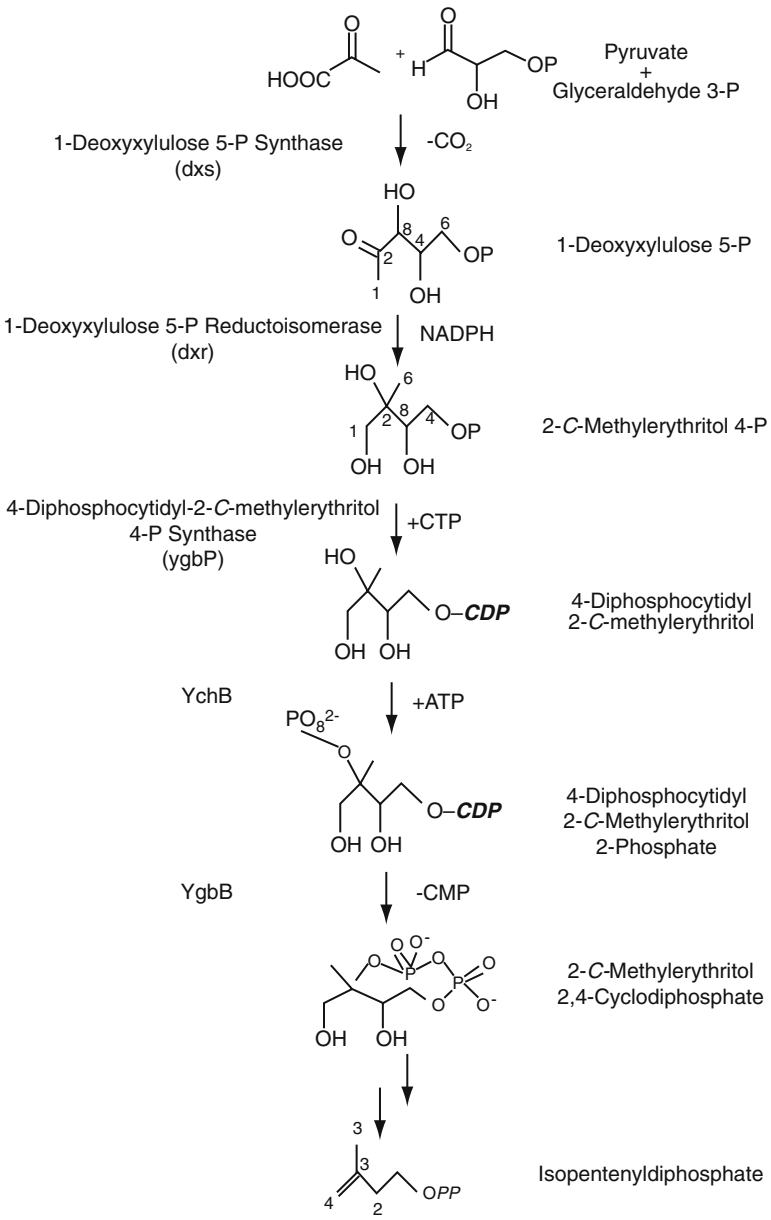

Fig. 3. The 2-methylerythritol 4-phosphate (MEP) pathway to isopentenyl diphosphate.

be the only quinone species in *Zymomonas* membranes (Strohdeicher et al., 1990).

Although aerobically growing cultures apparently do not produce extra ATP for biomass synthesis, oxidative phosphorylation can be measured in nongrowing cells and membrane vesicles (Kalnenieks et al., 1993). The energy coupling sites of the electron transport chain vary depending on the aeration of the culture. In the membranes of anaerobically cultivated cells, ATP synthesis is linked mainly to the site I (i.e., NADH dehydogenase I; Kalnenieks et al., 1995). On the other hand, for aerobically cultivated cells the buildup of membrane potential (Kim et al., 1995) and oxidative phosphorylation (Kalnenieks et al., 1996) takes place in the respiratory chain region downstream from site I. There are two kinetically distinguishable NADH oxidase activities in *Z. mobilis* membranes: one with a low K_m for NADH (around 7 μM) and another one with a higher K_m (around 60 mM; Kim et al., 1995; Kalnenieks et al., 1996). This points to the presence of two different membrane-bound NADH dehydrogenases, as was also found for other bacterial respiratory chains (e.g., for *E. coli*; Calhoun et al., 1993). It has been suggested that the NADH oxidase with the low K_m corresponds to the energy-coupling NADH: ubiquinone oxidoreductase of type I, and that the oxidase with the high Km represents the energy non-generating, type II NADH: ubiquinone oxidoreductase (Kalnenieks et al.,

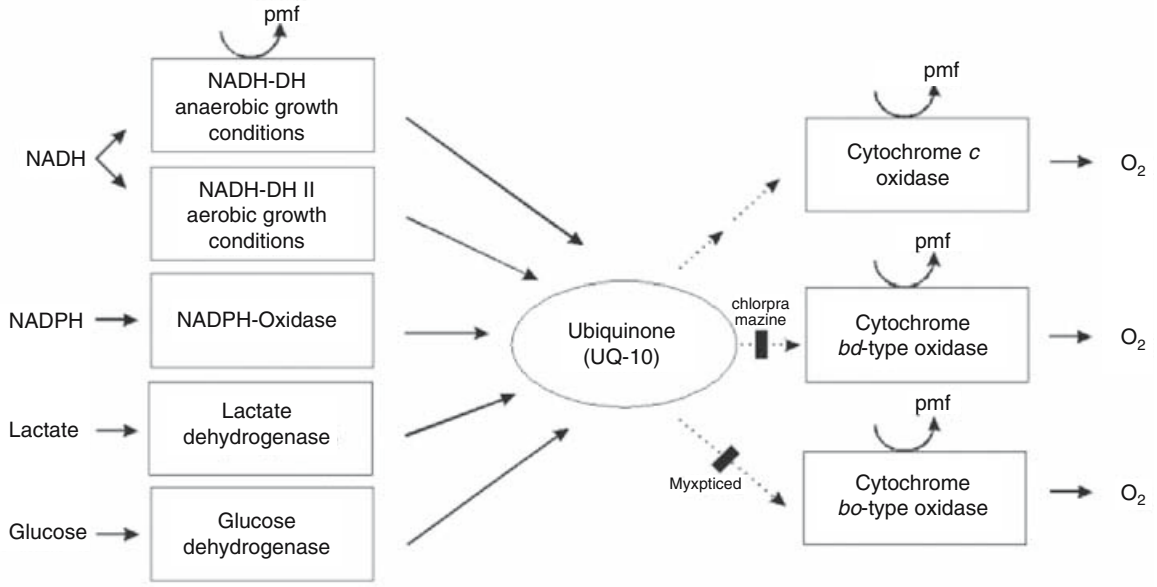

Fig. 4. Electron transport chain of *Zymomonas mobilis*; pmf, proton motive force.

1996). Spectral features of several types of cytochromes (b, c and d) have been observed in membrane preparations (Belaich and Senez, 1965; Pankova et al., 1985; Kalnenieks et al., 1998). In spite of high rates of respiration, the cytochrome content in *Z. mobilis* is comparatively low, being approx. 2–3 times lower than that in *E. coli* (Kalnenieks et al., 1998). From spectroscopic data it has been postulated that electrons, coming from the quinone pool, are distributed between three branches, terminated by 1) cytochrome *bd*, 2) a cytochrome *bo*-like component and 3) a cytochrome *a*-type terminal oxidase (Kalnenieks et al., 1998). At present it can be outlined that the respiratory chain of *Z. mobilis* is composed of several membrane oxidoreductases and of branched electron pathways to oxygen (Fig. 4). The presence of a respiratory chain in *Z. mobilis* supports the suggestion made by Swings and De Ley (1977) that this organism may have originated from aerobic ancestors.

Pyruvate Dehydrogenase Multienzyme Complex

In *Z. mobilis*, the formation of acetyl-CoA from pyruvate is catalyzed by a pyruvate dehydrogenase (PDH) complex (Bringer-Meyer and Sahm, 1993). The occurrence of this enzyme complex in *Z. mobilis* is surprising because anaerobically growing bacteria usually employ pyruvate formate lyase (EC 2.3.1.54) or pyruvate ferredoxin oxidoreductase (EC 1.2.7.1) for acetyl-CoA synthesis (Sawers and Watson, 1998; Chabriere et al., 1999). The formation of acetyl-CoA from pyruvate represents a main junction of catabolic and anabolic pathways. Up to 98% of the pyruvate is converted to the fermentation end products ethanol and CO_2, whereas only a small part of the pyruvate is oxidatively decarboxylated by the reaction of the PDH complex to acetyl coenzyme A, CO_2 and NADH (Sahm et al., 1992). Inasmuch as *Z. mobilis* lacks the 2-oxoglutarate dehydrogenase complex and other enzymes of the tricarboxylic acid cycle, the PDH complex plays an exclusively anabolic role in this organism (Bringer-Meyer and Sahm, 1993). Bacterial pyruvate dehydrogenase complexes are composed of multiple copies of three different enzymes: pyruvate dehydrogenase (E1p; EC 1.2.4.1), dihydrolipoamide transacetylase (E2p; EC 2.3.1.12) and lipoamide dehydrogenase (E3; EC 1.8.1.4; Reed and Hackert, 1990). In *Z. mobilis*, the PDH complex consists of four polypeptides, similar to the situation found in Gram-positive bacteria, with an E1α subunit of 38.6 kDa, an E1β subunit of 49.8 kDa, an E2 subunit of 48 kDa and an E3 subunit of 50 kDa. The E2 core of the complex is arranged to form a pentagonal dodecahedron, again resembling the quaternary structures of PDH complexes of Gram-positive bacteria and eukaryotes (Neveling et al., 1998a; Neveling et al., 1998b). The structural genes (*pdhA*αβ, *pdhB*, *lpd*) encoding the PDH complex of *Z. mobilis* are located in two distinct gene clusters, *pdhA*αβ and *pdhB*-orf2-*lpd* (Neveling et al., 1998b). Like the dihydrolipoamide acetyltransferases of *S. cerevisiae* and numerous other organisms, the product of the *pdhB* gene of *Z. mobilis* contains a single

lipoyl domain. In addition, the E1β subunit was found to contain an amino-terminal lipoyl domain, a property that is exceptional among PDH complexes (Neveling et al., 1999).

Genetics and Metabolic Engineering

Plasmids, Gene Transfer, Mutagenesis and Mutant Isolation

Most strains of *Z. mobilis*, including the type strains (ATCC10988 = ZM1; ATCC29191 = ZM6; ATCC31821 = CP4), contain several natural plasmids (in a size range from 1.6 kb to >40 kb; Rogers et al., 1982; Tonomura et al., 1982; Scordaki and Drainas, 1987). Plasmid profiling has been used to clarify uncertain strain status (Yablonsky et al., 1988; Degli-Innocenti et al., 1990). For the most part, the *Z. mobilis* plasmids remain cryptic, although some functions as antibiotic or heavy metal resistances have been assigned to them (Tonomura et al., 1982; Walia et al., 1984; Scordaki and Drainas, 1987; Ogale and Deobagkar, 1988). For genetic engineering, various broad host range plasmids (from several incompatibility groups), shuttle and/or expression vectors have been constructed (Byun et al., 1986; Brestic-Goachet et al., 1987; Conway et al., 1987a; Cho et al., 1989; Reynen et al., 1990)—some of them with inducible promoter features (e.g., *lacI*q/P*tac* vectors; Arfman et al., 1992; Reipen et al., 1995). Using plasmid R68.45, chromosome transfer between donor and recipient strains of *Z. mobilis* was reported (Skotnicki et al., 1982; Stokes et al., 1983). No bacteriophage has been reported for *Z. mobilis* yet. This, however, need not mean that no phages exist for *Zymomonas* but rather indicates the lack of in-depth investigations. A physical map of ZM4 strain has been constructed recently which allowed to estimate the genome size to about 2,085 kb (Kang and Kang, 1998) and the full genomic sequence of this strain is currently underway.

Mutant isolation and enrichment still poses remarkable problems (Sprenger et al., 1993b; Pencreac'h et al., 1996; Pappas et al., 1997) and up to now, no working method for gene disruption or even gene replacement has been reported, although an integrative shuttle vector has been reported recently (Delgado et al., 1995). Auxotrophic mutants have been won spontaneously, or after induction by UV light, by chemical mutagens (alkylating agents such as ethyl methane sulfonate [EMS] or nitrosoguanidine; Typas and Galani, 1992), or following transposon mutagenesis (Pappas et al., 1997). Mutants range from auxotrophs for amino acids or vitamins (Goodman et al., 1982; Eddy et al., 1988; Karunakaran and Gunasekaran, 1989; Pencreac'h et al., 1996; Estevez et al., 1997) to mutants with altered metabolic markers (fructose utilization; Bringer-Meyer et al., 1985; Suntinanalert et al., 1986; glucokinase; DiMarco and Romano, 1985; Aitabdelkader et al., 1996; alcohol dehydrogenases; Wecker and Zall; 1987; mannitol degradation; Buchholz et al., 1988; sorbitol formation; Kirk and Doelle, 1993). Furthermore, various strains with tolerance versus elevated temperatures (e.g., 42°C), molasses, salts or ethanol have been isolated as well as flocculent strains (see references in Ingram et al., 1989). Mutants resistant to allyl alcohol were found to be deficient in both alcohol dehydrogenases (Wecker and Zall, 1987; O'Mullan et al., 1995). These strong acetaldehyde-forming mutant strains grow and ferment poorly and are dependent on the presence of oxygen (Wecker and Zall, 1987).

Methods for introduction of foreign genes mainly rely on conjugation from *E. coli* donor cells (see references in Ingram et al., 1989; Sprenger, 1993a) or on electroporation (Okamoto and Nakamura, 1992; Lam et al., 1993; Liang et al., 1998), the latter at still low frequencies. Successful chemical transformation using spheroplasts (Yanase et al., 1986) or a chemical ($CaCl_2$) method with whole cells (Browne et al., 1984; Su and Goodman, 1987), yielding up to $1.8 \cdot 10^5$ transformants/μg of plasmid DNA, have been reported, but reproduction of these results has been difficult in other laboratories. *Zymomonas mobilis* is inherently resistant to nalidixic acid, which kills *E. coli* cells. In conjugations from *E. coli* donors to *Z. mobilis* recipients, nalidixic acid is therefore employed to discriminate against the donor cells (Uhlenbusch et al., 1991; Arfman et al., 1992). The other inherent antibiotic resistances limit the marker genes mainly to chloramphenicol and tetracycline. However, spontaneous resisters against both antibiotics also are found. The strain ZM6 (ATCC29191) and its derivatives are more sensitive to ampicillin than are the strains ZM1 (ATCC10988) and CP4; therefore replicons with Amp^R-genes may be utilized in ZM6 and its derivatives.

Metabolic Engineering

Nearly as soon as the paramount large-scale, ethanol-producing capabilities of *Z. mobilis* became widely known (Rogers et al., 1982), attempts to broaden the limited substrate and product range of *Z. mobilis* were started. Genetic and metabolic engineering of *Z. mobilis* have attracted many groups to study the unusual efficiency of sugar metabolism and ethanol produc-

tion and to improve the limited substrate and product range of the organism. Several reviews during the last years have dealt with these issues in depth and are recommended for further reading (Ingram et al., 1989; Conway, 1992; Johns et al., 1992; Sprenger, 1993a; Doelle et al., 1993; Sprenger et al., 1993b; Sprenger, 1996). Successful examples are summarized in Table 3. The main goals were to enlarge the substrate spectrum to the utilization of constituents of abundantly available and renewable carbon sources from wood and straw (e.g., hemicelluloses with D-xylose, L-arabinose and D-mannose as main monosaccharide constituents), whey (lactose), starch (maltose) and cellulose (cellobiose).

Many groups have worked especially on lactose as a carbon source, however, only with limited success. Main problems were that this disaccharide needs to be taken up by an energy-consuming step (proton-symporting lactose permease LacY), genes for galactose catabolism have to be co-introduced, and finally inhibitory by-products (such as galactonic acid or galactitol) may limit the growth and ethanol formation from lactose (literature reviewed in Sprenger, 1993a). More recently, catabolism of D-xylose, L-arabinose and D-mannose has been achieved with good ethanol yields (Feldmann et al., 1992; Zhang et al., 1995; Deanda et al., 1996; Weisser et al., 1996; de Graaf et al., 1999). Sugar uptake of D-xylose and D-mannose is gratuitous as the GLF transporter takes both sugars at good rates (Weisser et al., 1996; Parker et al., 1997). Introduction of a single phosphomannose-isomerase gene (*pmi*) from *E. coli* resulted in a mannose-positive phenotype of *Z. mobilis*, as another gratuitous reaction (involving fructokinase) took care of the mannose phosphorylation step (Weisser et al., 1996). Successful transfer of the plasmid-borne *pmi* gene could be monitored by growth of the *Z. mobilis* exconjugants on mannose as sole C-source; this can now be used as an alternative to the antibiotic-resistance markers, which are limited (Weisser et al., 1996). For D-xylose and L-arabinose catabolism, the peripheral enzymes have been introduced from various heterologous hosts into recombinant strains of *Z. mobilis* (Liu et al., 1988; Feldmann et al., 1992; Zhang et al., 1995; Deanda et al., 1996). After this was achieved it became evident that additional activities of the central pentose-phosphate metabolism are required (Feldmann et al., 1992; Zhang et al., 1995). Whereas transketolase is present at very low activity, transaldolase appears to be absent from *Z. mobilis* (Feldmann et al., 1992; Zhang et al., 1995). Introduction and expression of transketolase and transaldolase from *E. coli* were necessary to isolate xylose-positive clones of *Z. mobilis* (Zhang et al., 1995; de Graaf et al., 1999). In the same line, L-arabinose-fermenting clones have been isolated (Deanda et al., 1996). The path of ^{13}C-labeled xylose in recombinant *Z. mobilis* cells has recently been followed by NMR techniques (de Graaf et al., 1999; Kim et al., 2000).

To enlarge the product spectrum of *Z. mobilis*, an L-alanine dehydrogenase gene from *Bacillus sphaericus* has been introduced and was expressed from the strong *pdc* promoter. This led to a portion of pyruvate being diverted from the normal ethanologenic route to a fermentative L-alanine route. To reduce the overwhelming PDC activity, a thiamine-auxotroph strain was used which was then starved for thiamine in the

Table 3. Examples of successful metabolic pathway engineering in *Zymomonas mobilis*.

Heterologous gene(s)	Donor	Enzymes expressed	Pathway	Refs.
lacZY, galEKT	*E. coli*	lactose permease, β-galactosidase, galaktokinase, UDP-Gal-Epimerase, UDP-Glc-Transferase	lactose degradation galactose degradation	Buchholz et al. 1989
araBAD, tktA, talB	*E. coli*	L-arabinose isomerase, L-ribulokinase, L-ribulose 5-phosphate 4-epimerase, transketolase, transaldolase	L-arabinose degradation	Deanda et al. 1996
xylAB, tktA, talB	*E. coli*	xylose isomerase, xylulokinase transketolase, transaldolase	D-xylose degradation	Zhang et al. 1995
xylAB, tktA, talB	*Klebsiella pneumoniae*	xylose isomerase, xylulokinase	D-xylose degradation	Feldmann et al. 1992 de Graaf et al. 1999
	E. coli	transketolase, transaldolase		
pmi (manA)	*E. coli*	phosphomannose isomerase	D-mannose degradation	Weisser et al. 1996
alaD	*B. sphaericus*	L-alanine dehydrogenase	L-alanine formation	Uhlenbusch et al. 1991
?	*B. brevis*	α-glucosidase	ethanol formation from maltose (no growth)	Strzelecki et al. 1993
crtBEIY	*Erwinia uredovora*	carotene biosynthetic enzymes	β-carotene production	Misawa et al. 1991

medium to reduce the activity of thiamine-diphosphate-requiring PDC. This resulted in a major rerouting of pyruvate to L-alanine (Table 3; Uhlenbusch et al., 1991). The strong isoprenoid-forming pathway of *Z. mobilis* was successfully altered into a carotenoid pathway by introduction of *crt* genes from *Erwinia* species (Table 3; Misawa et al., 1991).

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Prokaryotes (2006) 5:222–231
DOI: 10.1007/0-387-30745-1_11

CHAPTER 3.1.10

The Manganese-Oxidizing Bacteria

KENNETH H. NEALSON

The "manganese-oxidizing group" is a phylogenetically diverse assemblage, which is characterized by the ability to catalyze the oxidation of divalent, soluble Mn(II) to insoluble manganese oxides of the general formula MnO_x (where X is some number between 1 and 2). This results in the accumulation of conspicuous and easily detectable extracellular deposits of insoluble brown or black manganese oxides. Many different organisms have the ability to catalyze Mn oxidation, including a diverse array of bacteria, fungi, algae, and even eukaryotes (Ghiorse, 1984b). Among the prokaryotes, the ability to oxidize Mn is also quite widespread (Ehrlich, 1981; Ghiorse, 1984b, 1988; Marshall, 1979; Nealson, 1983); included are members of many phylogenetic and physiological groups: e.g., cyanobacteria, a diversity of heterotrophic rods and cocci, the sheathed (*Leptothrix*-like) and budding (*Hyphomicrobium*-like) bacteria, some purported autotrophic strains related to *Pseudomonas* species and the still-controversial *Metallogenium* group. The anaerobic lactobacilli, which utilize the Mn oxidation reaction as a protection against oxygen toxicity (Archibald and Fridovich, 1981, 1982) are not included, as they do not precipitate extracellular Mn oxides, but rather accumulate millimolar levels of protein-associated Mn in the cytoplasm. This chapter focuses on the process of Mn oxidation and also considers why so many bacteria have been identified as Mn oxidizers. It also offers suggestions that may help to clarify this complex area.

Since there is no evidence of any advantage that Mn oxidation confers on bacteria, one might well ask the reason for the widespread distribution of this trait. The answer may lie in the Mn oxidation reaction itself. Under the conditions characteristic of most of the environments in which microbes are abundant, Mn is a very active element. Some critical features of Mn chemistry are summarized in Fig. 1 and are also discussed in more detail elsewhere (Ghiorse, 1988; Mulder and Dienema, 1981; Nealson et al., 1988, 1989; Pankow and Morgan, 1981).

The oxidation of Mn(II) to Mn(IV) is thermodynamically favored under aerobic conditions, with a negative free energy of approximately 16 kcal/mol (Stumm and Morgan, 1981; Ehrlich, 1981; Nealson et al., 1988). However, the large activation energy of Mn(II) oxidation renders Mn(II) very stable in most aquatic environments (Stumm and Morgan, 1981). The activation energy barrier can be overcome by raising the pH (see Fig. 1) or by the addition of Mn-binding components, including Mn oxides themselves, which are excellent chelators of Mn(II) (Stumm and Morgan, 1981). The catalysis of Mn(II) oxidation by Mn oxides (autooxidation) makes it difficult to distinguish between chemically and microbially catalyzed Mn oxidation, especially in natural environments where organic chelators and Mn oxide particles may be abundant.

Mn is, therefore, an element whose distribution and chemical speciation is kinetically controlled, thus allowing for the intervention of microbes and microbial products into the system. Some of the ways in which microbes might oxidize Mn(II) are shown in Table 1. If the pH or Eh of the environment is raised, if oxidants are produced by cells, or if binding of Mn(II) occurs so as to lower the activation energy, Mn(II) oxidation can rapidly proceed. With this in mind, it is not surprising that so many different bacteria have been identified as Mn(II) oxidizers, since the mechanisms of Mn oxidation are quite diverse (Ghiorse, 1988; Nealson et al., 1988, 1989). A true understanding of the "Mn-oxidizing bacteria" will likely await the time when it is possible to identify those reactions that confer some advantage to the bacteria and to disregard those that occur simply because of the dynamic chemistry of Mn(II). With regard to this, some of the recent studies of the mechanism of Mn(II) oxidation by cells, which include the isolation of Mn(II)-binding proteins (both intra- and extracellular) and polysaccharides, are particularly encouraging (Ghiorse, 1988; Nealson et al., 1989).

Habitats

Manganese-oxidizing bacteria are ubiquitous; they can be isolated from nearly any habitat. It is the experience of many workers that habitats containing high levels of Mn and those in which Mn cycling is an active process tend to have high numbers of Mn-oxidizing bacteria. However, such correlations do not always hold (Gottfreund and Schweisfurth, 1983; Schuett and Ottow, 1977). Some examples of habitats from which abundant manganese oxidizers have been identified by morphology alone and/or isolation include: deep-sea manganese nodules (Ehrlich et al., 1972; Schuett and Ottow, 1977); hydrothermal vent plumes (Cowen et al., 1986); oxic/anoxic interfaces in fjords (Tebo et al., 1984); desert varnish (Hungate et al., 1987); manganese deposits in water pipes (Tyler and Marshall, 1967a, 1967b); manganese-rich surface films of shallow lakes (Ghiorse, 1984a), and freshwater lake sediments and ferromanganese deposits (Chapnick et al., 1982; Gregory and Staley, 1982; Kuznetsov, 1975; Maki et al., 1987).

A feature that is common to many of these habitats is a ready and continuous supply of

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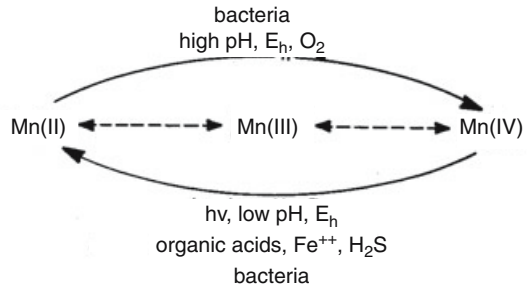

$$Mn^{2+} + 1/2\ O_2 + H_2O \longrightarrow MnO_2(s) + 2H^+ \quad (1)$$

$$Mn^{2+} + 1/4\ O_2 + 3/2\ H_2O \longrightarrow MnOOH(s) + 2H^+ \quad (2)$$

$$3Mn^{2+} + 1/2\ O_2 + 3\ H_2O \longrightarrow Mn_3O_4(s) + 6H^+ \quad (3)$$

CATIONIC FORMS	Mn^{2+}	Mn^{3+}	Mn^{4+}
EXAMPLES OF COMPOUNDS FORMED	$MnCl_2$, $MnSO_4$, etc.	Mn_2O_3, MnOOH, etc.	MnO_2
GENERAL PROPERTIES	A. Forms soluble salts	A. Form insoluble oxides and oxyhydroxides, e.g., Manganates (MnO_X, 1 X 2)	
	B. Forms insoluble minerals 1. $MnCO_3$ 2. $MnPO_4$	B. Manganese oxides adsorb trace metals and radionuclides, e.g., Cd, Co, Cu, Ni, Mn	
	C. Biologically available 1. Required trace element 2. Specific transport systems 3. Toxic at high concentrations	C. Unavailable as trace nutrients 1. Nontoxic 2. Potential electron acceptor	

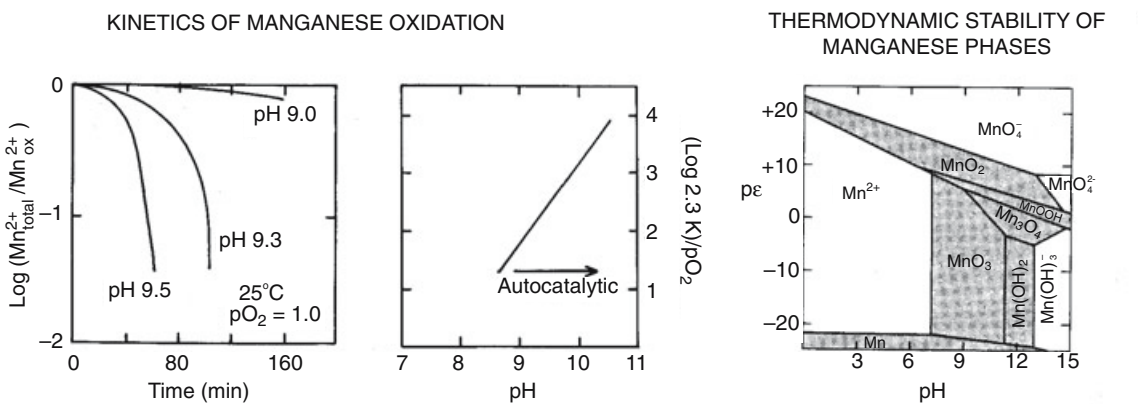

Fig. 1. Manganese chemistry and biochemistry. The top of the figure presents the major features of manganese cycling, including some of the well-known reactions leading to Mn(II) oxidation, the forms of Mn found in nature, and the general properties of these forms. The lower part of the figure shows the effect of pH on reaction kinetics and a thermodynamic phase-stability diagram for manganese (based on Stumm and Morgan, 1981).

Mn(II), which has been observed to typically occur in either of two ways (Fig. 2). The first (Fig. 2, part I) is in conjunction with oxic/anoxic interfaces. Under anaerobic conditions, Mn (III) and Mn(IV) can be rapidly reduced by a variety of indirect and direct mechanisms, such as excretion of organic or inorganic reductants, or by bacterial respiration (Nealson et al., 1989). This results in a solubilization of the Mn, which can then diffuse into the oxic zone, making an ideal natural enrichment condition for Mn(II)-oxidizing bacteria. Such interfaces are common in virtually all Mn-containing sediments and in stratified lakes and fjords.

Table 1. Possible mechanisms of Mn(II) oxidation by bacteria.

1. Indirect
   - A. Free radical or oxidant production
     - i. Hydrogen peroxide
     - ii. Superoxide
     - iii. Hydroxyl radical
   - B. Modification of redox environments
     - i. Oxygen production
     - ii. pH increase
       - a. CO_2 consumption
       - b. Acid consumption
       - c. Ammonia release
   - C. Production of Mn(II) chelators
     - i. Organic chelators
     - ii. Mn oxides
2. Direct
   - A. Mn-binding components
     - i. Proteins
     - ii. Glycocalyxes
     - iii. Cell wall components
   - B. Mn-oxidizing enzymes (oxidases)

Mn(II) is also continuously supplied by inputs of anoxic (reduced) water into aerobic environments (Fig. 2, part II). This occurs in deep-sea hydrothermal plumes in which chemically reduced Mn(II) is introduced into the deep sea and in ground water or hot spring inputs to oxic lakes, where chemically or biologically reduced Mn(II) is continuously introduced into lake waters.

In stratified aquatic environments (Fig. 2), microbial manganese oxidation can be a process of major biogeochemical importance. The freshly produced Mn oxides are returned as solid precipitates to the sediments or to anaerobic waters where they may be used for the anaerobic oxidation of other agents, including Fe(II), H_2S, and organic matter (see Nealson et al., 1989). Manganese is well suited for such a role, as it does not readily form insoluble sulfides and thus

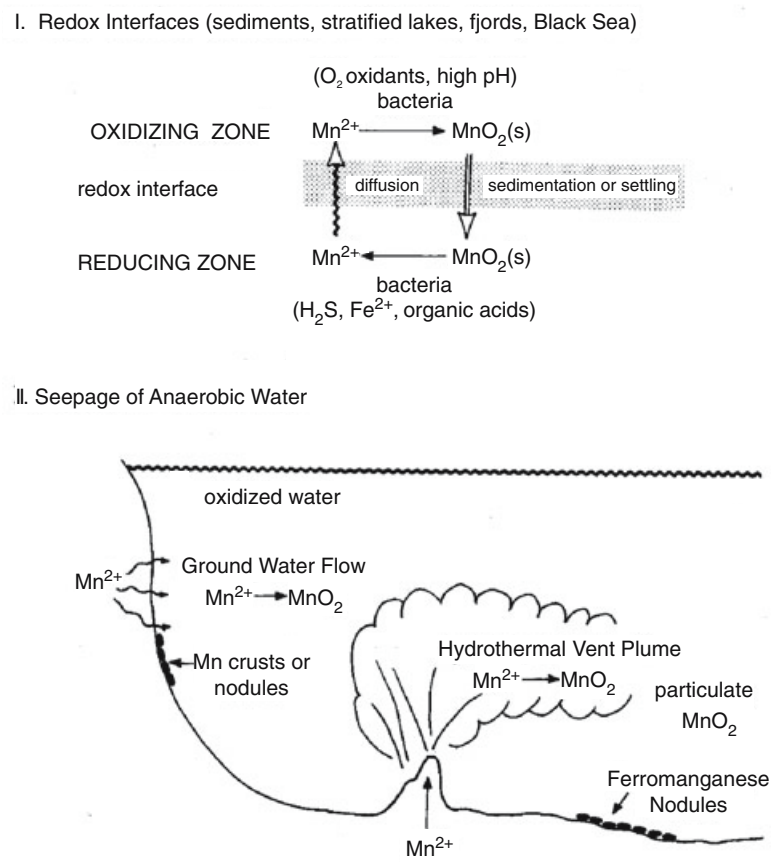

Fig. 2. Habitats of manganese-oxidizing bacteria: the two well-characterized general environments where one might expect to encounter natural enrichments of Mn-oxidizing bacteria.

remains in solution in marine anaerobic (sulfidic) zones (Stumm and Morgan, 1981).

While it has been known since the time of Beijerinck (1913) that pure cultures of bacteria catalyze Mn(II) oxidation, direct measurements of microbial Mn(II) oxidation in nature were, until a few years ago, very rare. In recent years, however, many research groups have utilized a variety of poisoned control experiments to implicate Mn(II)-oxidizing bacteria as the major catalysts of Mn(II) precipitation in many environments, including fjords, lakes, and sediments (see Ghiorse, 1988; Nealson et al., 1988). From these studies it can now be confidently stated that microbial Mn oxidation is a major biogeochemical process in freshwater, marine, estuarine, and soil habitats.

A detailed description of the sheathed bacteria (Mulder and Dienema, 1981) reveals that these organisms (*Leptothrix*-like) are found in abundance in the same environments as are other manganese oxidizers and that many of these bacteria are in fact characteristically encrusted with iron or manganese oxides. (See also The Sheathed Bacteria in the second edition)

Metallogenium

Perhaps the most controversial and enigmatic group of Mn oxidizers are those called *Metallogenium* (Zavarzin, 1981). These are Mn oxide-containing stellate structures of various morphologies that have been identified in environments throughout the world for many years (Emerson et al., 1989; Kuznetsov, 1975; Maki et al., 1987; Schweisfurth and Hehn, 1972; Zavarzin, 1968, 1981). The stellate morphotypes have been identified as organisms by some workers (Dubinina, 1970; Zavarzin, 1968, 1981) and disputed as such by others (Emerson et al., 1989; Maki et al., 1987; Schweisfurth, 1978; Schweisfurth and Hehn, 1972). The problem can best be demonstrated with the work of Maki et al. (1987), who identified abundant *Metallogenium* morphotypes in the deep, stratified waters of Lake Washington. These stellate precipitates contained no structural features of living cells and were not associated with the zone of rapid Mn precipitation in the lake. The conclusion reached was that they were not living particles, although it could not be proven that they did not have their origin as part of a living structure. There is thus little doubt that structures resembling *Metallogenium* exist, and they are abundant in many environments, especially in lake systems. However, there is little proof that *Metallogenium*-like structures are formed directly by, or associated with, any particular organism.

Emerson et al. (1989) reported that extracellular proteins and polysaccharides from a basidiomycetous fungus were capable of initiating the formation of *Metallogenium*-like structures in laboratory culture, and have suggested that similar extracellular catalyses occur in nature. While these studies do not prove that all *Metallogenium* structures are formed this way, they do provide a mechanism whereby such morphotypes can form without direct association with living cells. The *Metallogenium* question must be regarded as an open one, the answer to which might lead to new understanding of the relationship between Mn oxidizing organisms and the environmental chemistry of Mn.

Isolation and Enrichment of Mn-Oxidizing Bacteria

As discussed above, natural enrichments for Mn(II) oxidizers occur in environments in which manganese cycling is active, and in such habitats further enrichments may not be required. For example, Tyler and Marshall (1967a, 1967b) observed and isolated *Hyphomicrobium* species directly from ferromanganese deposits on hydroelectric pipes, and Ghiorse (1984a) observed and isolated *Leptothrix* species as the causative agent of manganese surface films in a shallow lake. In both these cases, large numbers of similar organisms were visible and closely associated with the manganese oxides; under such conditions it was easy to conclude that the organisms participated in the formation of the metal precipitates.

In other cases, while Mn(II)-oxidizing bacteria can be easily isolated, they are neither so abundant nor so obviously responsible for the observed precipitates. Furthermore, the isolation of Mn oxidizers indicates the "potential" for Mn oxidation rather than the "demonstration" of the direct involvement with precipitation of metals in nature. Since so many organisms are capable of Mn oxidation (and by so many different mechanisms), caution must be exercised in the interpretation of reports of high numbers of Mn oxidizers. Another important point is that the chemical methods used to identify Mn oxides (see below) are very sensitive, and it may well be that many organisms which are identified as Mn oxidizers in culture never accumulate such precipitates in nature.

Laboratory Enrichments

Reports of laboratory enrichments for Mn oxidizers are rare; perhaps because there is no known selective advantage for Mn oxidation, enrichments are not often attempted. Nealson (1978) reported long-term enrichments in which $MnCl_2$ was added to a variety of different marine sediment samples. After periods of

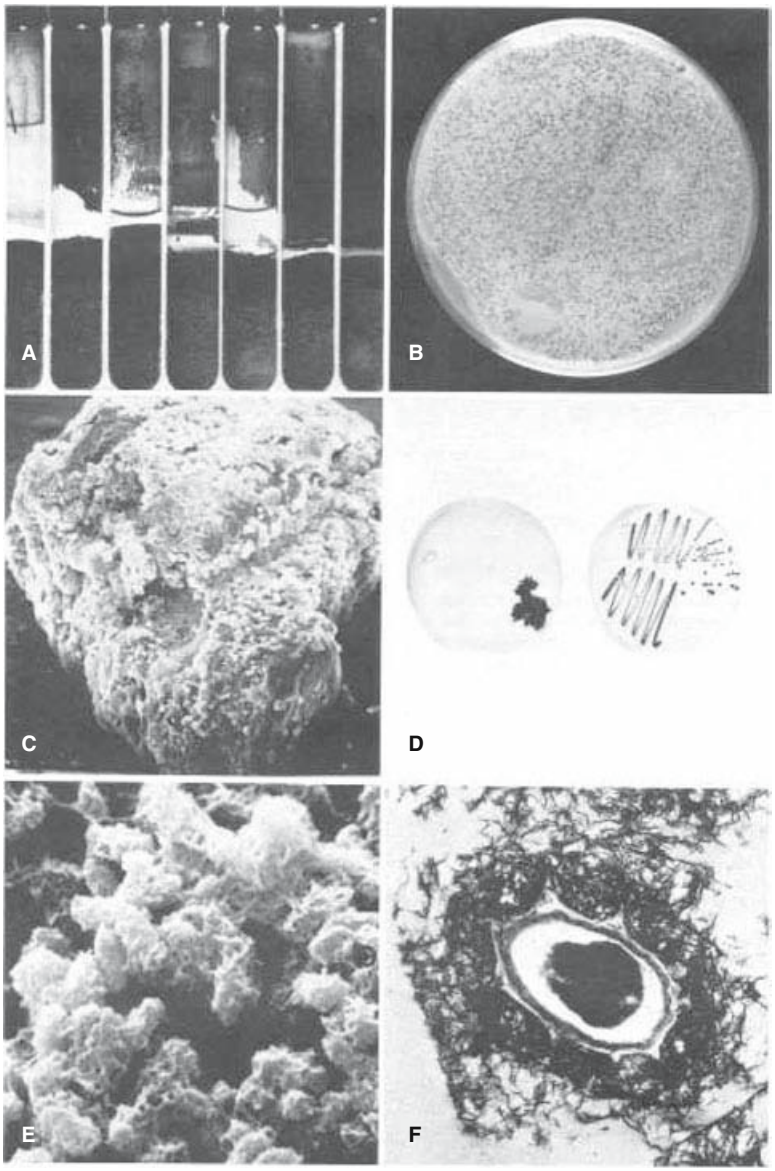

Fig. 3. Many faces of manganese-oxidizing bacteria. This figure shows six different views and size scales of Mn-oxidizing bacteria. (A) Black crusts and Mn-coated sand grains seen in enrichment culture tubes after several months of enrichment with 100 μM Mn(II). (B) Colonies resulting from plating of 0.1 ml of enrichment culture E-3—these colonies were stained with benzidinium HCl; the dark colonies are positive for Mn oxidation. (C) Scanning electron microscope (SEM) magnification of Mn-encrusted sand grain from enrichment culture. Mn coating appears to be bacterial in nature. (D) Left plate, outgrowth of Mn-oxidizing bacteria from sand grain placed on agar plate; right plate, pure culture isolate (called SG-1) from sand grain outgrowth; dark color of these colonies is due to Mn oxides precipitated around the cells. (E) High resolution SEM of pure culture of SG-1. (F) Transmission electron microscope (TEM) picture of thin section of spore of strain SG-1; crystalline material is Mn oxide.

enrichment of 3 months to 1 year, zones of Mn oxidation could be seen in nearly all enrichments, as Mn oxide films on tubes (Fig. 3A), as Mn-oxide-encrusted sand grains (Fig. 3A), or as zones of darkening in the sediment enrichments. Plating of these samples yielded very high numbers of morphologically and taxonomically similar bacteria (Fig. 3B; Nealson, 1978). These results suggest that there is a selective advantage for bacteria capable of Mn oxidation, and such approaches might be suggested as one method for finding those bacteria that should be properly placed in the "group" of true Mn-oxidizing bacteria (i.e., those that for one reason or another derive advantage from Mn oxidation).

Cultivation

Mn-oxidizing bacteria can be easily obtained in pure culture and grow well in liquid or solid media, so long as Mn(II) toxicity is avoided. As a guideline, some commonly used media are given below, but it may be equally helpful to discuss some of the general principles that need to be considered for growth of the Mn oxidizers.

Carbon Source Simple carbon sources (acetate, succinate, glycerol, etc.) are usually used for isolation of Mn oxidizers; for many of these organisms, Mn oxidation in culture occurs only after growth has slowed or ceased. Furthermore, some carbon sources will lower the pH of the

medium or repress Mn-oxidation ability (Nealson, 1978). Many of the media use very low levels of carbon, and in some media amino acids serve as the sole source of organic carbon. With newly isolated organisms, we routinely test several carbon sources for their ability to support growth and Mn oxidation.

pH Since the oxidation of Mn is strongly pH dependent (Fig. 1), it is important to control or at least monitor the pH of the medium. By the addition of a strong buffer near neutrality, one can avoid indirect Mn oxidation due to pH changes. We routinely add 10–100 mM Hepes buffer at a pH of 7.0–8.0 (see below).

Vitamins In general the Mn oxidizers are not fastidious organisms, although many workers routinely add a vitamin supplement (see below) or small amounts of yeast extract to the medium.

Mn Source The kind of Mn(II) salt used (e.g., acetate, carbonate, chloride, sulfate, etc.) appears to make little difference. It is, however, advisable to filter-sterilize the Mn(II) solution and to add it to the medium after autoclaving. Autoclaving can lead to partial oxidation and formation of Mn oxide particles which can then bind Mn(II) and catalyze its abiological oxidation.

Mn Concentration Mn(II) in the environment rarely exceeds 1–5 micromolar, and above 10 micromolar it can be toxic to bacteria (Chapnick et al., 1982). In rich media, such as the K medium given below, Mn can be added at relatively high (i.e., millimolar) concentrations without causing toxicity. However, as the medium become less rich and there are fewer organic compounds to chelate the Mn(II), the Mn concentration must be lowered to prevent growth inhibition. With newly isolated organisms, we routinely test for growth on media with both 10 and 100 micromolar Mn.

Continuous Culture One method for avoiding Mn toxicity involves the use of continuous cultures (chemostats). It is possible to begin experiments using a heterotrophic medium (with a carbon source like succinate or acetate) containing low levels (5–20 μM) of Mn(II). The Mn(II) concentration is monitored in the outflow of the chemostat, and when it has decreased to values of less than 1 μM, the Mn(II) concentration in the medium reservoir of the chemostat can then be slowly raised to 1 mM or more (Kepkay and Nealson, 1987). This allows studies of the relationship between the metabolism of the carbon source and Mn(II) at different concentration ratios. Continuous cultures could also be used for the isolation of organisms from environments where natural enrichments exist—this should enhance the possibility of finding Mn oxidizers in which the oxidation of Mn(II) yields biologically useful energy.

Autoclaving All the media listed below can be sterilized by autoclaving, but in those cases where solutions of metals or vitamins are involved, it is advisable to filter sterilize the stock solutions and add to the growth medium after sterilization. This avoids chemical precipitation and complexation with organics in the medium.

Representative Media for Mn Oxidizers

K Medium (Krumbein and Altmann, 1973)

This is a rich medium that supports the growth of a variety of different heterotrophic Mn oxidizers. The ingredients added per liter of seawater, freshwater, or distilled water are:

$FeSO_4 \cdot 7H_2O$ (may be omitted)	0.001 g
$MnSO_4 \cdot 4H_2O$ (or $MnCl_2$)	0.2 g
Peptone	2.0 g
Yeast extract	0.5 g
Hepes buffer (N-2-hydroxyethylpiperazine_ N′-2-ethanesulfonic acid, pH 7.5, added as 1M stock)	10 mM

Succinate Minimal Medium (after Kepkay and Nealson, 1987)

This is a basal medium for the study of Mn binding and metabolism, and it is made up with a distilled water (or artificial seawater) base. It contains in addition to the seawater or freshwater base:

NH_4Cl (KNO_3 used in some instances)	9.0 mM
K_2HPO_4	0.4 mM
$NaHCO_3$	2.0 mM
$FeSO_4$	5.0 μM
Hepes buffer (pH 7.8)	10 mM
Vitamin mix (solution #1 below)	10 ml
Sodium succinate (or other carbon source)	10 mM
$MnC1_2$ (concentration varied as needed)	10 μM

PC Medium (Tyler and Marshall, 1967a)

This is a low-nutrient medium used for the isolation of Mn-oxidizing Hyphomicrobia species. It may be made with distilled water or with water from the environment under study. It contains per liter of basal liquid:

$MnSO_4 \cdot 4H_2O$	0.02 g
Yeast extract	0.05 g

PYGV Medium (Ghiorse and Hirsch, 1979)

This is another low-nutrient medium used for the isolation and study of slow-growing budding and sheathed bacteria (Pedomicrobium and Leptothrix). It may take several days or longer for colonies to become visible on this medium, but there is also little trouble with fast-growing heterotrophs that tend to outgrow the Mn oxidizers on richer media. The medium contains per liter of distilled water or of artificial seawater:

Peptone	0.25 g
Yeast extract	0.25 g
Glucose	0.25 g
Vitamin mixture (solution no. 1 below)	10 ml
Mineral salts (solution no. 2 below)	20 ml

Solution no. 1: Concentrated Vitamin Solution

> The following vitamins are added to 1 liter of double-distilled water with stirring, filter sterilized, and stored at 5°C in the dark: 2.0 mg biotin; 5.0 mg niacin; 5.0 mg thiamine ·HCl; 5.0 mg p-aminobenzoic acid; 5.0 mg Ca-pantothenate; 5.0 mg pyridoxin ·HCl; 0.1 mg cyanocobalamin; 5.0 mg riboflavin; and 2.0 mg folic acid.

Solution no. 2: Hutner and Cohen-Bazire's salts

> 10 mg nitrilo triacetate (NTA) is dissolved by neutralization with KOH in 900 ml distilled water. The following salts are then added: 3.34 g $CaCl_2 \cdot 2H_2O$; 99 mg $FeSO_4 \cdot 7H_2O$; 29.7 g $MgSO_4 \cdot 7H_2O$; 12.67 g $NaMoO_4 \cdot 2H_2O$; and 50 ml metal solution "44" (solution no. 3, below). The final volume is adjusted to 1 liter with distilled water, and the solution, which should be clear, is stored at 5°C.

Solution no. 3: Metal Salt Solution "44"

> The following salts are added singly to 1 liter of double-distilled water to which a few drops of H_2SO_4 have been added: 250 mg ethylenediaminetetraacetate (EDTA); 1,095 mg $ZnSO_4 \cdot 7H_2O$; 154 mg $MnSO_4 \cdot H_2O$; 20.3 mg $CoC1_2 \cdot 6H_2O$; 500 mg $FeSO_4 \cdot 7H_2O$; 39.2 mg $CuSO_4 \cdot 5H_2O$; and 17.7 mg $Na_2B_4O_7 \cdot 10H_2O$.

Identification

Mn-oxidizing bacteria are usually identified by detection of brown or black solid Mn oxides that are deposited around or near the cells. However, this property is not definitive. First, many bacteria produce other dark pigments, and second, several different crystalline forms of Mn oxides are possible, and these have different colors and consistencies. Furthermore, other metals, particularly iron (Ghiorse and Hirsch, 1979), are often deposited with Mn oxides, lending yet different colors and consistencies to the precipitates.

In order to circumvent these problems, two manganese-specific spot-test indicators are commonly used to qualitatively establish the occurrence of Mn oxides. The first, benzidinium hydrochloride (Feigl, 1958), gives a deep blue color in the presence of Mn oxides. The reagent is prepared by dissolving 5 g of benzidinium ·HCl in 35 ml of glacial acetic acid. This solution is then diluted to a final volume of 500 ml with distilled water and stored at 5°C until needed. Colonies can be directly tested for the presence of Mn oxides by flooding the plates with 10 ml of reagent. Colonies of Mn oxidizers are identified by a dark blue color (see Fig. 3B).

There are some disadvantages to the use of the benzidinium reagent: 1) the reaction is interfered with by iron oxides, which can give weak false positives; 2) the reagent is toxic and has carcinogenic properties; and 3) the reagent mixture is bactericidal, so that colonies must be replicated before they are tested for Mn oxidation. Because of the toxic nature of this compound, it is recommended that it *not* be used unless there are no alternatives.

A second method utilizes the reagent leukoberbelin blue (LBB) (Krumbein and Altmann, 1973), a redox indicator which upon interaction with Mn(III) or Mn(IV) is oxidized and changes from colorless to a blue form. Mn oxides are reduced during this reaction, so that treatment with LBB can be used to gently remove oxides from Mn-encrusted cells (Nealson and Tebo, 1980). The LBB reaction is less susceptible to interference by iron than is the benzidinium reaction, and because it is less bactericidal, it can be used directly on cells or colonies to indicate Mn oxidation during growth on solid medium (Krumbein and Altmann, 1973). The reagent is made as follows: 4.0 g of berbelin blue-I (N, N′-dimethylamino-*p*,*p*′-triphenylmethane-*o*′-sulfonic acid) is dissolved in 80 ml of boiled distilled water. Prior to distillation, the water is acidified with a few drops of concentrated phosphoric acid. Then 0.3 ml concentrated NH_4OH is added, and the final volume is adjusted to 100 ml. This stock solution, which should be slightly acidic, can be stored for up to one year at 5°C in the dark. The working solution for use as a spot test reagent, stable for only a few days, is made by diluting the stock solution 1/100 into a weak (1 mM) acetic acid solution.

Mn oxidation can be quantified by monitoring the disappearance of soluble Mn(II) from the growth medium, either colorimetrically via the formaldoxime method of Brewer and Spencer (1971) or by measurement via atomic absorption spectroscopy (AAS). Alternatively, the oxidized Mn can be measured by separation of the insoluble oxides on membrane filters, dissolution (reduction) of the Mn oxides, and measurement of the resulting soluble Mn(II). Dissolution can be accomplished by any of several methods: 1) treatment with strong acid (6 M HCl) for several days; 2) treatment with a mixture of 5 parts concentrated HNO_3 to 3 parts concentrated HCl in a teflon bomb for 3 hours at 105°C; 3) leaching for several hours with concentrated nitric acid containing 1% $(NH_2OH)Cl$ (hydroxylamine hydrochloride); or 4) leaching for concentrated nitric acid containing 3% (w/v) H_2O_2. The method of choice will depend on the nature of the precipitates being analyzed and the subsequent analyses to be performed. We routinely use a 1% (w/v) NH_2OH solution without the HCl because it is easy, rapid, and causes minimum interference during AAS analysis.

Mn(II)-oxidizing bacteria can also be identified microscopically. However, the following comments should be made with regard to potential pitfalls: In the cases of the budding or appendaged Mn oxidizers, it is often possible to observe cell structures within and protruding

from the Mn oxides (Ghiorse and Hirsch, 1979; Tyler and Marshall, 1967a, 1967b), but with other types the oxides must be removed before the causative bacteria can be identified. Treatment with LBB can be used to remove the Mn oxides while working with natural precipitates under the light microscope, thus revealing whether cells are associated with the oxides. Alternatively, the oxides can be sectioned and examined by transmission electron microscopy, which reveals intimate associations between the cells and their Mn oxide coatings (see Fig. 3) (Ghiorse, 1984a; Ghiorse and Hirsch, 1979; Nealson and Tebo, 1980). Because of the nature of Mn chemistry, cases may also arise in which bacteria associated with Mn oxide precipitates have nothing to do with manganese oxidation, so caution must be exercized in interpreting such microscopic results.

Perhaps the least-valuable method for identification involves scanning electron microscopy (see Fig. 3C, 3E, and Ghiorse, 1988). Cells coated with Mn oxides can not readily be identified as bacteria. On the other hand, bacteria that are clearly identifiable as Mn oxidizers may be recognizable because they are not coated with Mn oxides (Nealson and Tebo, 1980). Since SEM analysis involves fixation of the cells or natural material before viewing, such analyses, in the absence of confirming studies with either light or transmission electron microscopy, can lead to misinterpretation, especially when natural Mn precipitates are examined.

On the other hand, when pure cultures of Mn-oxidizing bacteria are available, SEM, especially when coupled with other spectral methods, such as Energy dispersive X-ray analysis (EDAX) (Nealson and Tebo, 1980) or laser microprobe mass spectral analysis (Ghiorse and Hirsch, 1979), can add valuable information and insights into the properties of the Mn-oxidizing organism under study.

Applications

Toxic levels of Mn can be a problem in municipal water supplies and public and private wells; this occurs during summer months when the oxygen level falls and reduction of Mn oxides occurs. Once the Mn has been reduced and solubilized, it tends to be quite stable, even in the presence of oxygen, and one potential use of Mn-oxidizing bacteria (or products that these bacteria produce) is to remove dissolved Mn(II) from such environments (Czekalla et al., 1985). Understanding the mechanisms whereby these bacteria catalyze Mn oxidation may provide chemical or biological methods for dealing with this problem on a seasonal or even a permanent basis. Fresh Mn oxides are also potent chelators of other metals, and they have been used for the removal of radium from water supplies (Moore and Reid, 1973); the addition of manganese bacteria may provide a superior method for forming such fresh oxides in situ for such uses.

Mn oxides are also excellent electron acceptors for anaerobic respiration (see Nealson et al., 1989), and the possible application of Mn-oxidizing bacteria and Mn to stimulate respiratory carbon mineralization in sedimentary environments (especially under controlled conditions) may be worth considering. These bacteria in effect offer a natural system for "pumping" (via precipitation and sedimentation of Mn oxides) electron acceptor equivalents into an anaerobic environment (see Fig. 2).

Controversy and Perspectives

It is appropriate to end this chapter with a discussion of some of the current controversies surrounding the Mn-oxidizing bacteria. Perhaps the major question involves the advantages, if any, of Mn(II) oxidation to the bacteria. The advantages of Mn(II) oxidation, which have been discussed by Ghiorse (1984b), can neither be proven or eliminated by the data now available. The first advantage is the use of Mn(II) as an energy source for chemolithotrophic growth. While there is sufficient energy in the oxidation of Mn(II) to MnO_2 (Ehrlich, 1976), indisputable proof of Mn chemolithotrophy has been difficult to obtain. Several recent reports, when taken together, suggest that a resolution to this question may be near. Kepkay and Nealson (1987) reported the occurrence of growth of a marine *Pseudomonas* sp. (S-36), both mixotrophically on succinate plus Mn(II) and autotrophically on Mn(II), in Mn-limited chemostats. In the latter case, bicarbonate was the sole carbon source for growth. Ultrastructural studies of the same organism, S-36 (Tebo, 1983), revealed internal membranes and structures resembling carboxysomes in cells which had been grown under conditions of manganese-limited growth and with bicarbonate as the carbon source. Under these conditions, the carboxysomes, which are believed to be the sites of CO_2 fixation, are consistent with (but are not proof of) an autotrophic mode of metabolism. Finally, Tebo and Haygood (1989) have recently reported the presence of the gene coding for the large subunit of ribulose-bisphosphate carboxylase in marine Mn-oxidizing bacteria. These organisms should be excellent candidates for future study as Mn chemolithotrophs.

Adams and Ghiorse (1985) reported that survival of *Leptothrix discophora* in stationary-

phase cultures was prolonged by the addition of Mn(II) to the medium, and Mn(II) may provide a significant advantage to bacteria in natural habitats.

Bromfield (1978) discussed the possibility that Mn(II) oxidation may provide a protective mechanism against otherwise toxic levels of Mn(II) that might accumulate in the environment. Such a function is also discussed by Ghiorse (1984b) and is consistent with the observation that large numbers of bacteria capable of Mn(II) oxidation are found in habitats where Mn can reach toxic levels.

It now seems clear that there may be one or more advantages that accrue to those bacteria which oxidize manganese. For instance, it seems likely that the question of Mn chemolithotrophy will be resolved in the near future, as several lines of evidence suggest that such organisms do exist. Resolution of this question will enhance our understanding of the importance of Mn oxidation to bacteria and vice versa and give a new appreciation to the ecophysiology of the Mn oxidizers.

Second it also seems likely that there are other advantages that accrue to organisms that can oxidize Mn(II), including both unknown metabolic advantages and protective advantages. The elucidation of these will also aid in the definition of the "group" of Mn-oxidizing bacteria.

As discussed above, a careful study of the *Metallogenium* group is needed in order to understand how, or whether, these structures are true organisms, if they really fit into the biogeochemical scheme of Mn, and if they should be classified with the Mn oxidizers as a group.

With the answers to the above questions in hand, it may be possible in the future to specify which bacterial types (physiological and ecological) should be properly placed into this "group."

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Prokaryotes (2006) 5:232–249
DOI: 10.1007/0-387-30745-1_12

CHAPTER 3.1.11

The Genus *Paracoccus*

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Introduction

At the time that Henk van Verseveld and Adriaan Stouthamer (1991) described *Paracoccus* in the second edition of *The Prokaryotes*, only two species were recognized: the type species (*P. denitrificans*) and *P. halodenitrificans* (Kocur, 1984). Of those two, *Paracoccus halodenitrificans* was subsequently excluded because it was shown to be a member of the genus *Halomonas* in the γ-subclass of the Proteobacteria (Dobson and Franzmann, 1996; Miller et al., 1994; Ohara et al., 1990; Urakami et al., 1990). In the past decade, a number of new species of *Paracoccus* have been described and a major critical review of the diverse biotypes of *P. denitrificans* and similar organisms was undertaken, leading to a clearer definition of the type species. At the time of writing, a total of 14 species of *Paracoccus* have been proposed (Table 1). These include two well-studied species of facultatively chemolithoautotrophic sulfur bacteria, *Thiosphaera pantotropha* and *Thiobacillus versutus*, which have been reclassified as species of *P. pantotrophus* and *P. versutus*. *Thiosphaera pantotropha* strain GB17 was initially believed to be a strain of *P. denitrificans* (Ludwig et al., 1993), until it and several biotypes of *P. denitrificans* were reclassified as a separate species, *P. pantotrophus* (Rainey et al., 1999). *Paracoccus denitrificans* and *P. pantotrophus* have continued to be the focus of much study of the molecular biology, respiratory mechanisms, and regulation of carbon and nitrogen dissimilation in *Paracoccus*.

The treatment of *Paracoccus* in the edition second of *The Prokaryotes* (van Verseveld and Stouthamer, 1991) concentrated on *P. denitrificans*, and we refer the reader to that treatment, and other more recent studies of *P. denitrificans*, for in-depth information concerning the physiology and molecular biology of *P. denitrificans* (and in some cases *P. pantotrophus*). More relevant reviews include those by Stouthamer (1992), Stouthamer et al. (1997), Baker et al. (1998) and van Spanning et al. (2000).

History of the Type Species, *Paracoccus denitrificans*

The original isolate by Beijerinck and Minkman (1910) is still extant as the type strain of the genus (Goodhew et al., 1996; Rainey et al., 1999). It was originally named *Micrococcus denitrificans* and shown to grow anaerobically in pure culture in a bouillon medium supplemented with ammonium nitrate (10 g · liter^{-1}), reducing nitrate to dinitrogen (N_2) and nitrous oxide (N_2O) in approximately a 2 : 1 ratio (Beijerinck and Minkman, 1910). Subsequently many workers used derivatives of that strain and another isolated by Koster (Goodhew et al., 1996), and *Paracoccus denitrificans* became a model organism for the study of its cytochrome system and electron transport mechanisms, denitrification, methylotrophy, and various aspects of metabolic and molecular regulation. It became apparent that a number of seemingly distinct biotypes were in use in various laboratories, and the isolation of other species with similar properties (initially named *Thiobacillus versutus* and *Thiosphaera pantotropha*) helped lead to a questioning of the relatedness of these to *P. denitrificans* and between the biotypes of *P. denitrificans*.

As a consequence of studies of their cytochromes and the history of *P. denitrificans* cultures by Goodhew et al. (1996), of their chromosomes by Winterstein and Ludwig (1998), and of their 16S rRNA sequences and DNA:DNA hybridization among *P. denitrificans* strains (Rainey et al., 1999), it became clear that the physiologically similar strains described as *P. denitrificans* actually fell into two distinct groups that differed so significantly that they justified separation into distinct species. These were *P. denitrificans* and *P. pantotrophus* comb. nov (Rainey et al., 1999): the type strain of *P. denitrificans* is ATCC 17741^T (LMD 22.21; Beijerinck and Minkman, 1910; Ludwig et al., 1993). The type strain of *P. pantotrophus* is ATCC 35512^T (LMD 82.5), which is the original isolate of *Thiosphaera pantotropha* (Kuenen and

Table 1. List of the currently recognized species of *Paracoccus*, representative strains available in culture collections, and their accession numbers for 16S rRNA gene sequences.

Species	Type strain numbers	16S rRNA gene sequence accession numbers	References[a]
P. denitrificans[Tb]	ATCC 17741[T], LMD 22.21[T], DSM 413[T]	Y16927, Y16928, Y16929	Rainey et al. (1999) Goodhew et al. (1996)
P. pantotrophus	ATCC 35512[T], LMD 82.5[T]	Y16933, X69159	Rainey et al. (1999) Ludwig et al. (1993)
P. versutus	ATCC 25364[T], DSM 582[T]	Y16932, Y16931	Rainey et al. (1999)
P. aminophilus	JCM 7686[T]	D42239	Katayama et al. (1995)
P. aminovorans	JCM 7685[T]	D32240	Katayama et al. (1995)
P. alcaliphilus	JCM 7364[T]	D32238	Katayama et al. (1995)
P. thiocyanatus	IAM 12816[T]	D32242	Katayama et al. (1995)
P. solventivorans	DSM 6637[T]	Y07705	Siller et al. (1996)
P. kocurii	JCM 7684[T]	D32241	Katayama et al. (1995)
P. marcusii	DSM 11574[T]	Y12703	Harker et al. (1998)
P. alkenifer	DSM 11593[T]	Y13827	Lipski et al. (1998)
P. carotinifaciens	IFO 16121[T]	AB006899	Tsubokura et al. (1999)
P. methylutens	VKM B-2164[T]	AF250334	Doronina et al. (1998)
P. kondratievae	VKM B-2222[PT]	AF250332	Y. A. Trotsenko and T. P. Tourova (personal communications, 2000)

Symbols: [T]indicates type strain of each species; ATCC, American Type Culture Collection; LMD, Delft Collection of Microorganisms; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; JCM, Japan Collection of Microorganisms; IAM, Institute of Applied Microbiology, Tokyo, Japan; IFO, Institute of Fermentation, Osaka, Japan; VKM, All-Russian Collection of Microorganisms.

[a]References are for details of culture methods and sources of cultures.

[b]*P. denitrificans* is the type species of the genus.

Robertson, 1989; Robertson and Kuenen, 1983). Culture collection strains that (by this work) are indicated to be identical to each of these type strains are listed in Table 2. Winterstein and Ludwig (1998) have shown that the genome of *P. denitrificans* ATCC 13453, DSM 413 and strain Pd 1222 consists of three chromosomes, whose DNA molecules are approximately 2.1, 1.1 and 0.64 Mb in size. In contrast, *P. denitrificans* DSM 65 and *Thiosphaera pantotropha* LMD 82.5 contain four large DNA species that were 2.2, 1.50, 1.71–1.77 and 0.5 Mb in size. This observation is wholly consistent with our confirmation of the first three species as *P. denitrificans* and the assignment of the last two to the new combination, *P. pantotrophus*.

While it has long been recognized that the strains of *P. denitrificans* comprised several biotypes within a supposedly heterogeneous species (Jordan et al., 1997; Van Verseveld and Stouthamer, 1991), our work and that of Goodhew et al. (1996) revealed a more significant factor underlying this heterogeneity. This is that the validity of reference strains held by different international culture collections as derivatives of the type strain ATCC 17741[T] is uncertain. The results show that at some stage some culture collection strains supposedly derived from *P. denitrificans* LMD 22.21 (the original isolate of Beijerinck and Minkman, 1910) and ATCC 17741[T] must have been replaced with other strains (i.e. of *P. pantotrophus*). It is clear that where comparison with a type strain is of crucial significance, as in defining a genotype or the identity of a strain with the type strain, reference to the authentic type strain held by the collection is essential. It is obviously highly desirable that cross-checking by culture collection curators of the authenticity of type strains and the authenticity of cultures reportedly derived from them is undertaken.

Classical Taxonomic and Physiological Characteristics of Species of the *Paracoccus* Genus

Morphologically, all the species are coccoid, between 0.4–0.9 μm in diameter, or coccobacilli, up to 2 μm in length, and occur as single cells, pairs or clusters. All are Gram-negative and most species are non-motile. All species grow aerobically on a wide range of organic substrates and some are capable of anaerobic growth with nitrate or nitrous oxide as the terminal oxidant, producing dinitrogen as the final product. None is known to be able to grow fermentatively. The optimum temperature for growth of all the species is in the range 25–37°C, and the pH for good

Table 2. Partial list of *Paracoccus* strains derived from the original isolate of *P. denitrificans* and of those strains transferred to the new species, *P. pantotrophus* comb. nov.[a]

Paracoccus denitrificans	Paracoccus pantotrophus
P. denitrificans ATCC 17741^T	*T. pantotropha* ATCC 35512^T
P. denitrificans LMD 22.21^T	*T. pantotropha* LMD 82.5^{Tb}
P. denitrificans DSM 413^T	*T. pantotropha* LMD 92.63
P. denitrificans ATCC 19367	*P. denitrificans* DSM 65[c]
P. denitrificans ATCC 13543	*P. denitrificans* LMG 4218
P. denitrificans IFO 13301	*P. denitrificans* LAM 12479
P. denitrificans DSM 1404	*P. denitrificans* JCM 6892
P. denitrificans DSM 1405	*P. denitrificans* DSM 11072
P. denitrificans NCIMB 8944	*P. denitrificans* DSM 11073
P. denitrificans strain Pd 1222[d]	*P. denitrificans* DSM 11104

Symbols: LMG, Ghent Collection of Microorganisms, Ghent, Belgium; NCIMB, National Collection of Industrial and Marine Bacteria; other symbols defined in Table 1.

[a]Transfer was based on 16S rRNA gene sequence similarities and DNA-DNA hybridization (Rainey et al., 1999) and cytochrome *c* profiles (Goodhew et al., 1996).

[b]*Thio. pantotropha* strain GB17, as used by Ludwig et al. (1993).

[c]Formerly regarded as the type strain of *P. denitrificans*.

[d]A derivative of *P. denitrificans* DSM 413.

growth ranges between pH 6.5–8.5 for different species, except for *P. alcaliphilus* (which is moderately alkaliphilic with an optimum of pH 8–9 and grows at pH 9.5, but only weakly at pH 7.0; Urakami et al., 1989). All the species examined to date contain ubiquinone-10 as the respiratory quinone, as expected for members of the α-3 subclass of the Proteobacteria, with small amounts of ubiquinone-9 and ubiquinone-11 also being reported for some species. Typically the major fatty acids are 18:1 and 18:0 straight-chain acids, 19cyc, and 10:0(3-OH), 12:1(3-OH), and 14:0(3-OH) hydroxy-acids (Katayama et al., 1995; Lipski et al., 1998). Most, if not all, strains may accumulate poly-β-hydroxybutyrate under carbon-sufficient growth conditions and are generally catalase and oxidase positive. The mol% G+C content of the DNA of the species described to date ranges between 63–71 mol%. When the genus *Paracoccus* was created (Davis et al., 1969), its sole member was the *Micrococcus denitrificans* of Beijerinck and Minkman (1910), a distinguishing feature of which was autotrophic growth on carbon dioxide using hydrogen oxidation as the source of metabolic energy (Kornberg et al., 1960). This property is not universal among the 14 species now named. Indeed, a number of the characters commonly used to define the general features of the genus (Van Verseveld and Stouthamer, 1991; Van Spanning et al., 2000) derive from the properties exhibited by the most-studied strains of *P. denitrificans*, *P. pantotrophus* and *P. versutus* and do not apply to some of the more recently-described species. Thus, the ability to use the oxidation of hydrogen or inorganic sulfur compounds to support autotrophic growth, the ability to grow either autotrophically or methylotrophically on methanol or methylamine, or the ability to grow by anaerobic denitrification do not now appear to be exhibited by all the species and are thus not common taxonomically diagnostic features of all *Paracoccus* isolates (Table 3).

The chemolithoautotrophic growth on inorganic sulfur compounds (e.g. thiosulfate) of three species of *Paracoccus* has been studied in some detail: *P. versutus*, *P. pantotrophus* and *P. thiocyanatus*. The type species, *P. denitrificans*, is also defined as being able to grow on thiosulfate. This property is in need of further study, as the analyses of Goodhew et al. (1996) and Rainey et al. (1999) showed that numerous strains of *P. denitrificans* were in fact strains of *P. pantotrophus*, and most comprehensive work on thiosulfate oxidation has been carried out with *P. versutus* and *P. pantotrophus* strain GB17 (Kelly et al., 1997; Friedrich, 1998). The type strain of *P. denitrificans* was received from C.B. van Niel by Davis et al. (1969) and became ATCC 17741, which Davis et al. (1969) thought likely to be the same as strains ATCC 19367 and ATCC 13543. We showed that this was true (Rainey et al., 1999) and that these strains were all identical to the original Beijerinck isolate (LMD 22.21). However, some strains supposedly also derived from the van Niel strain (via the strain Stanier 381: DSM 65 and LMG 4218) were in fact *P. pantotrophus* (Goodhew et al., 1996; Rainey et al., 1999). Strains DSM 413 and Pd 1222 are also representatives of the type

Table 3. Distinguishing properties of the 14 species of the genus *Paracoccus*.

	Species[a]													
Character	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Denitrification	+	–	–	–	–	+	+	+	+	–	+	+	–	+
Pigmentation	–	–	+	+	–	–	na	–	+	–	–	–	–	–
Motility	–	–	–	+	–	–	–	–	–	–	–	+	–	–
Urease	+	+	na	–	–	na	na	–	–	–	–	–	+	+
Growth on:														
$H_2 + CO_2$	+	–	na	na	–	–	na	na	+	–	+	+	+	+
Thiosulfate + CO_2	+	na	na	na	na	na	na	–	w	na	+	+	–	+
Thiocyanate + CO_2	–	na	na	na	na	na	na	na	+	na	na	–	–	na
Methanol	+	+	–	na	–	–	+	–	–	–	–	+	+	+
Methylamine	+	+	–	na	+	+	–	+	–	+	–	+	+	+
Formate	+	–	+	na	–	–	–	+	w	–	–	+	+	na
Trimethylamine	w	–	–	na	+	–	–	+	–	+	–	–	–	–
Xylose	–	+	na	na	+	–	–	–	–	–	na	–	–	–
Fructose	+	+	+	na	–	–	–	–	+	+	+	+	+	+
Sucrose	+	–	+	na	–	–	–	–	–	–	na	+	+	–
Glycerol	+	+	+	na	+	–	–	w	–	+	na	+	+	+
Mannitol	+	+	+	+	–	–	–	–	+	+	na	+	+	+
Inositol	+	+	+	na	–	–	w	–	–	–	na	+	+	+

Symbols: +, present; –, absent; na, data not found in the published literature; w, weak growth.
[a]List of species (numbered in the same sequence as in Table 4): 1. *P. denitrificans*; 2. *P. alcaliphilus*; 3. *P. marcusii*; 4. *P. carotinifaciens*; 5. *P. aminophilus*; 6. *P. solventivorans*; 7. *P. alkenifer*; 8. *P. kocurii*; 9. *P. thiocyanatus*; 10. *P. aminovorans*; 11. *P. pantotrophus*; 12. *P. versutus*; 13. *P. methylutens*; 14. *P. kondratievae*.

strain, *P. denitrificans* LMD 22.21, having been derived through the "Morris strain" (Goodhew et al., 1996). In an early study, Friedrich and Mitrenga (1981) showed that strain Stanier 381 (= *P. pantotrophus*) oxidized and grew on thiosulfate, while the Morris strain (= *P. denitrificans*) did not. The original Beijerinck isolate of the type strain, LMD 22.21, was, however, shown to grow autotrophically on thiosulfate (Robertson and Kuenen, 1983), but no other studies of the use of inorganic sulfur compounds by authentic strains of *P. denitrificans* seem to have been published.

Growth of the type species, *P. denitrificans*, and of *P. versutus* and *P. kondratievae* on methanol or methylamine has been proved to be autotrophic, using the complete oxidation of the one-carbon compounds to carbon dioxide to provide metabolic energy and the fixation of carbon dioxide by the Calvin cycle for biosynthesis (Bamforth and Quayle, 1978; Van Verseveld and Stouthamer, 1978; Kelly et al., 1979; Kelly and Wood, 1982; Van Verseveld and Thauer, 1987; Doronina et al., 2001). The type strain of *P. pantotrophus* (formerly *Thiosphaera pantotropha*; Robertson and Kuenen, 1983) does not grow on methanol or methylamine, but spontaneous mutational events during prolonged incubation with methanol resulted in the appearance of a strain able to grow on methanol, using ribulose bisphosphate carboxylase to fix carbon dioxide (Egert et al., 1993). In contrast, three *Paracoccus* strains isolated by Jordan et al. (1995) and subsequently shown to be strains of *P. pantotrophus* (Jordan et al., 1997; Rainey et al., 1999) were capable of good growth on methanol without prior selective methods.

While a number of other *Paracoccus* species also grow on one-carbon compounds (Table 3) and some (*P. aminovorans*, *P. aminophilus* and *P. kocurii*) use some or all of mono-, di-, and tri-methylamines, trimethylamine-*N*-oxide, formamide, *N*-methylformamide, *N*,*N*-dimethylformamide, and formate as growth substrates, experiments proving that all these are also autotrophic have not been reported. This information is quite important to obtain, as the possibility of the occurrence of alternative assimilatory pathways for one-carbon compounds (such as the serine pathway) has not yet been excluded. The existence of such pathways in any of these species would have considerable taxonomic importance. Of relevance is the observation that significant activities of hydroxypyruvate reductase, a key enzyme of the serine pathway, were found in *P. versutus* grown on formaldehyde (Kelly and Wood, 1984) and in *P. methylutens* grown on methylamine or dichloromethane (Doronina et al., 1998), although the major pathway for carbon fixation was the Calvin cycle in both species.

Phylogenetic Relationships Between the 14 Named Species of *Paracoccus*

16S rRNA gene sequences are available for the type strains of all 14 species of the genus *Paracoccus* and a number of additional strains. Phylogenetic analyses have demonstrated that all the described species of the genus *Paracoccus* form a coherent cluster within the α-3 subclass of the Proteobacteria with the closest relatives being members of the genus *Rhodobacter* (Tsubokura et al., 1999). Comparison of the 16S rRNA gene sequences of the type strains of each of the 14 species of the genus *Paracoccus* described to date shows the 16S rRNA gene sequence similarities within the genus to be in the range 93.5 to 99.8% (Table 4). *P. methylutens* shows the lowest 16S rRNA gene sequence similarity (93.5 to 97.5%) to the other species of the genus while *P. carotinifaciens* and *P. marcusii* share 99.8% sequence similarity. The phylogenetic dendrogram shown in Fig. 1 demonstrates the relationships between the species of the genus *Paracoccus*. The majority of species are found to comprise distinct lineages with long branches not closely related to their next neighbor. The lack of close relationships between the species is also seen through the bootstrap values, which clearly indicate low confidence in most of the branching points within the dendrogram. With the exception of the relationships between (i) *P. marcusii* and *P. carotinifaciens*, (ii) *P. solventivorans* and *P. alkenifer*, and (iii) *P. pantotrophus*, *P. versutus* and *P. methylutens* no other branching points are well supported by the bootstrap analyses. Considering the overall structure of the phylogeny based on the 14 described species, the high degree of divergence between the majority of these species and the fact that some true species share high levels (<99.5%) of 16S rRNA gene sequence similarity, it is clear that there is room to add numerous new species to this genus based on sequence analyses before the phylogenetic structure becomes saturated. Because of the use of different methodologies in studies to determine the degree of DNA reassociation between *Paracoccus* species and strains it is difficult to make any correlation between 16S rRNA gene sequence similarity values and percentage DNA reassociation values. However, from the DNA reassociation data of Rainey et al. (1999), it is clear that strains sharing high 16S rRNA gene sequence similarities can still be distinct species. Hybridization between DNA from *P. pantotrophus* DSM 65 and *P. versutus* DSM 582 showed 54% reassociation while the 16S rRNA gene sequences from these two strains showed <99.0% similarity. This comparison indicates that 16S rRNA gene sequence data are useful in assigning new isolates to the *Paracoccus* genus, but that high similarity values between 16S rRNA sequences do not necessarily indicate species identity. Additional tests such as assessment of DNA-DNA hybridization are also required to establish identity at the species level.

The "ae2" editor (Maidak et al., 1996) was used to align the 16S rRNA gene sequences of *Paracoccus* spp. available from the public databases (accession numbers indicated in parentheses). The programs of the PHYLIP package including "dnadist" and "neighbor" were used for the phylogenetic analyses (Felsenstein, 1993). The tree topology was reanalyzed using 1,000 bootstrapped data sets and the programs "seqboot," dnadist and "consense" of the PHYLIP package (Felsenstein, 1993). Bootstrap values from the analyses of 1,000 data sets (expressed as percentages) are shown at the branching points. The 16S rRNA gene sequence of *Rhodobacter capsulatus* was used as an outgroup in the

Table 4. The 16S rRNA gene sequence similarity values for the species of the genus *Paracoccus*.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*P. denitrificans*	100													
2	*P. alcaliphilus*	97.4	100												
3	*P. marcusii*	96.4	96.7	100											
4	*P. carotinifaciens*	96.2	96.5	99.8	100										
5	*P. aminophilus*	97.8	96.4	96.7	96.6	100									
6	*P. solventivorans*	96.4	95.8	95.4	95.3	96.0	100								
7	*P. alkenifer*	95.5	95.8	95.0	94.9	94.9	98.1	100							
8	*P. kocurii*	95.7	96.0	95.0	94.9	95.1	96.9	95.7	100						
9	*P. thiocyanatus*	97.8	96.7	95.8	95.7	96.8	96.4	96.1	96.4	100					
10	*P. aminovorans*	97.9	97.4	96.6	96.4	97.1	96.0	95.9	95.3	97.4	100				
11	*P. pantotrophus*	98.2	96.4	95.0	94.8	96.7	96.7	95.4	96.5	97.1	96.4	100			
12	*P. versutus*	97.9	96.2	95.0	94.8	96.7	96.7	95.4	96.5	97.1	96.7	99.7	100		
13	*P. methylutens*	96.2	94.7	93.6	93.5	95.0	95.3	94.0	95.4	95.7	95.0	97.5	97.5	100	
14	*P. kondratievae*	97.1	95.2	95.0	94.9	96.0	96.1	94.7	96.3	96.6	97.0	97.5	97.5	96.2	100
	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14

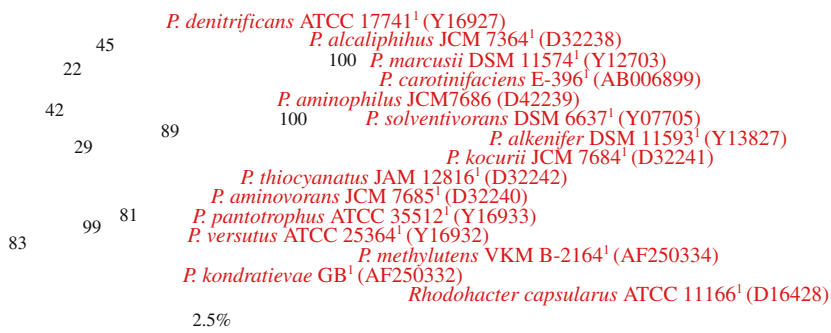

Fig. 1. Phylogenetic dendrogram derived from 16S rRNA gene sequences of all the species of the genus *Paracoccus* described to date.

Table 5. Sources from which species of *Paracoccus* have been isolated.

Species	Sources of isolates
P. denitrificans	Garden soil, arable soil, field soil, sewage sludge, meadow soil, horse manure, cow dung, canal and well mud, unspecified soils
P. pantotrophus	Denitrifying, sulfide-oxidizing effluent treatment pilot plant (Gist-Brocades, The Netherlands), *Quercus* tree soil (Kew Gardens, England)
P. marcusii	Orange-pigmented contaminant (presumed airborne) on a nutrient agar plate
P. thiocyanatus	Activated sludge enriched with thiocyanate (isolated by H. Murooka; Katayama-Fujimura et al., 1983)
P. solventivorans	Soil from 1-m depth at a defunct natural gas company
P. alkenifer	Biofilters treating waste gas from an animal rendering plant
P. carotinifaciens	Orange-pigmented isolate (on nutrient agar) from soils in the Kanagawa prefecture (Japan)
P. versutus	Mud (used in enrichment culture for *Thiobacillus denitrificans*; Taylor and Hoare, 1969; Taylor et al., 1971)
P. kocurii	Pilot activated sludge system used to treat waste water from a semiconductor manufacturing process
P. alcaliphilus	Not reported (Urakami et al., 1989)
P. aminophilus	Soil (Japan)
P. aminovorans	Soil (Japan)
P. methylutens	Groundwater
P. kondratievae	Maize rhizosphere

analyses. The scale bar represents 2.5 nucleotide substitutions per 100 nucleotides.

Ecological Aspects and Enrichment Culture for Isolation of the Species of *Paracoccus*

Earlier reviews of *Paracoccus* have been devoted almost exclusively to *P. denitrificans* and showed it to occur in many terrestrial situations (Van Verseveld and Stouthamer, 1991). The sources from which 13 of the 14 species discussed here were isolated are given in Table 5 and serve to illustrate the ubiquitous distribution of *Paracoccus* species in terrestrial environments. While the enrichment media used for the isolation of these species were diverse (Table 6), none of these could be regarded as exclusively selective for the species recovered, especially as two species were first obtained on nutrient agar, so it is concluded that there is an element of serendipity in the successful isolation of novel or existing species of *Paracoccus*. To date, no strains seem to have been reported from the marine environment, although the tolerance of 3% (w/v) NaCl by some species (*P. alkenifer* and *P. methylutens*) indicates they would survive in sea water.

All the species of *Paracoccus* can grow heterotrophically, with most species being able to use a very wide range of simple and complex organic substrates. Some species exhibit distinctive and potentially exploitable novel metabolic traits. For example, some species can grow in one or more of the following modes: as methylotrophs on one-carbon compounds, as autotrophs at the expense of oxidizing hydrogen or inorganic sulfur compounds, as autotrophs on thiocyanate or carbon disulfide, and as mixotrophs on compounds such as methylated sulfides (when both methylotrophic and chemolithotrophic energy mechanisms operate). The type species and some others are facultative denitrifiers. This metabolic diversity can enable enrichment culture to be directed towards the selection of specific metabolic types.

The procedures advocated for the enrichment culture of a number of *Paracoccus* species (Van Spanning et al., 2000) depend on selection either of the autotrophic/methylotrophic species in oxic media with hydrogen, methanol or methylamine

Table 6. Substrates used for the enrichment and isolation of the *Paracoccus* species listed in Table 5.

Species	Substrates used for the original pure culture isolations of the strains	References
P. denitrificans	Tartrate and nitrate, anaerobic with denitrification	Beijerinck and Minkman, 1910
P. pantotrophus	Mixotrophic, anaerobic chemostat with 10mM thiosulfate, 10mM acetate and 32mM nitrate	Robertson and Kuenen, 1983
	Aerobic batch enrichment with carbon disulfide	Jordan et al., 1995
P. marcusii	Nutrient agar, aerobic	Harker et al., 1998
P. thiocyanatus	Thiocyanate, autotrophic growth conditions, aerobic	Katayama et al., 1995
P. solventivorans	Acetone, aerobic	Siller et al., 1996
P. alkenifer	Not indicated	Lipski et al., 1992, 1998
P. carotinifaciens	Nutrient agar, aerobic	Tsubokura et al., 1999
P. versutus	Thiosulfate, aerobic organism	Taylor and Hoare, 1969 Taylor et al., 1971
P. kocurii	Tetramethylammonium chloride, with yeast extract, aerobic	Ohara et al., 1990
P. alcaliphilus	Not reported	Urakami et al., 1989
P. aminophilus	*N*,*N*-dimethylformamide, aerobic	Urakami et al., 1990
P. aminovorans	*N*,*N*-dimethylformamide, aerobic	Urakami et al., 1990
P. methylutens	Dichloromethane, aerobic	Doronina et al., 1998
P. kondratievae	Methanol	

(in the presence of carbon dioxide) or of denitrifying species under anoxic conditions with nitrate as the respiratory oxidant and organic compounds as carbon and energy sources. The former procedure could be applied to the enrichment of all species except *P. marcusii*, *P. thiocyanatus*, wild type *P. pantotrophus*, and possibly *P. carotinifaciens*. Growth coupled to heterotrophic denitrification is proved to be exhibited only by eight of the 14 species here described (Table 3). These include *P. thiocyanatus* and *P. pantotrophus*, thus providing a route for selection of these species. *P. marcusii* and *P. carotinifaciens* are incapable of denitrification and might not be enriched by either route.

The type culture isolated by Beijerinck (Beijerinck and Minkman, 1910) was obtained from an anaerobic culture provided with sodium potassium tartrate (Seignettesalz; Rochelle salt) and potassium nitrate (10 $g \cdot l^{-1}$ of each). Nitrous oxide (N_2O) is both an intermediate and a product of nitrate reduction by *P. denitrificans* and can be used as a sole respiratory oxidant by *P. versutus* (Wood and Kelly, 1983) and some other species (Van Spanning et al., 2000). Successful enrichment of some *Paracoccus* species can be achieved anoxically in liquid media supplied with tartrate or succinate and incubated under a nitrous oxide atmosphere, but such enrichments will obviously not be exclusively selective for *Paracoccus* species (Pichinoty et al., 1977a; Pichinoty et al., 1977b).

Growth of most mesophilic facultatively autotrophic hydrogen-oxidizing bacteria is slow or impossible under anaerobic denitrifying culture conditions (Van Verseveld and Stouthamer, 1991). Thus the ability of *P. denitrificans* to grow autotrophically using hydrogen oxidation coupled to nitrate reduction provides a means of enriching for this type of organism. Of the 14 species described, to date only four have been shown capable of both hydrogen oxidation and denitrification (although not necessarily at the same time: *P. denitrificans*, *P. thiocyanatus*, *P. pantotrophus*, *P. versutus*; Table 3), meaning that such a selective regime would select for a maximum of only four of the 14 *Paracoccus* species. Some species are also capable of good autotrophic growth on formate (Table 3), and of those, three are also denitrifiers (*P. denitrificans*, *P. versutus* and *P. kocurii*). Anaerobic enrichment with formate, carbon dioxide and nitrate (or nitrous oxide) could thus provide a selective route for the enrichment at least of *P. denitrificans* and *P. versutus* (Wood and Kelly, 1983). *P. denitrificans* is also capable of (slow) autotrophic growth on methanol under denitrifying conditions (Bamforth and Quayle, 1978), providing a further route for its selective isolation from environments also containing (strictly aerobic) facultative methylotrophs such as *Methylobacterium*.

A number of *Paracoccus* species can grow autotrophically using thiosulfate oxidation to provide energy, while at least some strains of *P. pantotrophus* can use carbon disulfide and methylated sulfur compounds (Jordan et al., 1997), and *P. thiocyanatus* can grow as an aerobic autotroph on thiocyanate (Katayama et al., 1995). These substrates have served to produce successful enrichment cultures from which novel species of *Paracoccus* have been isolated. Since such aerobic enrichment conditions are also elective for some autotrophic chemolithotrophs such as *Thiobacillus thioparus*, further screening to discriminate target species would be required: to date, no *Paracoccus* species have been found

which can use the oxidation of tetrathionate to support growth, whereas this compound is a substrate for most "thiobacilli" (Kelly et al., 2000).

Media for the Isolation and Culture of *Paracoccus* Species

It has been made clear in the preceding sections that media absolutely specific for the isolation of each of the Paracoccus species do not exist, as most strains are versatile heterotrophs with some sharing methylotrophic and lithotrophic properties with other unrelated genera. We give below the composition of some media which will enrich for and serve as growth media for *Paracoccus* species able to grow anaerobically with hydrogen and nitrate, or lithotrophically with thiosulfate, but which will also support growth of other hydrogen bacteria or chemolithotrophs. For routine isolation or culture media, the basal salts mineral media typically used in any microbiology laboratory are likely to be suitable for the growth of *Paracoccus* species on most substrates, subject to the provision of any required vitamin supplement (Table 7). Commercially available rich nutrient media will support the growth of all the known species for routine maintenance and cultivation. When a lithotrophic oxidation substrate such as thiosulfate is used, the principal technical problem is the acidification of the culture by the sulfuric acid produced as a product of oxidation. This acidification can be ameliorated in batch cultures without pH control by the use of strongly buffered media (e.g. that for *P. versutus* described below), subject to determining the tolerance of any given species to the relatively high concentrations of phosphate (or other buffer compounds) employed.

For Routine Culture on Chemoorganotrophic or Methylotrophic Substrates

We suggest a basal salts medium containing the following constituents ($g \cdot l^{-1}$):

K_2HPO_4, 3; KH_2PO_4, 2; NH_4Cl, 2; $MgSO_4 \cdot 7H_2O$, 0.2; sodium molybdate, 0.2; $FeSO_4 \cdot 7H_2O$, 0.2; with KNO_3, 5, for anaerobic cultivation. For autotrophic growth, $NaHCO_3$ ($1\ g \cdot liter^{-1}$) should be added. The molybdate and iron can be replaced with a suitable trace metal solution (e.g. one of those described below), and for strains whose potential vitamin requirement has not been tested, yeast extract ($0.1\ g \cdot l^{-1}$) or a vitamin mixture (see below) should also be added. Known requirements (e.g. thiamine) can typically be provided at $0.2\ mg \cdot l^{-1}$. Growth substrates can be added at the desired concentrations, such as 10–20 mM for sugars, organic acids (as their salts), alcohols, and other heterotrophic substrates and 10–50 mM for substrates such as formate, methanol, methylamines, or thiosulfate. Toxic substrates need testing initially at lower concentrations, such as 0.5–5 mM for dimethylsulfide, carbon disulfide, carbonyl sulfide, sodium sulfide, or formaldehyde and 5–30 mM for thiocyanate when tested as an autotrophic substrate.

The Molecular Hydrogen-Nitrate System (Vogt, 1965) for Isolation and Culture of *Paracoccus denitrificans* and Hydrogen-Oxidizing Species of *Paracoccus*

Three separate solutions are prepared, sterilized separately and mixed after cooling to prevent precipitation of phosphates:

Solution 1 ($g \cdot liter^{-1}$): $Na_2HPO_4 \cdot 12H_2O$, 9; KH_2PO_4, 1.5; NH_4Cl, 1; $MgSO_4 \cdot 7H_2O$, 0.2; KNO_3, 1; trace elements solution, 2 ml.

Solution 2 ($mg \cdot 100\ ml^{-1}$): Ferric ammonium citrate, 50; $CaCl_2 \cdot 2H_2O$, 100.

Solution 3 ($g \cdot 100\ ml^{-1}$): $NaHCO_3$, 5.

The trace elements solution, which is not sterilized prior to mixing with Solution 1, contains ($mg \cdot liter^{-1}$): $ZnSO_4 \cdot 7H_2O$, 100; $MnCl_2 \cdot 4H_2O$, 30; H_3BO_3, 300; $CoCl_2 \cdot 6H_2O$, 200; $CuCl \cdot 2H_2O$, 10; $NiCl_2 \cdot 6H_2O$, 20; $Na_2MoO_4 \cdot 2H_2O$, 30.

Solution 2 (10 ml) and solution 3 (10 ml) are added to solution 1 (1 liter). Erlenmeyer flasks containing 10 ml of the combined medium are incubated in an anaerobic jar filled with 85% H_2 and 15% CO_2 and shaken at 30°C. An appropri-

Table 7. Requirement for vitamin supplements exhibited by some species of *Paracoccus* for growth in minimal media.

Organism	Carbon substrate	Vitamin required
P. alcaliphilus	Methanol or glucose	Biotin
P. aminophilus	Various	Thiamine
P. aminovorans	Various	Thiamine
P. kocurii	Methylamines	Thiamine
P. methylutens	One-carbon compounds	B_{12} or yeast autolysate
P. thiocyanatus	Thiocyanate	Thiamine

ate oxygen absorbent may be included in the gas jar. Pure cultures may be maintained on the same medium supplemented with 1.7% (w/v) of a suitable agar.

Enrichment, Isolation and Maintenance Media for Thiosulfate-Oxidizing *Paracoccus* Species (e.g., *P. versutus* and *P. pantotrophus*; Kelly and Wood, 1998)

Four salt solutions are prepared:

Solution 1: (g ·liter $^{-1}$): $Na_2HPO_4 \cdot 2H_2O$, 39.5; KH_2PO_4, 7.5.

Solution 2: 4% (w/v) NH_4Cl.

Solution 3: 4% (w/v) $MgSO_4 \cdot 7H_2O$.

Solution 4: Trace metal solution (g · liter $^{-1}$): EDTA (disodium salt), 50; NaOH, 9; $ZnSO_4 \cdot 7H_2O$, 11; $CaCl_2$, 5; $MnCl_2 \cdot 6H_2O$, 2.5; $CoCl_2 \cdot 6H_2O$, 0.5; ammonium molybdate, 0.5; $FeSO_4 \cdot 7H_2O$, 5.0; $CuSO_4 \cdot 5H_2O$, 0.2. Adjust pH to 6.0 with 1 N NaOH and store, unsterilized, in a dark bottle.

Thiosulfate-agar Medium for Maintenance Slopes or Plates Solution 1, 200 ml; 4% NH_4Cl, 10 ml; 4% $MgSO_4 \cdot 7H_2O$, 2.5 ml; Solution T, 10 ml; 1 N NaOH, 11.5 ml; $Na_2S_2O_3 \cdot 5H_2O$, 5.0 g; agar, 15.0 g; phenol red (saturated solution), 10 ml. Initial pH should be 8.4.

Liquid Culture Medium This is prepared and sterilized in three parts to avoid precipitation of phosphates and any decomposition of growth substrates. The recipe given is for a final volume of 1 liter.

Part I (ml): 4% NH_4Cl, 20; 4% $MgSO_4 \cdot 7H_2O$, 2.5; solution T, 10; distilled water, 550.

Part II (ml): Solution 1, 200; 1 N NaOH, 10. Autoclave separately Parts I and II at 115°C for 10 min, then mix them when they are cool.

Part III (200 ml): An aqueous solution of the growth substrate, which may be thiosulfate (for growth of *P. versutus* and *P. pantotrophus*), carbon disulfide (for growth of *P. pantotrophus*), or other acidigenic substrate or any normal organic substrate.

The pH of this medium may be modified. Omitting the NaOH lowers the pH from 8.4 to 7.3, whereas intermediate pH values are obtained by adding intermediate volumes of NaOH or altering the ratios of the acid and basic phosphate salts employed (Kelly and Wood, 1998).

A number of *Paracoccus* species are known to require vitamins for successful enrichment and culture (Table 7). The following vitamin B mixture may be used generally and might be used to isolate marine species of *Paracoccus*, inasmuch as a vitamin requirement is common among marine bacteria.

Vitamin B mixture (mg ·liter $^{-1}$): thiamine-HCl, 10; nicotinic acid, 20; pyridoxine hydrochloride, 20; *p*-aminobenzoic acid, 10; riboflavin, 20; calcium pantothenate, 20; biotin, 1; cyanocobalamin, 0.5–1.0. Adjust pH to 7.0 by addition of 0.1 M NaOH.

Summary Descriptions of the Species of *Paracoccus*

For sources and original isolation conditions and principal properties of these species, see Tables 3, 4, 5 and 6.

Paracoccus alcaliphilus Type Strain JCM 7364^T

This organism is alkaliphilic and a facultative methanol- and methylamine-user. It grows at pH 7.0–9.5, but not below pH 6.5 or above pH 10. Colonies are white to pale-yellow; granules of poly-β-hydroxybutyric acid are accumulated in the cells. Grows on several hexoses, pentoses and sugar-alcohols but not on sucrose, maltose, lactose and trehalose or on formate, di- and trimethylamine. Nitrate is reduced only to nitrite and neither denitrification nor fermentative growth occurs. Ammonium, nitrate, urea and peptone are used as nitrogen sources. Its major fatty acids are 18:1 and 10:0(3-OH) and 14:0(3-OH). Its % G+C is 64–66.

Paracoccus alkenifer Type Strain DSM 11593^T

This strain will grow over the range pH 6.0–9.0. It denitrifies and will grow on solvents such as acetone, ethanol and methanol, some organic acids and asparagine, but not on carbohydrates or amines. Typical mono-unsaturated fatty acids are 14:1*cis* 7 and 20:1*cis*13, while 12:1*cis*5, 16:0 and 19:0 cyclo11-12 are absent. It also contains the hydroxy-fatty acids 10:0(3-OH) and 14:0(3-OH), of which the first predominates. Its mol% G+C was not reported (Lipski et al., 1998).

Paracoccus aminophilus Type Strain JCM 7686^T

This strain grows on methylamine and *N*,*N*-dimethylformamide, as well as di-and trimethylamine, trimethylamine-*N*-oxide, formamide, and N-methylformamide, but not on methanol or formate. It grows between pH 6.0–9.0 but not at pH 5 or 10, with an optimum of pH 6.5–8.0. It grows at 30°C, but not at 37°C.

Colonies on rich medium (peptone-yeast extract-glucose) are white-pale yellow. Granules of poly-β-hydroxybutyric acid are accumulated. Nitrate is reduced only to nitrite, and no fermentative growth is seen. It grows on various sugars, alcohols and organic acids but not on fructose, disaccharides, sugar-alcohols, citrate, ethanol or butanol. Ammonium and peptone, but not urea or nitrate, are used as nitrogen sources. The major fatty acid is straight-chain unsaturated 18:1 and its main hydroxy-fatty acids are 10:0(3-OH) and 14:0(3-OH). Its mol% G+C is 63.

Paracoccus aminovorans Type Strain JCM 7685^T

The properties of this organism are mainly as given for *P. aminophilus*, except that *P. aminovorans* can grow on fructose, sorbitol, mannose and mannitol, but not on xylose. It grows at both 30 and 37°C, but not at 42°C. Ammonium and peptone, but not urea or nitrate, are used as nitrogen sources. The major fatty acid is straight-chain unsaturated 18:1 and its main hydroxy-fatty acids are 10:0(3-OH) and 14:0(3-OH). Its mol% G+C is 67.

Paracoccus carotinifaciens Type Strain IFO 16121^T

This organism is an orange-pigmented, astaxanthin carotenoid-producing species, which may be a strain of *P. marcusii*. Colonies are orange to red. Growth occurs at 10–33°C, optimum at 28°C, with no growth at 37°C. It grows at pH 6.0–9.0, with an optimum at pH 6.5–7.5. Obligately aerobic. It grows on glucose, mannose, maltose, mannitol and the organic acids gluconate and malate, but not on arabinose, citrate and various other acids. No information on use of one-carbon substrates is available. Ammonium, but not nitrate, is used as a nitrogen source. Its major fatty acid is straight-chain unsaturated 18:1; the major hydroxy-fatty acid is 10:1(3-OH). Its mol% G+C is 67.

Paracoccus denitrificans Type Strain ATCC 17741^T, LMD 22.21^T, DSM 413^T

The type species of the genus, exhibiting metabolic versatility, with respiratory growth aerobically or with denitrification on many substrates, facultative methylotrophy and autotrophy on formate, hydrogen, thiosulfate, methanol, methylamine, and formaldehyde. It produces white or cream-colored colonies with no carotenoid pigments. It stores poly-β-hydroxybutyric acid as an intracellular carbon reserve and does not require vitamins or other organic growth factors. Its optimum temperature is 30–37°C and optimum pH is 7.5–8.0. Numerous sugars, organic acids, amino acids, and alcohols are used as sole energy and carbon sources for growth. These incude ribose, arabinose, glucose, fructose, trehalose, sucrose, mannose, acetate, propionate, malonate, tartrate, lactate, pyruvate, succinate, malate, citrate, gluconate, *p*-hydroxybenzoate, serine, proline, histidine, asparagine, glutamine, ethanol, propanol, butanol, glycerol and sorbitol. It apparently cannot use xylose, rhamnose, lactose, glycogen, cellulose, benzoate, *p*-aminobenzoate, phenol, arginine, threonine, tryptophan, ethanolamine, tetrathionate or thiocyanate for growth. Anaerobic growth can be supported by the respiratory reduction of nitrate, nitrite, or nitrous oxide, with dinitrogen as the end product; nitrous oxide can also be a product of nitrate reduction. Ammonium, nitrate, urea and glutamate are used as nitrogen sources. It is probably desirable for the growth substrate range of an authentic strain of *P. denitrificans* (e.g. ATCC 17741 or LMD 22.21) to be reassessed as it is uncertain how much of the literature data of this kind was actually obtained with strains of *P. denitrificans* rather than strains of *P. pantotrophus*. Its mol% G+C is 64–67.

Paracoccus kocurii Type Strain JCM 7684^T

This species grows on tetramethylammonium, methylamine, and di- and tri-methylamine, trimethylamine-*N*-oxide, and formate, but not on methanol. Optimum temperature is 25–30°C, with no growth at 20 or 40°C; optimum pH is 6.6–8.2, with no growth below pH 6.1 or above pH 8.8. It grows only a restricted range of other organic compounds, including lactate, acetate, pyruvate, propionate, and butyrate. Ammonium, but not nitrate or glutamate, is used as a nitrogen source. The major fatty acids are 18:1 and 19:0cyc; the major hydroxy-fatty acids are 10:0(3-OH) and 12:0(3-OH). Its mol% G+C is 71.

Paracoccus kondratievae, Proposed Type Strain VKM B-2222^{PT} (STRAIN GB^{PT})

The description of this species has been provided to us prior to publication (Doronina et al., 2001). This species is alkaliphilic and thermotolerant, growing at 30–50°C and pH 7.5–10.5 with optima at 38–42°C and pH 8.0–9.0. It grows aerobically as a facultative chemolithotroph with hydrogen or thiosulfate and as a methylotroph on methanol, methylamine or formaldehyde, assimilating carbon dioxide by the Calvin cycle. It grows anaerobically on methanol with nitrate, shows heterotrophic denitrification, and can ferment glucose. It grows on glucose, fructose, galactose,

ribose, arabinose, adonitol, dulcitol, glycerol, inositol, mannitol, sorbitol, ethanol, acetate, malate, α-ketoglutarate, succinate, fumarate, alanine, aspartate, glutamate, sarcosine, serine, *N*,*N*-dimethylglycine, and betaine. It does not grow on methane, dimethylamine, trimethylamine, chloromethane, dichloromethane, dimethylsulfoxide, lactose, xylose, rhamnose, raffinose, sucrose, trehalose, propionate, citrate, pyruvate, or tartrate. It does not require vitamins but showed growth stimulation by yeast extract (0.01% w/v). Ammonium, nitrate, urea, methylamine and amino acids are used as nitrogen sources for growth. Its major fatty acids are cyclopropane 19cyc and 16:0 and minor fatty acids are 18:0 and 18:1. Its major phospholipids are phosphatidylethanolamine, phosphatidylglycerol, cardiolipin and phosphatidylcholine. DNA-DNA hybridization showed 37–43% similarity of *P. kondratievae* to *P. denitrificans* and *P. methylutens* and 20–30% similarity to *P. alcaliphilus*, *P. alkenifer*, *P. aminophilus*, *P. aminovorans*, *P. marcusii*, *P. pantotrophus*, *P. solventivorans* and *P. thiocyanatus*. Its mol% G+C is 62.5.

Paracoccus marcusii Type Strain DSM 11574^T

This species produces bright-orange colonies caused by the presence of large amounts of carotenoids, including astaxanthin. It is an obligate aerobe and does not reduce nitrate to nitrite. It grows on glucose, fructose, galactose, mannose, arabinose, maltose, cellobiose, lactose, melibiose, sucrose, turanose, trehalose, gentiobiose, gluconate, glucuronate, galacturonic, glycerol, erythritol, mannitol, sorbitol, xylitol, inositol, adonitol, arabitol, propionate, *cis*-aconitate, citrate, lactate, malonate, succinate, formate, malate and alanine. It could not grow on methanol, methylamine, trimethylamine, dimethylformamide, thiosulfate, acetate, trimethylamine or a very wide range of other compounds. The major fatty acid is straight-chain unsaturated 18:1, with some 18:0 and 10:0. Its mol% G+C is 66. The description of this species was validly published in 1998 (Harker et al., 1998) and predated the description of the physiologically and morphologically similar *P. carontinifaciens* (Tsubokura et al., 1999). Subsequent comparison of the 16S rRNA sequences of the two strains showed that *P. carontinifaciens* and *P. marcusii* share 99.8% identity (Table 4), and the two species show only a few differences in growth substrates used. *P. carotinifaciens* was reported as motile by means of peritrichous flagella, whereas *P. marcusii* is reportedly non-motile. Whether or not these are distinct species, as is the case for *P. versutus* and *P. pantotrophus* (cf., Table 4), or are both strains of *P. marcusii* requires investigation, including the assessment of DNA-DNA hybridization between them.

Paracoccus methylutens Type Strain (DM 12) VKM B 2164^T

This species is a facultatively methylotrophic species able to grow on dichloromethane, methanol, methylamine, formate, but not formaldehyde, or di- or tri-methylamine. Nitrate is reduced to nitrite. It grows at 10–37°C, optimally at 25–30°C; pH range is 6.5–9.5 with an optimum at pH 7.0–8.0. Aerobic. It grows on a wide range of organic substrates including glucose, mannose, arabinose, maltose, ethanol, acetate, citrate and Krebs' cycle acids, and propionate, but not acetamide, rhamnose, raffinose or trehalose. Ammonium, nitrate, methylamine and urea are used as nitrogen sources. Its major fatty acid is 18:1w7. Its mol% G+C is 67.

Paracoccus pantotrophus Type Strain ATCC 35512^T, LMD 82.5^T

Coccoid shaped cells may exhibit pleomorphism on very rich media. It is very similar physiologically to both *P. denitrificans* and *P. versutus* but can be distinguished from them by its inability to use citrate and its ability to grow as an anaerobic autotroph by denitrification on sulfide or thiosulfate as sole energy substrate. It grows autotrophically on reduced sulfur compounds and hydrogen and mixotrophically and heterotrophically on a wide range of organic compounds, but the wild type will not grow on one-carbon substrates. Aerobic and anaerobic heterotrophic growth is supported by glucose, fructose, mannose, lactate, pyruvate, acetate, succinate, fumarate, gluconate, glutamate, proline, aspartate, alanine, histidine, leucine, isoleucine, acetone, propan-1,2-diol, and propan-2-ol, but not by arabinose, lactose, methyl acetate, methanol, methyl ethyl ketone, propylene oxide, oxalate or pimelate. It grew aerobically but not anaerobically on benzoate and only anaerobically on propionaldehyde. Ammonium, nitrate, urea and glutamate but not methylamine are used as nitrogen sources. Its mol% G+C is 66.

Paracoccus solventivorans Type Strain DSM 6637^T

This species grows aerobically or with denitrification on acetone, acetoacetate, 2-butanone, 2-propanol, fumarate, gluconate, ribose, pyruvate, and a number of other compounds. Optimum pH is 7.0–8.0 and optimum temperature is 30–37°C.

Ammonium is used as a nitrogen source; other nitrogen compounds are not reported. Characteristic fatty acids are 12:1*cis*5 and 20:1*cis*13. Its mol% G+C is 68.5–70.

Paracoccus thiocyanatus Type Strain IAM 12816^T

As far as is known, this organism is unique among the *Paracoccus* species in being a chemolithoautotroph able to grow aerobically on thiocyanate as its sole energy substrate; it also grows autotrophically on thiosulfate and sulfur. Optimal growth is at pH 7.5–8.5 in rich medium (range pH 6.5–9.5) and pH 7.0–8.0 in thiocyanate medium (range pH 6.0–8.5). Optimum temperature was 30–35°C (range 15–40°C). It grows on a wide range of sugars, alcohols, organic acids and amino acids, but not on citrate, benzoate, maltose, lactose, sucrose, malate and several others. Growth with denitrification occurred only on organic substrates. Ammonium and glutamate, but not nitrate, urea or aspartate, serve as nitrogen sources. It exhibits the fatty acid profile typical of the genus, with 18:1 and 19cyc and 10:0(3-OH). Its mol% G+C is 66.5–67.6.

Paracoccus versutus Type Strain ATCC 25364^T, DSM 5m82^T

This species grows as an autotroph, mixotroph and heterotroph on a very wide range of substrates. Aerobic autotrophic growth occurs on thiosulfate, sulfide, sulfur, methanol, methylamine, formate and formaldehyde, but not on tetrathionate or thiocyanate. It grows anaerobically with denitrification on organic substrates but not with reduced sulfur compounds. It grows on many sugars and organic acids, but not on lactose or cellobiose. It grows optimally at pH 7.5–8.0 in complex media (range pH 6.5–9.5) and optimally at initial pH 8.0–8.8 in thiosulfate medium without pH control; optimum pH is 7.8 on thiosulfate with pH control. Optimum temperature is 30–37°C (range 17–40°C). Ammonium, nitrate, glutamate and aspartate, but not urea, may serve as nitrogen sources. Its mol% G+C is 67–68.

Cytochromes and Electron Transport Systems in *Paracoccus* Species

P. denitrificans and those other species that have been studied in detail show complex electron transport systems both for aerobic respiration and for denitrification (Lu and Kelly, 1984a; Van Verseveld and Stouthamer, 1991; Stouthamer, 1992; Baker et al., 1998), and the respiratory system of *P. denitrificans* has long been regarded as a model for the mitochondrion (Stouthamer, 1992; Baker et al., 1998). A major difference from the mitochondrial system is the presence in *P. denitrificans* of alternative terminal oxidases (ba_3, cbb_3, and aa_3), depending on the oxygen tension in the oxidase environment. The bacteria also exhibit a diversity of branched pathways for electron transport, enabling growth under various conditions of oxygen availability from fully aerobic to anaerobic denitrification. For detailed analysis of the genetics and functioning of these complex systems the reader is referred to Baker et al. (1998). At least 17 *c*-type cytochromes have been identified in *Paracoccus* species (Baker et al., 1998), which are located both in the cytoplasmic membrane and in the periplasmic space depending on the nature of the substrate and the growth conditions, and include those which are components of terminal oxidases, the bc_1 complex and nitrate and nitric oxide reductases. Some *c*-type cytochromes involved in inorganic sulfur oxidation in *P. pantotrophus* and *P. versutus* are unusual in their large size, in their multiple subunits and in containing at least two redox centres, with widely different midpoint potentials (Kelly et al., 1997; Friedrich, 1998). The roles of these separate centres have not yet been fully explained (Lu and Kelly, 1984a; Kelly et al., 1997).

Recently the cytochromes c_{551} and $c_{552.5}$ essential for the thiosulfate-oxidizing system of *Paracoccus versutus* (see following section) have been examined using electron paramagnetic resonance (EPR) spectroscopy (J. K. Shergill, A. C. White, W-P. Lu, D. P. Kelly, A. P. Wood, C. Joannou and R. Cammack, in preparation). Cytochrome c_{551} was estimated to contain approximately six atoms of Fe per mole, and EPR showed the presence of a single type of low-spin heme, with $gz = 3.22$ and $gx \sim 1.17$. The EPR and optical absorption spectra were consistent with methionine-histidine coordination. It was concluded that cytochrome c_{551} is a hexamer of a protein containing heme *c*. Cytochrome $c_{552.5}$ showed a more complex EPR spectrum indicating at least four different types of low-spin hemes (heme-1, $gz = 2.401$, $gy = 2.245$, $gx = 1.914$; heme-2, $gz = 2.516$, $gy = 2.302$, $gx = 1.875$; heme-3, $gz = 2.583$, $gy = 2.395$, and $gx = 1.834$; and heme-4, $gz = 3.5$). These hemes responded differently to changes in pH, reduction by dithionite and the presence of ethylene glycol in the medium, and it was found that heme-2 and heme-3 were interconvertible. A small and variable amount of high-spin heme also was observed. These EPR studies indicated that the cytochrome $c_{552.5}$ of *P. versutus* contained more redox centers than the two centers identified by optical redox measure-

ments, making this cytochrome among the most complex yet characterized.

Sulfur Compound Oxidation by *Paracoccus* Species

The oxidation of thiosulfate, sulfide or thiocyanate supports the autotrophic growth of several species, but only *P. versutus* and *P. pantotrophus* GB17 have been studied in detail (Kelly, 1999):

$$S_2O_3^{2-} + 2O_2 + H_2O = 2SO_4^{2-} + 2H^+$$
$$[\Delta G^\circ = -738.7 kJ \cdot mol^{-1}]$$
$$HS^- + 2O_2 = SO_4^{2-} + H^+$$
$$[\Delta G^\circ = -732.6 kJ \cdot mol^{-1}]$$
$$SCN^- + 2O_2 + 2H_2O = SO_4^{2-} + CO_2 + NH_4^+$$
$$[\Delta G^\circ = -824.5 kJ \cdot mol^{-1}]$$

In these species, thiosulfate is oxidized by a multienzyme system located in the cytoplasm (Lu et al., 1985; Kelly, 1989; Kelly et al., 1997), the mechanism of which is summarized by Kelly et al. (1997), Baker et al. (1998) and Friedrich (1998). Considerable progress has also been made in identifying the genes in *P. pantotrophus* controlling expression of the enzymes of the thiosulfate-oxidizing system (Wodara et al., 1994; Friedrich, 1998).

Phylogenetically, *P. versutus* and *P. pantotrophus* are very similar, although DNA-DNA hybridization showed them to differ at the species level (Fig. 1; Table 4; Rainey et al., 1999). The mechanism and genetic basis of thiosulfate and sulfide oxidation have been studied in depth only in these two species (Lu, 1986; Lu and Kelly, 1983; Lu and Kelly, 1984a; Lu and Kelly, 1984b; Lu et al., 1984c; Lu et al., 1985; Kelly, 1985; Kelly, 1988; Chandra and Friedrich, 1986; Mittenhuber et al., 1991; Wodara et al., 1994; Wodara et al., 1997; Kelly et al., 1997; Friedrich, 1998). It is thus likely that the mechanism and control of inorganic sulfur oxidation are likely to be very similar, if not identical, in the two species, enabling a composite model to be derived from the available data. Such a model may be an oversimplification as differences in detail may exist between the two species (Friedrich, 1998), although these differences may be seen to be less profound as further work is done.

The enzymes and some of the cytochromes of the thiosulfate-oxidizing multienzyme system are located in the periplasm (Lu, 1986; Lu et al., 1985), and periplasmic targeting sequences are seen in at least some of the proteins of the system (Baker et al., 1998; Friedrich, 1998). Schemes interrelating the components and the proposed mechanism of the thiosulfate-oxidizing system are given by Kelly et al. (1997), Baker et al. (1998), and Friedrich (1998).

The cluster of genes involved in coding for the thiosulfate oxidizing system was initially dissected using Tn5-*mob* mutagenesis (Chandra and Friedrich, 1986; Mittenhuber et al., 1991; Friedrich, 1998). To date, a sequence of six genes coding for enzymes and cytochromes involved in thiosulfate and sulfide oxidation have been identified. Collectively these have been defined as the Sox character (ability to oxidize inorganic sulfur) in *Paracoccus* and are coded (in downstream sequence): *soxA* to *soxF* (Friedrich, 1998). The six genes comprise a minimum of 6.3 kb pairs in the *P. pantotrophus* chromosome (Friedrich, 1998), although their location in the genome is uncertain. One strain, deficient in thiosulfate oxidation, was shown to lack the 450 kbp megaplasmid (Chandra and Friedrich, 1986; Baker et al., 1998), and the Sox locus was studied using a mutant containing a full complement of megaplasmids/minichromosomes (Mittenhuber et al., 1991). The genes identified or inferred to date, together with their gene products and the probable biochemical functions of these proteins in the thiosulfate-oxidizing system, are summarized in Table 8.

Dissimilatory Nitrogen Metabolism in *Paracoccus*

Respiratory denitrification has been studied in detail in *P. pantotrophus* and *P. denitrificans* (Berks et al., 1995; Baker et al., 1998) and its genetic regulation has been comprehensively analyzed (Baker et al., 1998). Nitrate reduction to nitrite is catalyzed by two nitrate reductases: one is a membrane-bound enzyme induced in the presence of nitrate under conditions of oxygen limitation; the other is a constitutive periplasmic molybdopterin enzyme with both heme and non-heme iron sites. Nitrite is reduced to nitric oxide (NO) by a periplasmic nitrite reductase, which contains two hemes as prosthetic groups and receives electrons for nitrite reduction from cytochrome *c*550 and a copper-containing pseudoazurin. The hemes are a typical *c*-type and an unusual *d*1 heme. Nitric oxide is reduced to nitrous oxide (N_2O) by a nitric oxide reductase anchored on the periplasmic side of the cytoplasmic membrane. This enzyme contains one large and one small protein subunit, the former binding heme *b* and the latter, heme *c*. Nitrous oxide is reduced to dinitrogen by a periplasmic nitrous oxide reductase which contains two different copper centers.

Table 8. Genes and gene products of the periplasmic thiosulfate-oxidizing multienzyme system of *P. pantotrophus* and *P. versutus*.

Sox gene	Gene product	Properties of mature gene product	Proposed identity of the gene product proteins
soxA	Sox A	29-kDa diheme cytochrome	Electron transport from thiosulfate; possible 29kDa subunit of *P. versutus* cytochrome $c_{552.5}$ (56-kDa dimer)
soxB	Sox B: 60.5kDa (with 16 amino-acid leader peptide)	59-kDa mature protein	Enzyme B (60kDa) of *P. versutus*; oxidation of covalently bound thiosulfate to sulfate
soxC	Sox C: 47.5kDa (with 40 amino-acid signal peptide)	43.7-kDa mature protein	Sulfite:cytochrome *c* oxidoreductase, possibly essential for full activity of the thiosulfate-oxidizing multienzyme system
soxD	Sox D	40-kDa protein	Periplasmic diheme cytochrome *c*; possible 43-kDa subunit of *P. versutus* cytochrome c_{551} (260-kDa hexamer)
soxE	Sox E	26-kDa protein	Periplasmic diheme cytochrome *c*
soxF	Sox F	12-kDa protein	Putative flavocytochrome *c* (possible sulfide dehydrogenase)
"sox Y"	Sox Y	12-kDa peptide	Component of a heterodimer with Sox Y
"soxZ"	Sox Z	16-kDa peptide	Enzyme A (16kDa) of *P. versutus*; probable thiosulfate-binding protein

P. pantotrophus has been reported to be able to carry out denitrification also under aerobic conditions, as well as being able to nitrify under heterotrophic conditions (Robertson et al., 1988; Arts et al., 1995). Periplasmic nitrate reductase was present in such aerobically grown bacteria and was the initiator of nitrate reduction; nitric oxide and nitrous oxide reductases were also active under aerobic conditions (Bell and Ferguson, 1991). The concerted action of the processes of nitrification and denitrification was indicated by the production (by cell cultures) of ^{15}N-labeled dinitrogen and nitrous oxide from ^{15}N-ammonia as well as from ^{15}N-nitrite (Arts et al., 1995). The physiological and ecological significance of these processes is not yet fully understood.

Glucose Dissimilation by *Paracoccus* Species

While central metabolic pathways such as the Krebs' cycle probably occur in all species of *Paracoccus*, different species exhibit a diversity of mechanisms for the aerobic dissimilation of glucose. As well as the Krebs' cycle, Forget and Pichinoty (1965) showed both the Entner-Doudoroff and oxidative pentose phosphate (hexose monophosphate) pathways to operate in *Paracoccus* denitrificans, but a later report that the glycolytic pathway is absent from *P. denitrificans* is not applicable to all species of *Paracoccus* (Slabas and Whatley, 1977; Van Spanning et al., 2000). The most detailed studies of glucose metabolism were conducted with *P. versutus*, in which all three of the Embden-Meyerhof-Parnas, Entner-Doudoroff and pentose phosphate pathways can operate simultaneously in glucose-grown bacteria (Wood et al., 1977; Wood and Kelly, 1978; Wood and Kelly, 1979; Wood and Kelly, 1980). The ratios of these pathways vary according to growth conditions, and during growth on other hexoses, pentoses and disaccharides or on mixtures of glucose and maltose, the Embden-Meyerhof-Parnas pathway is completely repressed, and the Entner-Doudoroff pathway predominates (Wood and Kelly, 1979; Wood and Kelly, 1980; Smith et al., 1980).

Chromosomes and Plasmids in *Paracoccus* Species

Only a few species of *Paracoccus* have been studied with respect to their plasmid complement. The earliest detailed study showed plasmids in three strains described as *P. denitrificans* (Gerstenberg et al., 1982). One was the "Morris" strain which can be assumed to have been correctly described as *P. denitrificans* (Goodhew et al., 1996; Rainey et al., 1999) and contained one megaplasmid of molecular mass exceeding 300 MDa. Another was the "Stanier" strain, which subsequently appeared in culture collections as both ATCC 17741 and DSM 65 (Goodhew et al., 1996). It is now known that ATCC 17741 is the type strain of *P. denitrificans* but that DSM 65 is a strain of *P. pantotrophus* (Rainey et al., 1999). This means that it is uncertain whether the "Stanier" strain used by Gerstenberg et al., 1982) was *P. denitrificans* or

P. pantotrophus. It contained two plasmids of molecular masses around 50 MDa and >300 MDa (Gerstenberg et al., 1982). The "Vogt" strain (DSM 415) contained only one <300 MDa megaplasmid and may thus also be *P. denitrificans* (Gerstenberg et al., 1982). *P. denitrificans* DSM 413 and *P. versutus* were also shown to contain megaplasmids <500 kb (Wlodarczyk and Piechuka, 1995; Jordan et al., 1997). We conclude that the evidence currently available shows the type strain (and its derivatives) of *P. denitrificans* may contain megaplasmids of at least 450 kb in size, but it is uncertain if they can also contain smaller (100 kb) plasmids, as none were present in strains DSM 413 or the "Stanier" strain (Jordan et al., 1997; Rainey et al., 1999). In contrast, some strains of *P. pantotrophus* do contain 85–110 kb plasmids as well as megaplasmids of 450 kb, but one strain (DSM 11072) lacked the 100 kb plasmid (Jordan et al., 1997; Rainey et al., 1999).

The genomic structure of *P. denitrificans* and *P. pantotrophus* is complex in that several very large DNA molecules comprise the genome and are presumed to be chromosomal elements (Baker et al., 1998; Winterstein and Ludwig, 1998). The authentic *P. denitrificans* strains ATCC 13543, DSM 413 and Pd 1222 contain three chromosomal DNA molecules: I, 1.83 Mb; II, 1.16 Mb; and III, 0.67 Mb (Winterstein and Ludwig, 1998), of which at least molecules I and II are linear DNA. The distribution among these molecules of genes coding for respiratory oxidases, cytochrome *c*550, methanol oxidation and *S*-formylglutathione hydrolase appeared "random," indicating that the three replicons together comprise the genome and are probably replicated concurrently (Winterstein and Ludwig, 1998). Two strains of *P. pantotrophus* (LMD 82.5 and DSM 65) contain four DNA molecules: 2.2, 1.5, 0.71 or 0.77, and 0.5 Mb, of which the 0.71 Mb molecule was circular (Winterstein and Ludwig, 1998). In addition, the *P. pantotrophus* strains showed the expected plasmid of molecular size 97 kb (LMD 82.5) or 60 kb (DSM 65).

While at least the three large replicons in both *P. denitrificans* and *P. pantotrophus* are likely to represent the chromosomal DNA (with a genome size of around 4 Mb), the genetic importance of the megaplasmids or mini-chromosomes of <450 kb in *P. denitrificans*, *P. pantotrophus* and *P. versutus* is unknown, as is that of the 107 bp pTAV1 plasmid of *P. versutus* (Jagusztyn-Krynicka et al., 1990; Bartosik et al., 1995), although loss of the 450 kb plasmid from *P. pantotrophus* was possibly linked to loss of the ability to carry out thiosulfate oxidation (see section on sulfur compound oxidation in this Chapter; Chandra and Friedrich, 1986).

Biotechnological Potential of *Paracoccus* Species

In common with other facultative denitrifiers, *P. denitrificans* can contribute to the removal of nitrate from wastewaters, and *P. pantotrophus* was isolated from a desulfurizing and denitrifying effluent-treatment system (Robertson and Kuenen, 1983). Experimentally, long-term continuous denitrification was obtained using *P. denitrificans* immobilized with a polyelectrolyte complex (Kokufuta et al., 1987). *Paracoccus* species are probably important components of many wastewater treatment system communities, having been found in denitrifying sand filters and activated sludge systems (Ohara et al., 1990; Katayama et al., 1995; Neef et al., 1996). Others have been isolated from biofilters treating effluent gases and from contaminated soils (Siller et al., 1996; Lipski et al., 1998). The ability of different species to degrade unusual and potentially polluting compounds indicates their potential role in natural or contrived bioremediation systems. Target compounds include methanol, acetone, dichloromethane and other solvents, tetramethylammonium compounds, methylamines, substituted formamides, thiocyanate, sulfides, organic sulfur compounds such as carbon disulfide, carbonyl sulfide and methanethiol, all of which are waste products of diverse commercial processes. As yet, no controlled system inoculated with specific *Paracoccus* strains seems to have been used on a commercial scale.

Exploitation of *Paracoccus* species as potential sources of bio-products has also received little attention to date. A mixture of co-immobilized cells of *P. denitrificans* and Corynebacterium was shown to be effective for the continuous production of L-phenylalanine from acetamidocinnamic acid (Nishida et al., 1987), and the production of relatively large amounts of astaxanthin by *P. marcusii* and *P. carotinifaciens* might enable these species to be exploited for the production of such pigments (Harker et al., 1998; Tsubokura et al., 1999).

Acknowledgements. We are grateful to Dr. T. P. Tourova (Moscow) and Dr Y. A. Trotsenko (Pushchino) for providing us with the 16S rRNA gene sequences of *P. methylutens* and *P. kondratievae* and for letting us see their manuscript on *P. kondratievae* prior to publication and to Dr. I. P. McDonald (Warwick) for assistance during the drafting of this study. We thank Dr. R. J. M. van Spanning (Amsterdam) and Dr. S. C. Baker (Oxford) for showing us unpublished manuscript material.

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DOI: 10.1007/0-387-30745-1_13

CHAPTER 3.1.12

The Genus *Phenylobacterium*

JÜRGEN EBERSPÄCHER AND FRANZ LINGENS

The genus *Phenylobacterium* comprises a single species called *P. immobile*, which is remarkable for its extremely limited nutritional spectrum. All strains isolated and described hitherto grow optimally only on artificial compounds like chloridazon, antipyrin, and pyramidon (formulas in Fig. 1). Chloridazon, formerly called pyrazon, is the active ingredient of the herbicide Pyramin®, which is used for the control of broadleaf weeds in sugar beet and beet root cultures. The fact that the breakdown of this herbicide is a microbial process was demonstrated by studies with soil samples, including heat-sterilized soil (Drescher and Otto, 1969; Frank and Switzer, 1969). Engvild and Jensen (1969) described the isolation of bacteria capable of growth on chloridazon as sole source of carbon and energy. At the same time and independently, Fröhner et al. (1970) isolated chloridazon-degrading bacteria that proved to be similar to the organisms of Engvild and Jensen. Meanwhile, more than 20 different strains have been isolated that can grow on the herbicide chloridazon and also on the structurally related analgesics antipyrin and pyramidon. All of these xenobiotic-degrading bacteria show a high degree of similarity with respect to different properties, and they were grouped together in one single species, named *Phenylobacterium immobile* (Lingens et al., 1985).

P. immobile is not closely related to any other Gram-negative bacterium, as demonstrated by 16S rRNA investigations (Ludwig et al., 1984). It was found to be a member of subgroup alpha-2 of the alpha subclass of the proteobacteria, standing phylogenetically isolated in this group (proteobacteria were formerly named "purple bacteria and their nonphotosynthetic relatives"). Lipopolysaccharide analysis (Weisshaar and Lingens, 1983), serological studies (Dorfer et al., 1985), investigations on the polyamine pattern (Busse and Auling, 1988), and ubiquinone analysis (R. M. Kroppenstedt, J. Eberspächer, and F. Lingens, unpublished observations) have confirmed the results on the phylogenetic position of *P. immobile*.

Habitat

P. immobile seems to be a typical inhabitant of the upper aerobic part of the soil. Different strains have been isolated from soil samples originating from various locations all over the world. Attempts to demonstrate the breakdown of chloridazon in soil or in mud samples under anaerobic conditions failed. Although in one case, a slow degradation of chloridazon in river water was observed, efforts to isolate chloridazon-degrading bacteria from this specific water sample were without success. However, we cannot rule out the possibility that phenylobacteria occur also in aquatic habitats.

Isolation and Cultivation

The technique for the enrichment of *P. immobile* is based on selective pressure exerted on a microbial soil population. This technique leads to the isolation of bacteria that are able to utilize synthetic molecules not normally encountered in nature as sole carbon source. As a synthetic substrate for selective enrichment, chloridazon, antipyrin, or pyramidon can be applied.

Isolation Procedure

A convenient method for the isolation of *P. immobile* starts with sampling about 300 g of soil or compost. Air-dried soil or soil with very low humus content was found to be less satisfactory. The soil sample is mixed with 0.5 g of chloridazon, antipyrin, or pyramidon, and the preparation is incubated at 30°C or at room temperature in a flower pot and regularly moistened with water. Degradation of the xenobiotic compound is followed by thinlayer chromatography. From the excess water that drains from the flower pot, about 0.05 ml is applied to a thin layer plate coated with silica gel containing a fluorescent indicator with maximum sensitivity under UV radiation of 254 nm. Decomposition is complete when the spot of the xenobiotic compound is no longer detectable under UV light and a new spot corresponding to the dephenylated heterocyclic moiety of the xenobiotic appears, usually after one to several weeks, depending on the soil. Then a 5-g sample of the active soil is placed into an Erlenmeyer flask containing 50 ml of mineral salts medium (for composition, see recipe below) supplemented with the xenobiotic as carbon source at a concentration of 0.4%. This culture is

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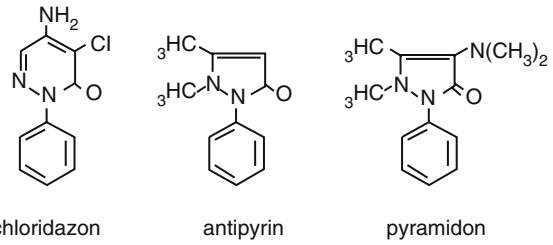

Fig. 1. The herbicide chloridazon and the structurally related analgesics antipyrin and pyramidon can be used for the enrichment of *P. immobile*. These xenobiotics are also the best growth substrates for the organism.

incubated on a rotary shaker at 30°C, and degradation is monitored by thin layer chromatography. When the decomposition of the xenobiotic is complete, 1 ml of the culture fluid is transferred into a new Erlenmeyer flask. After 5 to 10 transfers, a sample of the liquid culture is streaked onto agar plates containing the same medium. Single colonies, which normally appear after 1 to 3 weeks, are picked and again streaked onto agar. After 5 to 10 transfers, pure cultures can usually be obtained. In several cases, the isolation of a pure culture was more difficult than usual because *P. immobile* was closely associated with other nondegrading bacteria. In one case the other organism was identified as *Pseudomonas cepacia*.

Medium for *Phenylobacterium immobile*

Fröhner et al. (1970) found, for the first isolates of *P. immobile*, that vitamin B_{12} was an essential growth factor. This vitamin is thus routinely added to the mineral salts medium at 30 µg/liter. When grown on chloridazon or antipyrin *P. immobile* acidifies the culture fluid. Therefore a medium with reasonable buffer capacity was developed that due to the organism's osmotic sensitivity had to be kept at a low total salt concentration.

Medium for the Culture of *P. immobile*

The mineral salts medium has the following composition per 1 liter of deionized water:

$Na_2HPO_4 \cdot 12H_2O$	0.7 g
KH_2PO_4	0.3 g
$(NH_4)_2HPO_4$	0.7 g
$(NH_4)H_2PO_4$	0.3 g
$(NH_4)_2SO_4$	0.1 g
Trace element solution (see below)	1 ml
Vitamin B_{12} solution, 0.03 mg/ml	1 ml
$MgSO_4 \cdot 7H_2O$	0.25 g
$CaCl_2 \cdot 6H_2O$	0.05 g

To avoid precipitates the magnesium and calcium salts are each dissolved separately.

Trace element solution per 1 liter of deionized water:

$MnSO_4 \cdot 4H_2O$	400 mg
$ZnSO_4 \cdot 7H_2O$	400 mg
$FeCl_3 \cdot 6H_2O$	200 mg
$CuSO_4 \cdot 5H_2O$	40 mg
H_3BO_3	500 mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	200 mg
KI	100 mg
Biotin	100 mg

As carbon source, chloridazon, antipyrin, or pyramidon is added at a concentration of 0.4 to 1 g per liter. The pH of the medium is 7.0.

Cultivation

Optimum growth and maximum cell yield are achieved when *P. immobile* is grown at 30°C in mineral salts medium with chloridazon or antipyrin at 0.4 to 1 g per liter. Under these conditions, a doubling time of 7 to 8 hours is observed, and, depending on the strain, a yield of about 0.4 to 1.0 g bacteria (wet weight) per liter of culture fluid is obtained.

Complex media used for routine cultivation in bacteriology with 10 to 20 g peptone per liter, or the same amount of yeast extract plus meat extract, do not support the growth of *P. immobile*. These bacteria were found to be osmotically sensitive, as demonstrated by NaCl addition to the chloridazon-mineral salts medium. At a NaCl concentration of 5 to 7 g per liter, considerable growth inhibition was observed with total inhibition at 10 g per liter. *P. immobile*, however, does grow on complex media with 0.5 to 2 g per liter peptone plus yeast extract, but growth is considerably slower than on antipyrin or chloridazon.

The strictly aerobic bacteria are cultivated on a rotary shaker, and in large-scale fermentations they are well aerated with 50 liter per min of air in a 100-liter-fermentor. For large-scale fermentation conducted with the type strain *P. immobile* strain E, a scale-up ratio of 1:10, starting with a 1-liter culture inoculated from an agar plate, was found to yield good results. In this case, a fermentation time of 24 h (from inoculation of the 100-liter fermentor to the late log phase) and a cell yield of 1 g per liter were obtained.

Growth on Agar Plates

Mineral salts medium with either 0.2% chloridazon or 0.1% antipyrin as carbon source and supplemented with 15 g agar per liter allows good growth of *P. immobile*. However, it takes at least 4 to 7 days until the first tiny colonies are visible on the agar even when the plate is inoculated with a large number of bacteria. After 2 to 3 weeks, the colonies reach a size of 1 to 2 mm in diameter. A concentration of 2 g chloridazon per liter leads to the precipitation of fine chloridazon

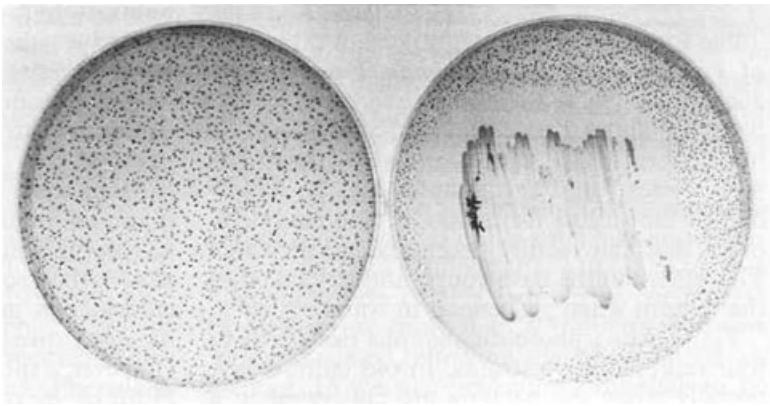

Fig. 2. Agar plates containing mineral salts medium with 2 g chloridazon per liter. On the plate at the right chloridazon crystals have been metabolized by *P. immobile*. The large crystal at the left margin of the bacterial smear is the dephenylated heterocyclic moiety of chloridazon, the main metabolite of chloridazon degradation.

crystals in the agar (Fig. 2). During growth on this agar, *P. immobile* removes the crystals by degradation, and as a result of bacterial growth, a clearance zone around the smear develops. In agar cultures of 4 weeks and older, new, and in most cases, relatively large crystals form within the bacterial smear. These crystals were identified as the dephenylated heterocyclic moiety of chloridazon, which is a dead-end metabolite of chloridazon degradation.

Purity of Cultures

Since none of the *P. immobile* strains grows on an ordinary complex medium, an inoculation of an agar plate containing the following medium is routinely used for testing purity:

Testing Medium

The complex medium contains per liter deionized water:

Nutrient broth (dehydrated)	10 g
Yeast extract	5 g
NaCl	5 g

Growth on this medium indicates contamination of the culture.

Preservation of Cultures

For short-term preservation, bacteria are regularly transferred on agar at intervals of 2 to 3 weeks. For long-term preservation, the bacteria suspended in skimmed milk are dropped onto silica gel grains and stored at 4°C. Good results were obtained with this method, when transfer is repeated every 2 to 3 years. However, even after a period of 10 years some of the strains were viable. Storage at –80°C of a concentrated bacterial suspension in fresh chloridazon-mineral salts medium supplemented with 15% glycerol also resulted in good viability after 2 years.

The following strains have been deposited in the Deutsche Sammlung für Mikroorganismen (DSM) and at the American Type Culture Collection (ATCC): strain E, type strain (DSM 1986, ATCC 35973); strain A_{12} (DSM 2115, ATCC 35972); strain J_1 (DSM 2116, ATCC 35974); strain K_2 (DSM 2117, ATCC 35975); strain N (DSM 2113, ATCC 35976); and strain Z_6 (DSM 2114, ATCC 35977). Two further strains have been deposited at the Czechoslovak Collection of Microorganisms (CCM): strain C_2 (CCM 3864) and strain R (CCM 3865).

Identification

The morphology and physiological properties of *P. immobile* are not especially remarkable (see Table 1). Nearly all biochemical tests are negative, and, with regard to this fact, *P. immobile* shows similarities with *Acinetobacter*. In fact, *Phenylobacterium immobile* was originally identified as an *Acinetobacter* species (Fröhner et al., 1970) but determination of the GC content clearly ruled out this identification.

The most distinguishing feature of *Phenylobacterium immobile* is its high nutritional specialization. This property is shared, although in a less pronounced way, by certain members of the pseudomonads, together with some morphological, physiological, and biochemical characteristics. The general definition of *Pseudomonas*, however, excludes nonmotile organisms, and all strains of *Phenylobacterium immobile* are nonmotile.

P. immobile is not closely related to any other Gramnegative eubacterium, as shown by partial sequence analysis of 16S rRNA from strain E (Ludwig et al., 1984). Phylogenetically, it was found to belong to subgroup alpha-2 of the proteobacteria (see "Phylogenetic Position" in this Chapter), with an isolated position in this group.

Table 1. Important characters for the identification of *Phenylobacterium immobile*.

Cells: Rods, coccal rods, or cocci; 0.7 to 1.0 by 1.0 to 2.0 µm; singly, in pairs or short chains; nonmotile; nonsporeforming; nonpigmented; no sheaths; no prosthecae.

Colonies: Develop slowly on chloridazon-mineral salts agar, small, colorless, circular, entire edges, convex; smooth or rough colonies possible.

Staining: Gram negative, not acid-fast, no capsule.

Physiology: Strictly aerobic, catalase positive, weakly oxidase positive; no growth at 4°C and 37°C, optimum growth at 28–30°C; no growth at pH 4 and 9, growth between pH 6.5 and 8, optimum pH 6.8–7.0; osmotically sensitive; vitamin B_{12} is a growth factor; NH_4^+ and NO_3^- used as N sources, no denitrification, no N_2 fixation.

Biochemical tests: Negative results in the indole reaction, methyl red, Voges-Proskauer, litmus milk, and urease test; no hydrolysis of gelatin, starch, agar, or esculin; weakly positive for H_2S from thiosulfate or cysteine; no acid or gas from 34 different sugars.

Carbon sources: Optimum growth on chloridazon, antipyrin, pyramidon, and L-phenylalanine (most strains after long lag phase only); no growth on simple carbon sources like sugars, alcohols, carboxylic acids (31 compounds tested), and amino acids (except phenylalanine and glutamate); poor growth on glutamate, pyruvate, fumarate, succinate, and malate; no growth on routine complex media unless medium is diluted (0.5 to 2 g peptone per liter).

GC content: 65–68.5 mol%.

Morphology

Table 1 summarizes some important properties of *Phenylobacterium immobile*. On chloridazon-mineral salts agar, after 2 to 3 weeks colonies are about 1 to 2 mm in diameter and do not adhere to the agar (see also "Growth on Agar Plates," and this chapter Fig. 2). Nearly half of the strains form smooth and shiny colonies that can readily be emulsified in water. The other strains have rough and dry colonies that clump when suspended in water.

Fig. 3 shows photomicrographs from cells of four representative strains. In old cultures, especially when the bacteria are cultivated in a medium that allows only poor growth, such as a dilute complex medium, pleomorphic forms, such as long rods, long chains of cells connected by small filaments, or club-shaped and elliptical forms, sometimes occur.

Gram staining, Ziehl-Neelsen staining, and capsule staining are negative, and electron microscopy of thin sections also reveals the typical Gram-negative cell wall pattern (Lingens et al., 1985). Strain K_2, which forms smooth colonies on agar, was treated with ruthenium red and electron microscopy of ultrathin sections revealed the presence of a microcapsule—or according to the definition of Costerton et al. (1981), a "flexible" capsule—surrounded by a slime layer of acid polysaccharides (Lingens et al., 1985). No microcapsule was detected in ruthenium-red-stained cells of strain E, which forms rough colonies on agar.

Physiological and Biochemical Characteristics

The osmotic sensitivity and nutritional specialization of *Phenylobacterium immobile* do not allow the use of routine media for biochemical characterization. Therefore, tests were performed in modified media (Lingens et al., 1985). The characteristics determined for all strains are shown in Table 1.

Utilization of Carbon Sources

All the different strains of *Phenylobacterium immobile* do not utilize all three xenobiotic substrates. Whereas chloridazon and antipyrin are well utilized by most strains, pyramidon is a growth substrate for only 7 of the 22 isolates. When pyramidon is added to media containing chloridazon or antipyrin, growth inhibition is observed among all of the isolates.

The pathway for the degradation of the three xenobiotics was elucidated by metabolic and enzymatic studies (Blecher et al., 1981; Eberspächer and Lingens, 1978; Miller et al., 1977; Sauber et al., 1977a; Sauber et al., 1977b). The pathway follows the well-known route for the oxidative dissimilation of aromatic compounds, and in the case of these xenobiotics only the aromatic nucleus is used as a carbon source, the heterocyclic moiety remaining unchanged.

More than 20 different heterocyclic or aromatic compounds that are structurally related to chloridazon or antipyrin were tested as possible carbon sources (Lingens et al., 1985). Most chloridazon analogs with altered heterocyclic moiety proved to be good growth substrates. However, a substitution at the aromatic nucleus, as for *o*-, *m*- or *p*-methylchloridazon, made the compound nondegradable by the organism. Of the aniline derivatives tested, N-methylacetanilide and N-methylformanilide were found to be poor growth substrates. Of 18 aromatic compounds that usually are utilized by various bacterial species, none

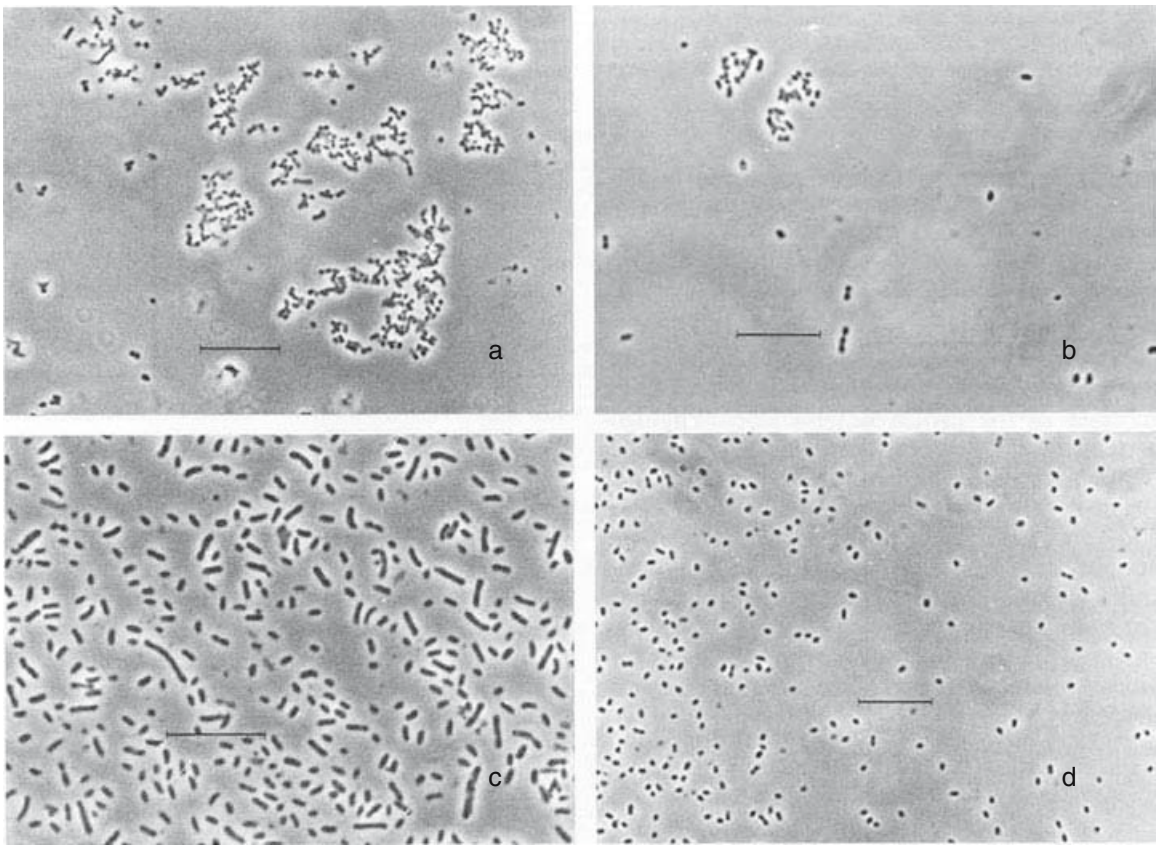

Fig. 3. Phase contrast photomicrographs of cells of *Phenylobacterium immobile*. (a) Strain A_{13}; (b) strain E, the type strain; (c) strain K_2; and (d) strain N. Bars = 10 µm.

supported growth of *Phenylobacterium immobile*. Among these compounds were benzene, toluene, phenol, catechol, benzaldehyde, benzoate, and a number of mono- and dihydroxylated benzoates.

One strain (strain N) was found to grow well on L-phenylalanine with a normal lag phase of 1 day. All other strains had lag phases of 2 to 3 weeks, but then they also grew well on phenylalanine. The long lag phases were only observed when the strains were transferred from chloridazon or antipyrin to phenylalanine for the first time, since after additional transfers the bacteria grew immediately. In liquid cultures during growth on mineral salts medium with phenylalanine, especially at higher concentrations (3 to 5 g per liter), a yellowish-green fluorescent pigment is produced. On chloridazon or antipyrin mineral salts media, a greenish-yellow nonfluorescent pigment is formed. Phenylalanine-induced cells also grow well on phenylpropionate, phenylpyruvate, and phenyllactate.

Nucleic Acid Data

GC content of the DNA of *P. immobile* was found to be between 65 and 66.5 mol%, although for one strain (C_2) a somewhat higher value of 68.5 mol% was determined. DNA hybridization tests revealed 100% homology of DNA preparations of four different strains with DNA of strain R. No homology was found using DNA from *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas fluorescens*, or calf thymus.

Depending on the strain, between one and six different plasmids were found, which varied in size from 8 to 300 megadaltons (Kreis et al., 1981).

From the type strain, 16S rRNA was isolated, and oligonucleotides of hexamer size and larger were sequenced (Ludwig et al., 1984). With this oligonucleotide catalog, similarity coefficients (S_{AB} values) to more than 400 microorganisms were calculated, allowing the phylogenetic allocation of *Phenylobacterium immobile* to be determined (see "Phylogenetic Position" in this Chapter).

Lipopolysaccharide, Peptidoglycan, and Polyamine Pattern

The composition of the carbohydrate moiety and of the lipid A from the lipopolysaccharide of the

type strain has been described by Weisshaar and Lingens (1983). Remarkable is the presence of one mole of 2,3-diamino-2,3-dideoxy-D-glucose as a constituent of the lipid A backbone. This diaminosugar was only found in members of subgroup alpha-2 of the alpha subclass of the proteobacteria (see "Phylogenetic Position" in this Chapter), whereas the lipid A of most Gram-negative bacteria contains glucosamine.

The detection of ester-linked 3-hydroxy-5-*cis*-dodecanoic acid as a major substituent in the lipopolyaccharide is also remarkable (Bellmann and Lingens, 1985a). This unusual fatty acid, not found in nature before, was used to demonstrate the presence of *Phenylobacterium immobile* in soil samples (Bellmann and Lingens, 1985b).

The peptidoglycan composition of the type strain was identical to that of a normal Gram-negative bacterium (Lingens et al., 1985). The type strain was found to contain only sym-homospermidine as a polyamine component (Busse and Auling, 1988).

Toxicity

Bacteria of *P. immobile* are harmless when tested in rats and rabbits (Kaiser et al., 1981). Tests included oral administration, intracutaneous and intraperitoneal injections of the bacteria, and inhalation experiments.

Enzymes

The type strain was found to possess all of the enzymes of the citric acid cycle. Properties of citrate synthase, of rhodanese (Layh et al., 1982), of arogenate (pretyrosine) dehydrogenase (Keller et al., 1982), and of *meta*-cleaving chloridazon-catechol-dioxygenases (Schmitt et al., 1984) were studied in more detail, and the taxonomic significance of these enzymes has been discussed.

Serology

Agglutination and immunofluorescence tests revealed the serological uniformity of the different strains (Layh et al., 1983). Slight differences in immune reactions allowed a classification of the strains into 5 serological subgroups. No relationship was found between *Phenylobacterium immobile* and 40 representative Gram-negative bacteria, including *Acinetobacter calcoaceticus*, *Azospirillum brasiliense*, *Paracoccus denitrificans*, different *Pseudomonas* species, *Rhizobium* species, *Rhodomicrobium vannielii,* and *Rhodopseudomonas capsulata.* A slight but significant immunofluorescence reaction was observed with *Pseudomonas vesicularis*, *Gluconobacter oxydans*, *Aquaspirillum itersonii*, and *Rhodospirillum rubrum* (Dorfer et al., 1985). Crossed immunoelectrophoresis revealed a serological relationship between *Phenylobacterium immobile* and *Pseudomonas diminuta* (J. Dorfer, C. Löffler, J. Eberspächer, and F. Lingens, unpublished observations).

Phylogenetic Position

Partial sequence analysis of 16S rRNA has revealed the isolated phylogenetic position of *Phenylobacterium immobile* (Ludwig et al., 1984). The highest S_{AB} values (0.51) were found with *Pseudomonas diminuta* and *Rhizobium leguminosarum*. Similarity coefficients of this magnitude indicate a rather remote relationship that would not be detectable by DNA-DNA hybridization. A comparison of the 16S rRNA nucleotide catalogs showed that *Phenylobacterium* is a member of subgroup alpha-2 of the alpha subclass of the proteobacteria (in the nomenclature of Stackebrandt et al., 1988). These organisms have also been called the "subgroup alpha-2 of the alpha purple bacteria" (Woese, 1987) or the "subgroup Ib of the purple nonsulfur bacteria and their nonphotosynthetic relatives." Members of this group belong to the "4th rRNA superfamily," (de Vos and de Ley, 1983). S_{AB} values of 0.49 and 0.46 were found with *Rhodopseudomonas viridis*, *R. capsulata*, *R. sphaeroides*, *Rhodomicrobium vannielii*, and *Aquaspirillum itersonii*. For representatives of the other major groups of Gram-negative bacteria, including *Acinetobacter calcoaceticus* and the two phylogenetically defined clusters of the genus *Pseudomonas*, S_{AB} values of 0.23 to 0.32 were obtained, indicating a wide phylogenetic distance.

In accordance with this finding, weak serological reactions of *Phenylobacterium immobile* were found only with members of the alpha subclass of the proteobacteria (Dorfer et al., 1985) and not with any other Gram-negative bacteria.

Furthermore, the demonstration of 2,3-diamino-2,3-dideoxy-D-glucose as a lipid A constituent of *Phenylobacterium immobile* supports its relationship to subgroup alpha-2 of the proteobacteria. This unusual sugar was also detected in *Rhodopseudomonas viridis*, *R. palustris*, *R. sulfoviridis*, *Pseudomonas diminuta*, *P. vesicularis*, and *Nitrobacter winogradskyi*, which are all members of the same phylogenetic group (Weckesser and Mayer, 1987).

Busse and Auling (1988) have shown that polyamines may serve as a useful chemotaxonomic marker within the proteobacteria. They found that the species of the alpha-2 subgroup have sym-homospermidine as the dominant

component of the polyamine pattern. Consistent with its phylogenetic position, *Phenylobacterium immobile* contains sym-homospermidine exclusively.

Ubiquinones are also useful chemotaxonomic markers, and members of the alpha subgroup of the proteobacteria were shown to contain ubiquinone composed of 10 isoprenoid units. Strains E, J_1, and Z_6 of *P. immobile* contain a ubiquinone of the Q-10 type (R. M. Kroppenstedt, J. Eberspäher, and F. Lingens, unpublished observations).

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Prokaryotes (2006) 5:257–265
DOI: 10.1007/0-387-30745-1_14

CHAPTER 3.1.13

Methylobacterium

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Introduction

The genus *Methylobacterium* is composed of a variety of pink-pigmented facultatively methylotrophic (PPFM) bacteria, which can grow on one-carbon compounds such as formate, formaldehyde and methanol as sole source of carbon and energy as well as on a wide range of multicarbon growth substrates. Most, but not all, strains can grow on nutrient agar and some can grow on methylated amines. Only one strain has been reported to utilize methane as sole carbon source.

Taxonomy

The first *Methylobacterium* strain to be described in the literature was isolated by Bassalik (1913) from earthworm contents and named *Bacillus extorquens*. Although they are common soil and environmental organisms, the PPFM bacteria were not isolated and studied extensively until the 1960s and 1970s, when interest in the study of the one-carbon assimilation pathways common to methylotrophic organisms and in the commercial applicability of these organisms first began.

Prior to 1960, the taxonomy of many of the isolates now assigned to *Methylobacterium* was still uncertain. Although regarded as Gram-negative, these organisms often stained Gram-variable. This Gram-variability, coupled with their morphological properties (mainly rods, which are occasionally branched and exhibit polar growth), has contributed to much of the confusion surrounding their checkered taxonomic history.

For example, Bhat and Barker (1948) assigned the *B. extorquens* isolate of Bassalik to the genus *Vibrio* as *V. extorquens*. Krasil'nikov (1959) and Bassalik et al. (1960) subsequently transferred the same species to *Pseudomonas* and *Flavobacterium*, respectively, before it temporarily came to rest, in the 8th edition of *Bergey's Manual of Determinative Bacteriology*, as *P. extorquens* incertae sedis (Doudoroff and Palleroni, 1974).

In a study of amine-utilizing bacteria, den Dooren de Jong (1927) described the pink, methylamine-utilizing species *Protaminobacter rubrum*, which De Vries and Derx (1953) later found to be very similar to organisms they had isolated from leaf nodules and leaf surfaces. On the grounds that all the organisms were Gram-negative, motile, branching rods, De Vries and Derx (1953) grouped *P. rubrum* with their isolates in the genus *Mycoplana* as *M. rubra*.

Peel and Quayle (1961), studying C-1 assimilatory pathways in methylotrophs, noted similarities between their own isolates, *Pseudomonas* AM1, and various other methylotrophic bacteria, including *V. extorquens*, *Protaminobacter ruber* (spelling amended, Breed et al., 1957) and *Pseudomonas methanica* isolated by Dworkin and Foster (1956). Peel and Quayle questioned the justification for classifying these organisms in different genera.

An early, but limited, taxonomic study by Stocks and McCleskey (1964) compared *V. extorquens*, *Protaminobacter ruber*, *Pseudomonas* AM1, and Harrington and Kallio's (1960) strain of *Pseudomonas methanica* with their own isolates and concluded that all were very similar and should be regarded as strains of *V. extorquens*, with the reservation that *Vibrio* might not prove to be the most suitable generic location.

In the more recent studies, Kouno and Ozaki (1975) isolated 59 different PPFM isolates from a variety of soil and water samples, and Austin et al. (1978) studied isolates from the phylloplane of *Lolium perenne*. In both these studies, there was agreement that the taxonomy of these organisms was obscure and needed further examination. Subsequently, Austin and Goodfellow (1979) concluded that their isolates were sufficiently different from *Protaminobacter ruber*, *Pseudomonas rhodos* (Heumann, 1962) and *P.* AM1 to merit placement in a new species, which they named *Pseudomonas mesophilica*.

Patt et al. (1974) isolated the first reported PPFM strain able to utilize methane and created

a new genus *Methylobacterium* to accommodate it. The new species *M. organophilum* was proposed for this single strain. Unfortunately, the genus description was based on the detailed examination of only one strain (*M. organophilum* xx), which has since lost its ability to utilize methane (R. S. Hanson, personal communication).

In a detailed taxonomic study, Green and Bousfield (1982) found *M. organophilum* strain xx to be phenotypically very similar to many of the strains of PPFM discussed previously, none of which could utilize methane. All of the 149 strains examined, using 140 biochemical, physiological, and morphological features, including *M. organophilum*, fell into either of two related (≥70% similarity) clusters, which were well separated from other facultative methylotrophs and nonmethylotrophic reference strains. As a result of this work, Green and Bousfield (1982) suggested that all the PPFM bacteria constituted a distinct taxon, which could be excluded from most of the genera to which they had previously been assigned. The genus *Methylobacterium* was chosen to accommodate this taxon (Green and Bousfield, 1981).

However, as the description of *Methylobacterium* (Patt et al., 1976) excluded organisms that could not utilize methane, an emended genus description was proposed (Green and Bousfield, 1983) that would allow the inclusion of all PPFM strains (whether they utilized methane or not), thus removing methane assimilation as an essential feature of the genus. At the same time, the description of *Methylobacterium* was more tightly circumscribed to prevent the genus from being used as a "dumping ground" for every facultative methane utilizer subsequently isolated, irrespective of its taxonomic relatedness to the PPFM bacteria.

As a result of this proposal the emended genus *Methylobacterium*, in addition to the type species *M. organophilum*, now contained three other validly named species: *Pseudomonas rhodos* (Heumann, 1962), renamed *M. rhodinum*; *Pseudomonas mesophilica* (Austin and Goodfellow, 1979), renamed *M. mesophilicum*; and *Pseudomonas radiora* (Ito and Iizuka, 1971), renamed *M. radiotolerans*.

Despite the overall similarities of the PPFM organisms and their recognition as a distinct taxon, there nevertheless remained doubts about the internal heterogeneity of the group (Green and Bousfield, 1983; Urakami and Komagata, 1981). This doubt was reinforced when subsequent work on representative strains of PPFM (Hood et al., 1987; Hood et al., 1988) involving DNA-DNA similarities and electrophoretic comparison of total soluble proteins revealed four major and several minor similarity (homology) groups. As a result, three new species of *Methylobacterium* were proposed (*M. rhodesianum*, *M. zatmanii* and *M. fujisawaense*) and several other probable centers of variation within the genus were recognized. Other workers have since described four new species. In 1993, Urakami et al. (1993) described a new species *Methylobacterium aminovorans*, which is involved in the biodegradation of tetramethylammonium hydroxide (TMAH) and *N*,*N*-dimethylformamide (DMF). In 1998, a strain that tolerates high (≥50 mM) thiocyanate and cyanate and is capable of utilizing these compounds as sole nitrogen source was isolated and described. This new species was named *Methylobacterium thiocyanatum* (Wood et al., 1998). The two most recently proposed species can grow on chlorinated methyl compounds as sole carbon and energy source. *M. chloromethanicum* (McDonald et al., 2001) and *M. dichloromethanicum* (Doronina et al., 2000) can utilise chloromethane (methyl chloride) and dichloromethane, respectively.

Two additional non-pink pigmented facultative methylotrophs claimed to be able to utilize methane as sole source of carbon and energy and to belong to the genus *Methylobacterium* ("*M. ethanolicum*" and "*M. hypolimneticum*"; see Lynch et al., 1980) have since been shown to be taxonomically unrelated to the PPFM (P. N. Green, unpublished observations).

Thus, twelve validated species of the genus *Methylobacterium* presently exist—the eleven discussed above plus *M. extorquens*, the type strain of which was recovered in one of the major DNA similarity (homology) groups and had already been described by Urakami and Komagata (1984) as *Protomonas extorquens*. As *Methylobacterium* was shown to have nomenclatural priority over *Protomonas* (Bousfield and Green, 1985), *P. extorquens* was subsequently transferred to the genus *Methylobacterium*.

Habitat

Members of the genus *Methylobacterium* are ubiquitous in nature and are thus found in a variety of habitats (Green and Bousfield, 1981; Green and Bousfield, 1983), including soil, dust, freshwater, lake sediments, leaf surfaces and nodules, rice grains, air, and hospital environments, and in various products and processes, e.g., as contaminants in pharmaceutical preparations such as face creams. As a common airborne organism, PPFM can occur in a wide variety of commercial processes where growth conditions are favorable, including various fermentation processes. Our identification service has also had isolates from pure water users such as silicon chip manufacturers.

The PPFM bacteria are strict aerobes and can be isolated from almost any freshwater environment where some dissolved oxygen exists; from tap water systems (Grfi and Bauer, 1973), where they produce pink ropy masses of growth; and from stratified lake systems (Hanson, 1980), where they occupy a special niche. The methane-oxidizing PPFM strains of *Methylobacterium organophilum* were isolated from the metalimnion of Lake Mendota, USA. Only in this stratified layer during the summer months, where methane was available in the presence of reduced oxygen tensions, did aerobic methane oxidation take place. Indeed, because of their ability to metabolize various breakdown products present in plant detritis such as methanol, methylamine, various other methylated compounds, and (in some cases) methane, the PPFM bacteria may play an important ecological role in the carbon cycle in nature. In addition, their ability to resist a certain degree of desiccation and to scavenge trace amounts of nitrogen and carbon (P. N. Green, unpublished observations) make them well suited for survival in stressful environments.

One such environment in which high numbers of PPFM bacteria are often found is roadside dust. Using selective media such as methanol salts agar (see Isolation), information has been obtained which suggests a correlation between levels of vehicular traffic and numbers of PPFM bacteria.

The association of *Methylobacterium* spp. with plants has been studied by various workers. In a numerical taxonomic study of phylloplane bacteria isolated from *Lolium perenne*, Austin and Goodfellow (1978) found pink chromogens (PPFM organisms) to be one of the major phenotypes isolated. Yoshimura (1982) found similar organisms in his study of pine-forest phylloplane bacteria and showed that their numbers varied quite dramatically with the seasons and with the accompanying environmental conditions.

After M. E. Rhodes-Roberts (personal communication) had isolated a PPFM strain (*Mycoplana rubra* NCIB 10409) from the sterilized leaf nodules of *Psychotria mucronata* and Corpe and Basile (1982) reported associations of similar organisms with lower plants, it became apparent that extra- and/or intracellular symbiotic or mutualistic associations may exist between plants and some strains of *Methylobacterium*. Although Corpe and Basile (1982) produced evidence to suggest that the PPFM bacteria present on mosses and liverworts may produce growth-stimulatory substances for these lower plants, there is presently no further evidence to support the theory of a symbiotic association. Also, radiotracer studies using $^{14}CO_2$ failed to demonstrate the uptake of labeled metabolites present in leaf exudates by a known *Methylobacterium* strain inoculated onto the surface of young *Vicia faba* plants (P. N. Green, unpublished observations). Thus, there remains the possibility that populations of PPFM attached to dust or soil particles are deposited on plant surfaces by the wind, and their numbers on leaf surfaces merely reflect the population size in the surrounding environment or as dictated by climatic conditions.

Because they are common airborne organisms, *Methylobacterium* spp. are also found as occasional contaminants in a variety of systems: domestic water supplies (mentioned above), media chills (refrigerated rooms on cabinets in which bacteriological culture media is stored prior to use), pharmaceutical products, fermentation vessels (especially in association with other C-1 compound-utilizing bacteria), and hospital environments (Gilardi and Faur, 1984). In the last case, they may pose a threat as opportunistic pathogens to seriously ill patients.

Isolation and Cultivation

Because of the ability of *Methylobacterium* spp. to grow on methanol as sole carbon and energy source and because of their characteristic pigmentation, these organisms are relatively easy to isolate. The following methanol mineral salts (MMS) medium is a suitable selective medium for *Methylobacterium*.

Methanol Mineral Salts Medium

The following are added per liter:

K_2HPO_4	1.20 g
KH_2PO_4	0.62 g
$CaCl_2 \cdot 6H_2O$	0.05 g
$MgSO_4 \cdot 7H_2O$	0.20 g
NaCl	0.10 g
$FeCl_3 \cdot 6H_2O$	1.0 mg
$(NH_4)_2SO_4$	0.5 µg
$CuSO_4 \cdot 5H_2O$	5.0 µg
$MnSO_4 \cdot 5H_2O$	10.0 µg
$Na_2MoO_4 \cdot 5H_2O$	10.0 µg
H_3BO_3	10.0 µg
$ZnSO_4 \cdot 7H_2O$	70.0 µg
$CoCl_2 \cdot 6H_2O$	5.0 µg

The MMS medium is sterilized by autoclaving at 121°C for 20 min and cooled to 50°C. A filter-sterilized vitamin solution (Colby and Zatman, 1973) is added if required, along with 0.1–0.2% (v/v) sterile methanol. The pH of the medium is adjusted to pH 7.0. Solidified media (MMS agar) are prepared by the addition of 1.5–2% Oxoid (purified) agar before autoclaving. No PPFM strain isolated to date has been shown to require vitamins or other added growth factors.

Although *Methylobacterium* strains can grow between 5° and 37°C, all grow well at 30°C, and

thus 25–30°C can be used for all isolation and subsequent growth experiments. These organisms are fairly slow growers, often taking 2–3 days at 30°C to produce clearly visible colonies or confluent growth and often taking more than 7 days for colonies to reach their maximum size of 1–3 mm in diameter. Growth is sometimes more luxuriant, with a deeper pigmentation, on Glycerol-Peptone (GP) agar.

Glycerol-Peptone Agar

The following are added per liter:

Agar	15 g
Glycerol	10.0 g
Peptone (Difco)	10.0 g

The pH is adjusted to pH 7.0

Although this medium is useful for subculturing stocks of pure *Methylobacterium* spp., it is less suitable for enrichment than the MMS medium, as other rapidly growing heterotrophs present in the sample can overgrow the PPFM bacteria. Certain antibiotics (see Identification) can also be considered for use in selective media, as can individual carbon sources for use in isolating specific groups or species of *Methylobacterium*. A summary of the properties of various species and isolates is given in Table 1.

If MMS agar is used as a selective medium, the vast majority of the pink colonies that reach diameters of more than 1 mm will be strains of PPFM organisms. Pink methylotrophic yeasts are not uncommon, but bacterial pink methylotrophs other than *Methylobacterium* species are rare.

Growth of PPFM organisms in liquid media is almost always characterized by a surface ring and/or thin pellicle, indicative of their aerobic nature.

When attempting to isolate PPFM strains from leaf surfaces, a leaf impression technique, using one of the above media, is recommended. Homogenization of whole leaves or embedding leaves in molten agar are alternatives, although they are not as successful as the impression technique.

Table 1. Substrate[a] utilized as sole carbon source to differentiate strains of *Methylobacterium*.

Species	D-Glucose	D-Fucose	D-Xylose	L-Arabinose	Fructose	L-Aspartate/L-Glutamate	Citrate	Sebaceate[b]	Acetate	Betaine	Methylamine	Trimethylamine	Methane	TMAH	DMF	Chloromethane	Dichloromethane	Growth on peptone-rich nutrient agar[c]
M. zatmanii	–	–	–	–	+	–	–	–	+	–	+	V	–	–	–	nd	nd	+
M. extorquens	–	–	–	–	–	V	–	–	+	+	+	–	–	–	–	nd	nd	V
M. rhodesianum	–	–	–	–	+	V	–	–	+	+	+	–	–	–	–	nd	nd	+
M. rhodinum	V	–	–	–	+	+	+	–	+	+	+	–	–	–	–	nd	nd	+
M. aminovorans	–	–	–	–	+	+	–	–	+	+	+	+	–	+	+	nd	nd	+
M. organophilum	+	–	–	–	+	–	–	–	+	–	+	+	V	–	–	nd	nd	+
M. chloromethanicum[f]	″	nd	″	″	″	″	″	nd	nd	″	+	″	″	nd	″	–	nd	″
M. dichloromethanicum[g]	–	–	–	–	+	+	–	–	+	+	+	–	–	nd	–	nd	+	+
M. thiocyanatum[d]	+	nd	nd	V	+	+[e]	+	nd	+	nd	+	–	nd	nd	nd	nd	nd	+
M. radiotolerans	+	+	+	+	–	+	+	+	+	+	–	–	–	–	–	nd	nd	+
M. fujisawaense	V	+	+	+	V	+	+	+	V	–	–	–	–	–	–	nd	nd	+
M. mesophilicum	V	+	+	+	–	+	+	V	–	–	–	–	–	–	–	nd	nd	–

Symbols: +, utilized as substrate; –, not utilized; V, variable result; and nd, no data.

Abbreviations: TMAH, tetramethylammonium hydroxide; DMF, *N*,*N*-dimethylformamide.

[a]Owing to the slow growth of some strains on certain substrates, carbon utilization tests were read after 14 days of incubation at 30°C (Green and Bousfield, 1982). Doubtful results were checked by twice subculturing in liquid medium.

[b]Most strains which utilize sebacate can also utilize pimelate, suberate, azelate and adipate.

[c]Nutrient agar e.g. Oxoid cm55.

[d]Taken from Wood et al. (1998).

[e]Tested for glutamate only.

[f]Taken from McDonald et al. (2001).

[g]Taken from Doronina et al. (2000).

If fungal contamination of samples from particular habitats is a problem when attempting to isolate strains of PPFM, 20 μg/ml of cycloheximide can be added to the medium.

Isolation and cultivation of PPFM bacteria involving single carbon susbstrates should be carried out on an appropriate salts basal medium (e.g. MMS where the methanol is replaced by the test substrate). For most substrates a final concentration of 0.1% is satisfactory. (For those strains utilising dichloromethane, see Doronina et al. 2000. Gaseous compounds methane and chloromethane are usually provided as head space gases in the proportions 50 : 50 methane : air and 2 : 98 chloromethane : air.)

Identification

All *Methylobacterium* strains are rods (0.8–1.0 × 1.0–8.0 mm), which occur singly or occasionally in rosettes (Patt et al., 1974; Heumann, 1962). They are often branched or pleomorphic, especially in older stationary-phase cultures (Fig. 1a). There is some evidence to suggest that they exhibit polar growth or a budding morphology (L. B. Perry, unpublished observations). All strains are motile by a single polar, subpolar or lateral flagellum, although some strains are not vigorously motile. Cells often contain large sudanophilic inclusions (poly-β-hydroxybutyrate) and sometimes also volutin granules (Fig. 1b). They are Gram-negative, although many strains stain as Gram-variable. Representative strains have a multilayered cell wall structure and the type of citrate synthase (Green and Bousfield, 1982) characteristic of Gram-negative bacteria. Most strains grow slowly and some not at all on nutrient agar. After 7 days of incubation at 30°C, colonies on GP agar are 1 to 3 mm in diameter and pale pink to bright orange-red, whereas colonies on MMS agar are a more uniform pale pink. The pigment is nondiffusible, is nonfluorescent, and probably is a carotenoid (Downs and Harrison, 1974; Ito and Iizuko, 1971). In static liquid media, most strains form a pink surface ring and/or pellicle.

All strains are strict aerobes and are catalase and oxidase (often weakly) positive. They are chemoorganotrophs and facultative methylotrophs, capable of growth on a variety of C-1 compounds. All grow on formaldehyde (often at micromolar concentrations), formate, and methanol; some grow on methylated amines. Only one species (*M. organophilum*) is reported to have utilized methane as sole carbon and energy source, but the ability has since been lost by the type (and only) strain. This organism's ability to assimilate methane was thought to be plasmid borne and easily lost if cultures were not maintained on an inorganic medium in a methane atmosphere (R. S. Hanson, personal communication). Representative PPFM strains have been reported to assimilate C-1 compounds via the

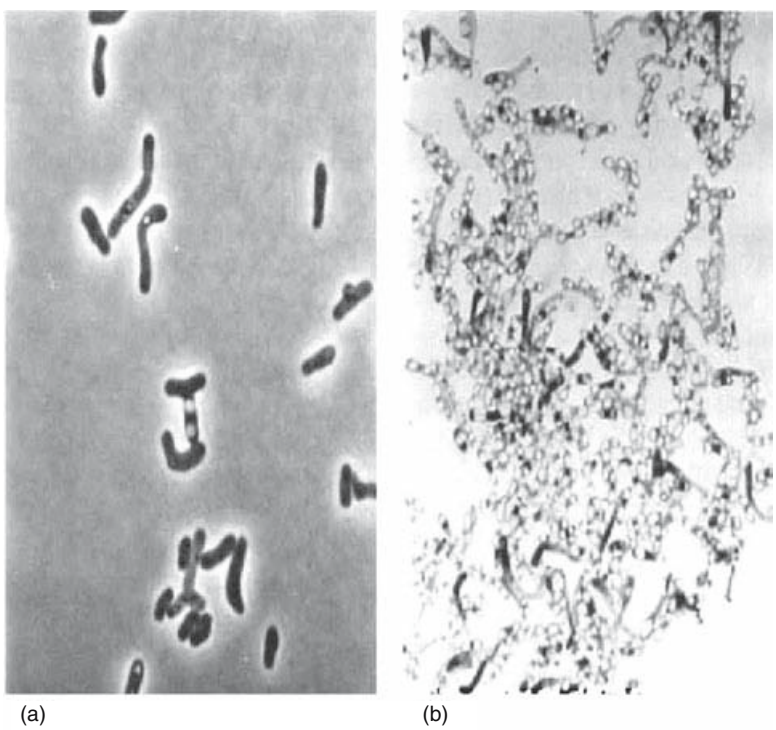

Fig. 1.

isocitrate lyase deficient (icl^-)$^-$serine pathway (Bellion and Spain, 1976; Quayle, 1972) and to have a complete tricarboxylic acid cycle when they are grown on complex organic substrates. The serine pathway is the major route for assimilating 1-carbon compounds such as methanol and methylated amines in *Methylobacterium* spp. Carbon is assimilated into the cell via formaldehyde and is incorporated via an icl^- variant of the pathway described by Quayle.

The optimum growth temperature for all *Methylobacterium* strains is in the range 25 to 30°C. Some strains will grow at 15°C or less, and some will at or above 37°C. Although growth is optimal around neutrality, some strains can grow at pH 4 and some at pH 10. Growth factors are not required by any strain although calcium pantothenate (Urakami et al., 1993) has been shown to stimulate the growth of some strains, and most strains do not degrade or hydrolyze casein, starch, gelatin, cellulose, lecithin or DNA. Urease is produced by all strains, and some strains have weak lipolytic activity. The enzymes β-galactosidase, L-ornithine decarboxylase, L-lysine decarboxylase and L-arginine dihydrolase are not produced. Indole (except for *M. thiocyanatum*) and hydrogen sulfide (H_2S) are also not produced. The methyl red and Voges-Proskauer tests are negative, although some strains reduce nitrate to nitrite.

Most strains are sensitive to the chemotherapeutic agents kanamycin, gentamycin, albamycin T, streptomycin, framycetin and especially the tetracyclines, whereas most are resistant to cephalothin, nalidixic acid, penicillin, bacitracin, carbenicillin, colistin sulfate, polymyxin B and nitrofurantoin.

Species within the genus *Methylobacterium* are differentiated mainly by the pattern of compounds they utilize as sole carbon and energy source (see Table 1). However, care should be taken to standardize such tests because they are notoriously difficult to duplicate between laboratories. All carbon utilization tests shown in Table 1 were carried out as described by Green and Bousfield (1982), who used faintly turbid suspensions of cells (which had been thrice washed in sterile saline) to inoculate media. All tests were read only after 14 days of incubation at 30°C, and growth was compared to a negative control containing no added carbon source. This long incubation time was necessary to allow for slow growth on certain compounds. Doubtful results should always be checked by twice subculturing in liquid media.

The following compounds were used by most (≥95%) strains of *Methylobacterium*: glycerol, malonate, succinate, fumarate, α-ketoglutarate, DL-lactate, DL-malate, acetate, pyruvate, propylene glycol, ethanol, methanol and formate. Some strains (see Green and Bousfield, 1982, and Table 1) can also utilize L-arabinose, D-xylose, D-fucose, D-glucose, D-galactose, D-fructose, L-aspartate, L-glutamate, adipate, sebacate, D-tartrate, citrate, citraconate, saccharate, monomethylamine, trimethylamine, trimethylamine-*N*-oxide, ethanolamine, butylamine, dimethylglycine, betaine, tetramethylammonium chloride, *N*-N-dimethylformamide, chloromethane and dichloromethane. None of strains appear to use any of the disaccharides or sugar alcohols examined (Green and Bousfield, 1982) (except for glycerol) or any of the following as sole carbon and energy source: propionate, DL-arginine, L-valine, glycine, geraniol, tryptamine, histamine, putrescine, *m*-hydroxybenzoate, testosterone, sarcosine, phenol, thiourea, tetramethylurea, hexane or benzene. Ammonia, nitrate and urea can serve as nitrogen sources. *Methylobacterium thiocyanatum* can utilize cyanate and thiocyanate as sole source of nitrogen for growth (Wood et al., 1998).

The fatty acid composition of PPFM cells is comprised largely (around 70–90%) of $C_{18:1}$ mono-unsaturated straight-chain acids, and the major isoprenoid quinone components are ubiquinones with 10 isoprene units (Urakami and Komagata, 1979; Urakami and Komagata, 1986). Representative strains have been shown to contain 3-hydroxy $C_{14:0}$ as the principal hydroxy fatty acid (Urakami and Komagata, 1987; Urakami et al., 1993), in addition to small amounts of $C_{16:1}$ and $C_{19:0}$ cyclopropane acids. Similarly, representative strains were shown to contain large amounts of cardiolipin (diphosphatidylglycerol), phosphatidylethanolamine and phosphatidylcholine, and a small amount of phosphatidylglycerol in their phospholipids (Urakami et al., 1993) and to contain bacterial hopanoids or sterols (Urakami and Komagat, 1986; Knani et al., 1994). The DNA base composition is 68.0–72.4 mol% G+C (Hood et al., 1987; Urakami et al., 1993).

DNA-DNA similarity studies (Hood et al., 1987) and electrophoretic comparison of total soluble proteins (Hood et al., 1987; Urakami et al., 1993) from representative strains have demonstrated the existence of a number of similarity (homology) groups within the genus *Methylobacterium*, several of which have been subsequently proposed as new species (Green and Bousfield, 1988; Urakami et al., 1993; Wood et al., 1998), which can be distinguished phenotypically (see Table 1). Genotypic studies (Wilfrum et al., 1986; Tsuji et al., 1990; Bratina et al., 1992) have confirmed that strains belonging to the genus *Methylobacterium* belong to a single, if somewhat heterogeneous, taxon that is clearly distinguished from other methylotrophic and non-methylotrophic genera.

Sato (1978), Sato and Shimizu (1979), and Nishimura et al. (1981) have shown that strains of *Methylobacterium* can form bacteriochlorophyll *a* under specific cultural conditions, thus suggesting a common link in their ancestry with the phototrophs.

Recent DNA-rRNA similarity studies by Dreyfus et al. (1988) have placed *Methylobacterium* in the rRNA superfamily IV of De Ley (1978), along with other members of the *Agrobacterium-Rhizobium* complex.

Preservation

All members of the genus *Methylobacterium* survive freeze drying or lyophilization. These organisms can also be cryopreserved in their liquid growth medium supplemented with a suitable cryoprotectant (e.g., 10–15% [v/v] glycerol).

Applications

Although *Methylobacterium* strains have potential for the production of single-cell protein from methanol, their bioconversion ratios (cell mass formed: substrate consumed) are inferior to those of other methylotrophs. Thus, no immediate future is seen for these organisms in this capacity. However, PPFM strains have been used in fermentation processes for the manufacture of various coenzymes (coenzyme Q_{10}); amino acids (L-lysine, L-tyrosine, L-phenylalanine and L-glutamic acid); and vitamins (vitamin B_{12}) and as a source of poly-β-hydroxybutyrate (Stirling and Dalton, 1985; Hou, 1984). Their carotenoid pigment, which has been tested as a colorant in the food industry, may also have commercial applications.

The ease with which PPFM strains can be isolated from environmental samples and their (albeit tentative) link with vehicular emissions suggest possible uses for these organisms as environmental pollution indicators. In particular, the ability of some strains to grow in the presence of particulate exhaust material (soot) is interesting. The evidence that a number of these organisms can grow on some of the polycyclic aromatic hydrocarbons and long-chain aliphatic hydrocarbons contained in exhaust emissions (P. N. Green, unpublished observations) suggests a possible role for these bacteria as biological monitors of vehicular pollution.

In addition, several PPFM strains (Ito and Iizuka, 1971) have exhibited resistance to gamma-ray irradiation 10 to 40 times higher than that tolerated by several other Gram-negative bacteria examined and in a similar resistance range to that tolerated by *Deinococcus (Micrococcus) radiodurans* under certain test conditions. This resistance, coupled with their easily identifiable pigmented colonies, may make some PPFM strains suitable candidates for irradiation-quality-control monitoring in the food and packaging industries.

The ability of *M. thiocyanatum* (and probably other *Methylobacterium* strains) to tolerate and degrade relatively high (≥50 mM) levels of cyanate and thiocyanate may have uses in the bioremediation of thiocyanate wastes from various manufacturing processes. Similarly, a use for relevant species in the biological treatment of industrial effluents (e.g. dicloromethane in wastewaters) remains a possibility awaiting further study.

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Prokaryotes (2006) 5:266–289
DOI: 10.1007/0-387-30745-1_15

CHAPTER 3.1.14

The Methanotrophs—The Families Methylococcaceae and Methylocystaceae

JOHN BOWMAN

Introduction

Methanotrophs are a group of bacteria possessing a highly specialized metabolism restricted to the utilization of methane and methanol and are a subset of the methylotrophs, bacteria and archaea able to utilize C1 compounds. Methanotrophs are by definition obligately methylotrophic and do not have the ability to grow on organic compounds possessing carbon-carbon bonds. Besides methane, the only other substrate generally utilized by methanotrophs for growth is methanol; however, a few strains can utilize methylamine and a narrow selection of other C1 compounds. Methanotrophs are an integral part of the natural ecosystem, consuming much of the methane that is biogenically (through methanogenesis) and non-biogenically (e.g., from hydrocarbon seeps, natural gas fields and coal mines) derived. This interception of methane helps maintain a balance of atmospheric methane. Methanotrophs can utilize methane as they possess an enzyme called methane monooxygenase (MMO) which occurs, depending on the methanotroph species, either in a particulate (membrane-bound) or soluble (cytoplasmic) form. In a process referred to as dissimilatory methane oxidation, MMO oxidizes methane to methanol and then methanol is further oxidized to formaldehyde, which methanotrophs use for cellular carbon. Excess formaldehyde is oxidized to CO_2 via formate. The pathway also provides cells with reducing equivalents and drives electron transport for generation of ATP. The MMO is a powerful catalyzer of oxidation reactions and has been found able to oxidize a wide range of carbon compounds. This has given methanotrophs a significant biotechnological potential that has been harnessed in applications including bioremediation and industrial processes.

There are two major groups of methanotrophs, Type I and II. Type I methanotrophs are split into two more groups (Type I and Type X). The biology of Type I and Type II methanotrophs differs in phylogeny, chemotaxonomy, internal ultrastructure, carbon assimilation pathways, and certain other biochemical aspects. The differences are summarized in Table 1. The Type I methanotrophs are housed within the family Methylococcaceae which has six genera: *Methylococcus* (the type genus), *Methylocaldum*, *Methylomonas*, *Methylobacter*, *Methylomicrobium* and *Methylosphaera*. The first two genera are also referred to as Type X methanotrophs, and this group is distinguished by certain physiological, biochemical and phylogenetic characteristics. The Type II methanotrophs are grouped in a family called the Methylocystaceae with *Methylocystis* (type genus) and *Methylosinus* as the member genera.

The taxonomy of the methanotrophs, which started with limited descriptions of several species groups, has undergone a series of developments (Whittenbury et al., 1970b). These studies were integral in advancing our understanding of methanotrophs. The subsequent usage of a systematic polyphasic approach (Murray et al., 1990) has resolved methanotrophic intra- and intergeneric relationships (Bowman et al., 1993b; Bowman et al., 1995). However it is clear the inherent biological and physiological diversity of methanotrophs remains untapped, as they appear to be ubiquitous in many environments. With the exploration of novel habitats using increasingly more sophisticated molecular techniques, the isolation of novel methanotrophs is inevitable. Thus, understanding of the role and ecology of methanotrophs and their intrinsic biology is still a highly active research area.

Phylogeny

The use of 16S ribosomal RNA-based phylogenetic analysis (Bratina et al., 1992; Brusseau et al., 1994; Bowman et al., 1995) has helped to resolve many nomenclatural problems which previously affected methanotrophs (Whittenbury and Krieg, 1984). On the basis of 16S rRNA sequence data, Type I methanotrophs belong in the gamma subdivision of the Proteobacteria; however, in most phylogenetic trees based on available sequences, the Family Methylococcaceae is made up of two separate groups (Figure

Table 1. Characteristics of methanotroph types.

Characteristics	Type I Methanotrophs	Type X Methanotrophs	Type II Methanotrophs
Family	*Methylococcaceae*		*Methylocystaceae*
Member genera	*Methylosphaera* *Methylobacter* *Methylomicrobium* *Methylomonas*	*Methylococcus* *Methylocaldum*	*Methylosinus* *Methylocystis*
Resting stages	*Azotobacter*-type cysts (or none)	*Azotobacter*-type cysts	Exospores or lipoidal cysts
Intracytoplasmic membranes	Type I	Type I	Type II
Soluble methane monooxygenase[b]	–[a]	–	+
Carbon assimilation pathway	RuMP	RuMP	Serine
Benson-Calvin cycle enzymes	–	+	–
Major fatty acid carbon chain length	16	16	18
Major quinone	Q-8 or MQ-8	MQ-8	Q-10
Mol% G+C (T_m)	43–60	56–65	60–67
Phylogenetic group (*Proteobacteria*)	Gamma	Gamma	Alpha

[a]Symbols: +, 90% or more of strains are positive; and –, 90% or more of strains are negative. RuMP pathway, ribulose monophosphate pathway.
[b]Absent in most Type I methanotrophs but is present in some strains of *Methylococcus* and *Methylomonas*.

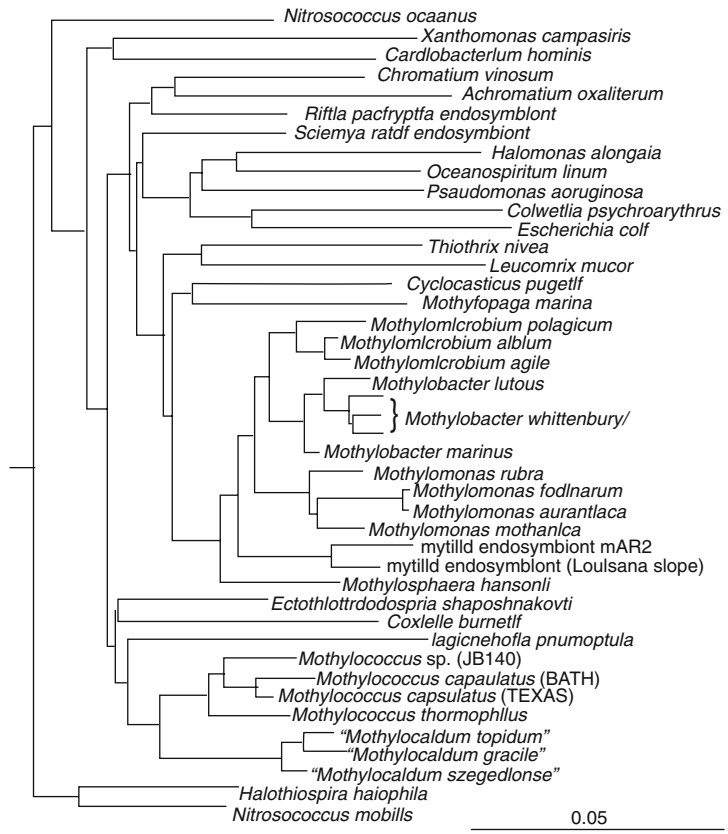

Fig. 1.

1). The first cluster includes the "Type X" methanotrophs—thermotolerant and thermophilic methanotrophs of the genera *Methylococcus* and *Methylocaldum*. This group deeply branches within the gamma subdivision, with the Ectothiorhodospiraceae, Chromatiaceae and Legionellaceae being amongst the closest relatives. The clade is separate from the remaining Type I methanotrophs, with evolutionary distances equal to other interposing genera. The second cluster contains mesophilic and psychrophilic type I methanotrophs, including the genera *Methylomonas*, *Methylobacter*, *Methylomicrobium* and *Methylosphaera*, as well as methanotrophic mytilid endosymbionts. This cluster is most closely related to *Methylophaga*

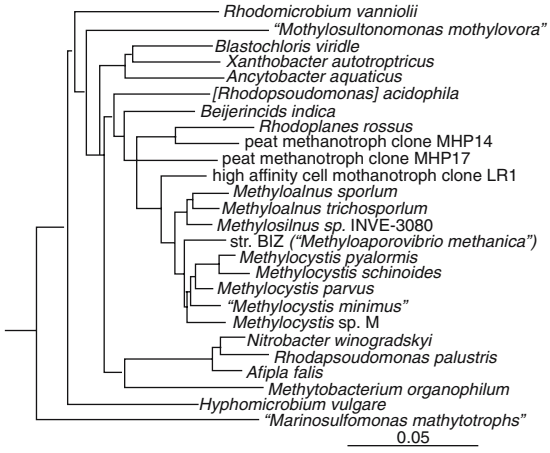

Fig. 2.

and *Cycloclasticus* and various chemoautotrophic endosymbionts.

Phylogenetic analysis based on 16S rRNA assigns *Methylocystis* and *Methylosinus* to the alpha subdivision of the Proteobacteria (Bratina et al., 1992; Brusseau et al., 1994). Owing to the close relationship, *Methylocystis* spp. is not clearly delineated from *Methylosinus* spp. (Figure 2). The Type II methanotrophs are more distantly related to an assemblage of facultatively methylotrophic, nitrogen-fixing and phototrophic bacteria including *Azorhizobium*, *Xanthobacter*, *Ancylobacter*, *Blastochloris*, *Rhodoplanes*, and *Beijerinckia*.

Taxonomy

Family Methylococcaceae

GENUS METHYLOCOCCUS AND GENUS METHYLOCALDUM Both *Methylococcus* and *Methylocaldum* represent the Type X methanotrophs, a subset of Type I methanotrophs with a thermotolerant or thermophilic growth tendency. The type species of *Methylococcus* is *Methylococcus capsulatus*, which was originally described by Foster and Davis (1966) and included strains isolated from sewage sludge. Subsequent numerical taxonomic analyses suggested several of the methanotrophic species groups of Whittenbury et al. (1970b) were related to *Methylococcus*, and new *Methylococcus* species descriptions were later published (Romanovskaya et al., 1978) including the species *Methylococcus bovis*, *Methylococcus chroococcus*, *Methylococcus luteus*, *Methylococcus vinelandii* and *Methylococcus whittenburyi* ("*Methylobacter capsulatus*"). However, later investigations using immunological analysis, protein electrophoresis, fatty acid analysis and genomic characteristics (Andreev and Galchenko, 1978; Galchenko and Nesterov, 1981; Bezrukova et al., 1983; Meyer et al., 1986, Bowman et al., 1991a; Bowman et al., 1991b) clearly demonstrated the genus was made up of two groups. This nomenclatural problem was resolved when only *Methylococcus capsulatus* and *Methylococcus thermophilus* were retained in *Methylococcus* with the description of *Methylococcus* appropriately emended to reflect the change (Bowman et al., 1993b). The other species were transferred to the genus *Methylobacter*, the original name coined for them by Whittenbury et al. (1970b). Other species have been described and include "*Methylococcus fulvus*" (Malashenko et al., 1972), which appears to be a synonym of *Methylobacter luteus* (Romanovskaya et al., 1978), whereas the single strain of the species "*Methylococcus mobilis*" (Hazeu et al., 1980) unfortunately has been lost.

Moderately thermophilic methanotrophs were recently described by Bodrossy et al. (1997) as the genus *Methylocaldum*. This group includes the species *Methylocaldum szegediense* (the type species), *Methylocaldum tepidum* and *Methylocaldum gracile*. The latter species was renamed from "*Methylomonas gracilis*" NCIMB 11128. Bodrossy et al. (1997) noted phenotypic similarity between *Methylococcus thermophilus* and *Methylocaldum* spp., though they appear to be phylogenetically distinct (evolutionary distance ~0.08). However, high levels of DNA:DNA hybridization have been recorded between *Methylococcus thermophilus* IMV-2Yu and *Methylocaldum gracile* NCIMB 11912 (Bowman et al., 1993b). As 16S rRNA sequences for these strains are quite divergent, the 16S rRNA sequence of *Methylococcus thermophilus* needs to be verified.

GENUS *METHYLOMONAS* The type species of the genus *Methylomonas* is *Methylomonas methanica*. The genus was first described officially by Whittenbury and Krieg (1984); however, this species has been known for quite some time as it was first isolated by Soehngen (1906), who named it "*Bacillus methanica*," making it the first recorded methanotroph. Orla-Jenson (1909) subsequently renamed it "*Methanomonas methanica*." Morphologically similar pink-pigmented strains were isolated also from methane:air enrichments of aquatic plants and other freshwater habits and were referred to as "*Pseudomonas methanica*" (Dworkin and Foster, 1956; Leadbetter and Foster, 1958). The name *Methylomonas methanica* was eventually coined by Whittenbury et al. (1970) for isolates very similiar to "*Pseudomonas methanica*," which they enriched from freshwater sediment.

Finally, the species was formally described by Romanovskaya et al. (1978), who at the same time described "*Methylomonas rubra*." Other pink-red pigmented groups of *Methylomonas* described by Whittenbury et al. (1970b) were regarded as variants of *Methylomonas methanica*. However DNA:DNA hybridization and phenotypic studies also suggested "*Methylomonas rubra*" was simply a synonym of *Methylomonas methanica* (Bowman et al., 1990). Additional species with a bright orange pigment isolated from coal-mine drainage water (*Methylomonas fodinarum*) and from sewage sludge and marshy soils (*Methylomonas aurantiaca*) also have been described (Bowman et al., 1990).

During the 1970s, with interest in biotechnology (particularly, the production of single-cell protein) expanding (Anthony, 1982; Hou, 1984), many methylotrophic bacteria were isolated which were grouped in the genus *Methylomonas*. Most of these species were able to utilize methanol and methylamine but not methane. In addition, practically all of these species were clearly misclassified, often lacking formal descriptions. Admittedly at that time, formal taxonomic arrangements were not available for the classification of methylotrophic bacteria. Thankfully most of these nomenclatural problems have now been resolved. Several invalid *Methylomonas* spp. are now recognized as belonging to *Methylobacillus glycogenes* (Urakami and Komagata, 1986b), including "*Methylomonas (Methanomonas) methylovora*" (Kuono et al., 1973), "*Methylomonas methanolica*," "*Methylomonas espexii*," "*Methylomonas methanocatalesslica*," and "*Methylomonas methanofructolica*" (Urakami and Komagata, 1986a). "*Methylomonas clara*" (Faust et al., 1977) has been shown to belong to *Methylophilus methylotrophus* (Jenkins et al., 1987).

A variety of methanotrophs were also grouped in *Methylomonas*, including "*Methylomonas methaninitrificans*" (Davis et al., 1964) and "*Methylomonas methanooxidans*" (Brown and Strawinski, 1958), both of which probably belong to the genus *Methylosinus* (Whittenbury et al., 1970a; Whittenbury et al., 1970b). The species "*Methylomonas margaritae*" (Takeda et al., 1974) and "*Methylomonas flagellata*" (Morinaga et al., 1976) possess traits very similar to those of *Methylomicrobium agile*; however, neither species has extant cultures. The marine species *Methylomonas pelagica* (Sieburth et al., 1987) was initially transferred to the genus *Methylobacter* (Bowman et al., 1993b) and then to the genus *Methylomicrobium* (Bowman et al., 1995).

GENUS *METHYLOBACTER* The representative type species of *Methylobacter* is *Methylobacter luteus*. The genus *Methylobacter* was formed following the emendation of the genus *Methylococcus* (Bowman et al., 1993b), and *Methylobacter* species are equivalent to the similarly named group first coined by Whittenbury et al. (1970b). Two species, *Methylococcus vinelandii* and *Methylococcus chroococcus*, have been shown by DNA:DNA hybridization to be synonyms of *Methylobacter whittenburyi*, while *Methylococcus bovis* is a synonym of *Methylobacter luteus*. Polyphasic taxonomic analyses have further refined the genus; species that cannot form cysts and have different fatty acid profiles were moved into the genus *Methylomicrobium* (Bowman et al., 1995) [RG1]. Recently two species "*Methylobacter psychrophilus*" (Omelchenko et al., 1996) and "*Methylobacter alcaliphilus*" (Khmelenina et al., 1997) were proposed for classification as strains, having been isolated from tundra soil and soda lake habitats, respectively. The genus contains the following validly described species: *Methylobacter luteus*, *Methylobacter whittenburyi*, and *Methylobacter marinus*.

GENUS *METHYLOMICROBIUM* The type species of *Methylomicrobium* is *Methylomicrobium agile* and it includes motile methanotrophs unable to form cysts. This genus was formed after polyphasic taxonomic analyses suggested that a number of *Methylobacter* species possessed sufficiently different traits (i.e., lack of encystment, more rod-like morphology, different fatty acid profile) to warrant the creation of a new genus (Bowman et al., 1995). The genus currently contains two terrestrial species, *Methylomicrobium agile* and *Methylomicrobium album*, and one marine species, *Methylomicrobium pelagicum,* previously called *Methylomonas pelagica* (Sieburth et al., 1987).

GENUS *METHYLOSPHAERA* A psychrophilic nonmotile, non-cyst-forming species from Antarctic lakes was found to form a distinct genus in the Methylococcaceae and is called *Methylosphaera hansonii* (Bowman et al., 1997).

Family Methylocystaceae

GENUS *METHYLOCYSTIS* The type species of the genus *Methylocystis* is *Methylocystis parvus*. The name *Methylocystis* was originally created by Whittenbury et al. (1970b); however, the actual description was published by Romanovskaya et al. (1978). Only *Methylocystis parvus* and "*Methylocystis minimus*" were included in the genus at the time, and several other species including "*Methylocystis methanolicus*," *Methylocystis echinoides* (Galchenko et al., 1977),"*Methylocystis pyreformis*," "*Methylocystis fuscus*" (Galchenko, 1977), and *"Methylocystis fistulosa"*

(Meyer, 1977) were ignored owing to lack of data, lack of a type strain and/or to the possibility that some were mixed cultures. The genus was not included in the Approved Lists of Bacterial Names (Skerman et al., 1980) but was subsequently revived and re-described in 1993 (Bowman et al., 1993b) and now includes *Methylocystis parvus* and the spinate, *Methylocystis echinoides*. Studies using protein electrophoresis (Galchenko and Nesterov, 1981) and immunotyping (Bezrukova et al., 1983) still suggest the diversity in *Methylocystis* is greater than what is currently recognized. Strains designated *Methylocystis echinoides* by Galchenko et al. (1977) were very similar to the strain IC 493S/5 (IMET 10491) of Haubold (1978) according to protein electrophoretic patterns (Galchenko and Nesterov, 1981). The strain IMET 10491 thus subsequently became the type strain of *Methylocystis echinoides* (Bowman et al., 1993b). "*Methylocystis minimus*" IMET 10519 (Whittenbury et al., 1970b; Romanovskaya et al., 1978) has been shown to be a synonym of *Methylocystis parvus* (Bowman et al., 1993b). Strain A of "*Methylovibrio soehngenii*" (Hazeu and Steenis, 1970) is probably just another strain of *Methylocystis parvus* (Anthony, 1982).

GENUS *METHYLOSINUS* The type species of the genus *Methylosinus* is *Methylosinus trichosporium*. As in the case of *Methylocystis*, the name *Methylosinus* was first coined by Whittenbury et al. (1970b) but was not published with a description until Romanovskaya et al. (1978) described the genus and two species, *Methylosinus trichosporium* and *Methylosinus sporium*. The genus was not included in the Approved Lists of Bacterial Names (Skerman et al., 1980) but was subsequently revived in 1993 (Bowman et al., 1993b). A variety of microorganisms have been confused with *Methylosinus* species including "*Blastobacter henrici*," which was misidentified as *Methylosinus trichosporium* (Trotsenko et al., 1989). "*Methylovibrio soehngenii*" strain B (Hazeu and Steenis, 1970) appeared to have a morphology identical to that of *Methylosinus sporium* (Anthony, 1982). Based on morphology, nitrogen-fixing methanotrophic strains designated "*Methylomonas methaninitrificans*" (Davis et al., 1964) and "*Methylomonas methanooxidans*" (Brown and Strawinski, 1958) are probably synonyms of *Methylosinus trichosporium* and *Methylosinus sporium*, respectively (Whittenbury and Krieg, 1984). Based on morphology and 16S rRNA sequences, "*Methylosporovibrio methanica*" strain 81Z (Tsuji et al., 1990) also appears to be equivalent to *Methylosinus sporium*. *Methylosinus*-like strains possessing a brilliant-red prodigiosin-like pigment (Strauss and Berger, 1983) also appear to belong to *Methylosinus sporium*.

Habitats

Methane is the next most important greenhouse gas after CO_2 and is the most abundant organic gas in the atmosphere. Methanotrophs' greatest significance is that they are the largest global methane sink and, as a result, are ubiquitous in nature. The largest and most active populations occur in environments with a stable gas exchange, in which both oxygen and methane are readily and continually available (Oremland and Cuthbertson, 1992; Reeburgh et al., 1993). Research indicates methanotrophs make up a high proportion of the total bacterial biomass in many freshwater aquatic environments, mostly within surface sediments (Boon et al., 1996; Ross et al., 1997). However their predominance in marine ecosystems is relatively unknown. Within these ecosystems methanotrophs represent a part of the basal trophic level and their carbon accumulates in the food web. Biogenic methane derived from methanogenesis is highly ^{13}C-depleted, and stable isotopic analyses indicate that carbon from methanotrophs reflects this. Measurement of ^{13}C isotope also suggests that a considerable proportion of carbon found in aquatic life at different trophic levels can be directly attributable to methanotrophic bacteria (Boschker et al., 1998). In other words, lower trophic levels such as zooplankton graze on methanotrophs and accumulate methanotroph carbon which is naturally ^{13}C-depleted, and as the zooplankton are consumed by higher life forms, this carbon can be traced up the food chain. Most generalist studies on methanotrophs in natural samples have measured methane oxidation rates as a very rough measure of population size and relative activity, however. Studies show in situ methane oxidation rates are affected by numerous biological and physical factors and are reviewed in some detail by Hanson and Hanson (1996).

Habitats in which methanotrophs are common include rice paddies; muddy soils of swamps and marshes; river, pond and lake surface sediments; meadow and deciduous forest soils; activated sewage sludge; some peat bogs; coal mine surfaces and drainage waters. Methanotrophs have in general not been isolated from "extreme" environments such as hypersaline lakes, hot springs and hydrothermal vents, even though some methanogenic archaea are able to grow in these habitats (e.g., *Methanopyrus*). For example, a study of a hypersaline mat environment failed to detect any methanotrophic activity (Conrad et al., 1995). However, methanotrophs have been isolated from Antarctic lakes, soda

lakes and tundra soils (Omelchenko et al., 1996; Khmelenina et al., 1997; Bowman et al., 1997) and thus some species possess psychrophilic and pH-based adaptations. In addition, methanotrophs (mostly of Type II) have been isolated from masonry of old buildings (Kussmaul et al., 1998).

Indirect immunofluoresence studies suggest Type I methanotrophs are common in surface sediments, the water column of various fresh and brackish water environments, and swampy soils (Reed and Dugan, 1978; Saralov et al., 1984; Ambramochkina et al., 1987; Malashenko et al., 1987; Galchenko et al., 1988; Galchenko, 1994). In addition 16S rRNA oligonucleotide signature probes (Brusseau et al., 1994) and isolation studies have demonstrated methanotrophs are associated with the roots and stems of aquatic macrophytes (Hanson et al., 1993; King, 1994; Calhoun and King, 1998). *Methylomonas* and *Methylococcus* spp. are found in significant populations in subtropical muds and swampy soils and in activated sludge (Bowman, 1992). *Methylococcus* and *Methylocaldum* strains have been isolated within and adjacent to thermal areas including thermal muds and springs which have temperatures up to 70°C or so (Malashenko et al., 1975a; Malashenko et al., 1975b; Bodrossy et al., 1997). The species "*Methylobacter psychrophilus*" was isolated from swampy tundra soils (Omelchenko et al., 1996), suggesting populations of methanotrophs in similar high latitude environments could be quite significant. In general little information exists on cold-adapted methane cyclers including both methanotrophs and methanogens. One place where such information does exist is Ace Lake in Eastern Antarctica, a marine-salinity meromictic water body in which methanogenesis occurs in the bottom water column and sediments (Franzmann et al., 1997). The lake supports a population of *Methylosphaera hansonii*, which concentrates in the oxycline of the lake (Bowman et al., 1997). It is possible this organism also may be common in surface sediments of the polar marine environments. Immunofluorescence studies suggest *Methylomicrobium pelagicum* is common in the upper mixing layers of temperate oceanic areas but is much less populous in estuarine waters (Sieburth et al., 1993). Conversely, *Methylobacter marinus* can be isolated from estuarine waters (Lidstrom, 1988). Various Type I methanotroph strains, intolerant to agar media (Lees et al., 1991), have been isolated from seawater off the coast of Britain.

Methylocystis and *Methylosinus* species have been isolated from a variety of soil and freshwater sediment habitats. *Methylosinus* strains tend to be isolated more regularly than *Methylocystis*, possibly because exospore formation makes them hardier. Indirect immunofluorescence studies suggest *Methylocystis* and *Methylosinus* can reach high populations in surface sediments of various freshwater and brackish water bodies (Reed and Dugan, 1978; Ambramochkina et al., 1987; Malashenko et al., 1987; Galchenko et al., 1988; Galchenko, 1994). *Methylosinus* has been found to dominate the culturable methanotroph population of groundwater (Bowman et al., 1993a) and rice paddy soils (Saralov and Babnazarov, 1982). Several strains of Type II methanotrophs have been isolated and detected on the root systems of the various aquatic macrophytes (Calhoun and King, 1998) and have been detected by gene probe methods in blanket peat bogs (McDonald et al., 1996).

Detection Direct plating procedures suggest methanotrophs are only a small proportion of the total viable microbial population in most samples. However direct plating methods are not very ideal for estimating methanotroph populations because of the poor transfer onto agar surfaces (usually less than 10% of cells survive subculture steps) and often rapid overgrowth by contaminating bacteria and fungi. Plating also is not aided by the intolerance to agar found in several methanotrophic strains from marine ecosystems. Most-probable-number counting based on liquid media, though generally labor- and materially intensive, gives a clearer indication of population levels (Bender and Conrad, 1994; Bowman et al., 1997; Escoffier et al., 1997) with the added advantage that methanotrophs can be isolated more easily from the highest dilutions.

Indirect immunofluorescence and ELISA techiques have been used extensively by research groups to detect specific species of methanotrophs, and the method appears useful in gaining a generalized concept of the distribution of methanotrophs (see above; Brigmon et al., 1998).

An alternative approach to detect methanotrophs involves use of specific phospholipid fatty acid (PLFA) biomarkers. For example, the PLFAs 16:1ω8c and 18:1ω8c (Nichols et al., 1985; Bowman et al., 1991a; Guckert et al., 1991) are very useful signatures for the detection and quantification of Type I and Type II methanotrophs, respectively, in environmental samples (Nichols et al., 1987; Sundh et al., 1995; Guezzenec and Fiali-Medioni, 1996; Boon et al., 1996). These fatty acids are practically methanotroph-specific. The detection of signature fatty acids relies on the confirmation of the double-bond geometry and isomeric state using gas chromatography-mass spectrometry. The quantity of the fatty acid also can be used to determine indirectly the biomass of methanotrophs in a given sample. However, a potential limitation

of this method is lack of sensitivity, as methanotroph populations in question must be relatively high (usually >1% of the total population) for lipid biomarkers to be reliably detectable. In addition, some methanotrophs, such as the species of *Methylococcus* and *Methylocaldum*, lack distinctive fatty acid biomarkers.

Nucleic acid oligonucleotide probe methods have been developed to detect methanotrophs in natural samples and have the advantage of detecting methanotrophs that have so far been resistant to cultivation, for example methanotrophic endosymbionts (Distel and Cavanaugh, 1994). Oligonucleotide probes have been developed from 16S rRNA sequences and shown to be specific for Type I and Type II methanotrophs (Brusseau et al., 1994). The method has been used for example to detect methanotrophs closely associated with aquatic plants such as duckweed (Hanson et al., 1993), freshwater lake water and sediment (Boon et al., 1996), peat (Dedysh et al., 1998; Edwards et al., 1998) and soil (Dunfield et al., 1999). A limitation of this method is that it depends on available sequence data and may underestimate populations that are present. To get around this problem gene probes based on methylotrophic functional genes have been developed also (Murrell et al., 1998). These include probes based on conserved genes of MMO and methanol dehydrogenase (McDonald and Murrell, 1997a; McDonald and Murrell, 1997b), which are known to be present in all methanotrophs and methylotrophs. Detection of these genes by PCR amplification has been useful in observing the distribution of methanotrophs in various environments including populations which so far have not been cultivated. For example methanotrophs detected in peat (McDonald et al., 1996) and seawater samples (Holmes et al., 1995b; Holmes et al., 1996) appear to belong to novel phylogenetic lineages distinct from other known methanotrophs. Finally more generalized molecular approaches for examining microbial communities can be applied to methanotroph studies. Jensen et al. (1998) utilized the DGGE (denaturing gradient gel electrophoresis) procedure to compare methane enrichments of agricultural soil and discovered that a number of novel groups of Type II methanotrophs were present. Also using DGGE, Dunfield et al. (1995) found a Type II methanotroph possessing a high affinity for methane and capable of oxidizing atmospheric methane (Bender and Conrad, 1992).

Endosymbionts Methanotrophic endosymbionts coexist with sulfur-oxidizing chemoautotrophic endosymbionts (Childress et al., 1986; Cavanaugh et al., 1987; Fisher et al., 1993) in a variety of unusual invertebrate communities associated with hydrocarbon seeps on the Pacific and Atlantic Ocean floor. Invertebrate hosts include deep-sea mytilid mussels (family Mytlidae; Childress et al., 1986) and the pogonophoran tubeworm *Siboglinum poseidoni* (Schmalijohann and Fluegel, 1987). Also cold water reefs of algae-free coral found in waters north of Norway, also associated with hydrocarbon seeps, are thought to use methane as a major nutrient and thus presumably contain methanotroph symbionts substituting for the algae (Hovland and Judd, 1988). That invertebrates can exist primarily on methane as a carbon source is shown by stable carbon isotope analyses (Southward et al., 1981; Cavanaugh, 1993). Methanotrophs growing in the gills of mytilids have been identified as Type I methanotrophs by the presence of key enzymes of the ribulose monophosphate pathway (RuMP), fatty acids, and hydrocarbons and by 16S rRNA oligonucleotide probes (Distel and Cavanaugh, 1994; Jahnke et al., 1995). Phylogenetic analyses indicate mytilid symbionts form a distinct lineage within the Methylococcaceae (Distel and Cavanaugh, 1994; Figure 1); however, cultivation of methanotroph symbionts has been unsuccessful to date. For more specific information consult the section of *The Prokaryotes* called "Symbiotic Associations between Prokaryotes and Animals" in Marine Chemesynthetic Symbioses in Volume 1.

Isolation and Cultivation

Media, Enrichment and Cultivation Methanotrophs can be enriched, isolated and cultivated in a mineral medium containing an inorganic nitrogen source and high purity methane in the headspace. The nitrate mineral salts (NMS) medium as described by Whittenbury et al. (1970a) is used with only minor modification of the mineral constituents. Nitrate salts are the usual nitrogen source for methanotroph cultivation; however, a low concentration of ammonia salts (<10 mM) can be substituted or used in combination with nitrates. High levels of ammonia are inhibitory to methanotrophs as it competitively inhibits MMO. Adding low levels of copper ensures good growth of all methanotrophs, as the membrane-bound form of MMO (see Physiology section) is a copper-containing enzyme. However, if copper is removed from the medium the isolation of methanotrophs able to form soluble MMO can be enhanced. Phosphates are also necessary for the growth of methanotrophs and in general media require 10–100 mM.

Unless otherwise specified, methanotrophs are always grown under methane. A small amount of sample is added to a liquid NMS medium (see below) in serum vials or in cotton-wool-stoppered flasks placed within air-tight contain-

ers. Agar plates can be incubated easily in containers, such as most desiccators and anaerobic jars, with an inlet tap or valve. An attached pressure gauge can be used if accurate methane additions are needed. Methane can then be added directly to vials and containers by first removing a portion of the headspace. The best methane : air ratio to use is equivocal but should be in a range of 1 : 10 to 1 : 1, as no dramatic difference in growth rates or yields occurs with the different ratios. Methane should be of high purity as natural gas could contain acetylene, which is a suicide substrate of MMO and will prevent growth even at very low concentrations. Static incubation proceeds at an appropriate incubation temperature. Most known species of methanotrophs, including those of *Methylocystis*, *Methylosinus*, *Methylomonas*, *Methylobacter* and *Methylomicrobium*, should be enriched and cultivated at 25–30°C. "*Methylobacter psychrophilus*" and *Methylosphaera hansonii* are psychrophiles, and their enrichment and cultivation should proceed at 2–10°C. In the case of the thermotolerant methanotrophs (*Methylococcus* and *Methylocaldum*), incubation should proceed at about 40–45°C, and depending on the source material, higher temperatures may be required. Enrichments take several days to several weeks, while pure cultures of most methanotrophs will form distinct colonies within 3–5 days. Some slow-growing species, such as *Methylosphaera hansonii*, may take up to 2 weeks to develop visible growth. Growth from the enrichments can then be directly plated onto mineral salts agar plates, which are then incubated under 1:1 methane:air or transferred to fresh liquid media for serial dilution. If samples contain large numbers of methanotrophs, turbidity in the enrichments will develop fairly rapidly (within 3–7 days) and static cultures often can develop a well-defined pellicle of growth, especially if *Methylomonas* species are enriched. For enrichments that rapidily develop turbidity, plating cultures early to achieve maximal biodiversity (if desired) is prudent, owing to the eventual domination of the culture by a single genotype. Unfortunately early plating does not make purification any easier. Pretreatment of samples can aid in the isolation of *Methylosinus* strains as they can be selected by exploiting the inherent heat- and desiccation resistance of their exospores. Soils and other samples should therefore be air-dried first and then heated to 80–85°C for 10–15 min before addition to enrichment media.

Methanotrophs also can be grown on methanol; however, this compound can often be toxic to methanotrophs due to accumulation of formaldehyde (see the Physiology section). Reliable growth on methanol can be achieved by incubating plates or liquid cultures in a sealed vessel containing a methanol-soaked tissue. The volatilized methanol is sufficient for growth. Alternatively, low levels of methanol can be used, though growth may be somewhat limited. Methanotrophs can be "trained" to tolerate higher levels of methanol by gradually building up the level of methanol (starting at 0.025% or so), with some strains able to tolerate levels as high as 5% (v/v). Specific strains of methanotrophs within a restricted number of species may use other C1 compounds (formate, methylamine, dimethylamine and formamide) for growth (see the Identification section).

Nitrate Mineral Salts Medium (NMS medium):

$MgSO_4 \cdot 7H_2O$	1 g
KNO_3	1 g
$Na_2HPO_4 \cdot 12H_2O$	0.717 g
KH_2PO_4	0.272 g
$CaCl_2 \cdot 6H_2O$	0.2 g
Ferric ammonium EDTA	5 mg
Trace element solution	1 ml

Trace Element Solution:

Disodium EDTA	0.5 g
$FeSO_4 \cdot 7H_2O$	0.2 g
H_3BO_3	0.03 g
$CoCl_2 \cdot 6H_2O$	0.02 g
$CuSO_4 \cdot 5H_2O$	0.03 g
$ZnSO_4 \cdot 7H_2O$	0.01 g
$MnCl_2 \cdot 4H_2O$	3 mg
$Na_2MoO_4 \cdot 2H_2O$	3 mg
$NiCl_2 \cdot 6H_2O$	2 mg

Preparation of trace element solution: Add components to distilled water and bring volume to 1 liter. Mix thoroughly. Neutralize pH with KOH.

Preparation of medium: Add components to 1 liter of distilled water (see below) and mix thoroughly. Adjust pH to 6.8 and distribute to culture vessels. If solid media are desired, add agar (Noble agar or agarose may also be used as well) to a concentration of 1.25 to 1.5% (w/v) and boil gently to dissolve the agar. Autoclave at 15 psi pressure (121°C) for 15 min to sterilize.

Artificial Sea Salts:

NaCl	24.32 g
$MgCl_2$	5.143 g
Na_2SO_4	4.06 g
$CaCl_2$	1.14 g
KCl	0.69 g
$NaHCO_3$	0.2 g
KBr	0.1 g
H_3BO_4	0.027 g
$Sr(NO_3)_2$	0.026 g
NaF	0.003 g
Na_2SiO_3	0.002 g
$FePO_4$	0.002 g
NH_4NO_3	0.002 g

Preparation of artificial sea salts solution: Add all components to 1 liter of distilled water and dissolve thoroughly. A slight precipitation of calcium salts may be encountered but will not affect its use in media.

Media modifications: For growth of marine methanotrophs (such as *Methylomicrobium pelagicum* and *Methylosphaera hansoni*), the medium is prepared with sea salts (artificial or natural seawater) instead of distilled water, and pH is adjusted to about 7.5. A few methanotrophs prefer "brackish" media rather than seawater media including "*Methylobacter alkaliphilus*" for which the NMS medium is amended with 2% NaCl and the pH is adjusted to 9.0. The growth of *Methylobacter marinus* requires preparation of NMS medium with tap water and emendation with 1–2% NaCl and, after autoclaving, addition of 1 ml of vitamin solution. Oddly this species will not grow in media prepared with distilled water, even with added NaCl and vitamins.

Vitamin Solution:

Pyridoxine · HCl	10 mg
Calcium pantothenate	5 mg
Nicotinamide	5 mg
Nicotinic acid	5 mg
Riboflavin	5 mg
Thiamine · HCl	5 mg
Biotin	2 mg
Folic acid	2 mg

Preparation of vitamin solution: Dissolve components in 1 liter of distilled water and then filter (0.45-µm or 0.22-µm) sterilize the solution. For long-term storage, the solution should be refrigerated and shielded from light.

Other growth substrates: If methanol is desired it should be added only after the liquid medium has cooled to about room temperature. Agar media should be cooled to about 50°C before addition of methanol. The methanol should be filter-sterilized before use.

About 0.1% (w/v or v/v) formate, formamide methylamine, dimethylamine, trimethylamine, or trimethylamine N-oxide should be added as needed before autoclaving.

Purification One of the most problematic areas of methanotroph study is obtaining pure cultures. In practically all situations methanotroph enrichments are heavily contaminated by non-methanotrophic (often methylotrophic) bacteria, which can easily overgrow and/or predate cultures. That is, predatory bacteria consume the methanotrophic bacteria, thus hampering isolation.

Because methanotrophs are relatively slow growers and plates are incubated in high humidity, fungal contamination is frequent unless containers are thoroughly cleaned with ethanol before each use. Combinations of fungicides such as cycloheximide and nystatin added to the medium are usually quite effective in reducing this problem. Cycloheximide and a suspension of nystatin can be added in a minimal amount of methanol (up to 0.25 ml per liter) to yield final concentrations of 200 U and 100 µg per ml of media, respectively. One approach to obtain pure cultures of methanotrophs is to use a plate microscope to observe colonies at an early stage of development (within 1–3 days). Colonies well separated from others can be picked with a sterile needle or loop and transferred to a clear section of the plate—well away from other developing microcolonies. Several colonies may have to be transferred in this way to obtain one successfully growing colony. It is necessary to make sure that the agar plates are fairly dry and excess liquid enrichment is not transferred onto the plate. This helps restrict the spreading of oligotrophic gliding bacteria and hyphomicrobia, both of which are a particular nuisance as they can rapidly overgrow the methanotroph colonies.

An alternative, rarely employed, straightforward approach to the isolation of methanotrophs utilizes NMS agar media containing a small amount of yeast extract (0.025% w/v) and methanol (0.025% v/v) (Malashenko et al., 1975) with incubation under a methane:air atmosphere. The enrichment cultures are serially diluted onto the media to the point of extinction. The yeast extract and methanol allow contaminants to reach larger colony sizes without affecting the growth of methanotrophs; indeed methanotroph growth may be considerably stimulated. Thus, methanotroph and contaminating bacterial colonies are more clearly visible. Single colonies on the spread plates are then transferred to liquid media. A number of passages from liquid media to spread plates and back to liquid media may be necessary. Several methanotrophs grow poorly or do not grow on agar. In some cases, highly purified agars, such as agar noble, used at lower concentrations may improve growth. Alternatively, silica gel may be used (Galchenko et al., 1975; Galchenko et al., 1977); however, preparation is often difficult and time consuming. For direct purification of these strains, a useful approach involves serially diluting in NMS liquid media in 96-well plastic titer trays (Bowman et al., 1997; Escoffier et al., 1997) as is done in most-probable-number counting experiments. Several strains can be purified in the same tray simultaneously. After sufficient incubation, the wells with the highest dilutions showing growth are examined microscopically. A number of separate transfers and dilutions may be required to obtain morphologically homogeneous cultures.

Some simple checks are necessary to assure the purity of methanotroph cultures. The checks include incubating methanotrophs on NMS agar or in liquid NMS media without methane; no growth should occur. In addition, strains should be plated in a complex organic medium such as nutrient agar (Oxoid or Difco) or R2A agar (Oxoid) and incubated with and without methane; again no growth should occur.

Identification

This section includes fairly detailed information on the morphology, ecophysiology, nutrition,

genotype and chemotaxonomy of methanotrophs (subdivided on the basis of family and genus) and can be used as a guide for identification. The information (unless otherwise indicated) comes from Bowman et al. (1993b). More specific information on metabolism and on physiologically related attributes, such as intracytoplasmic membranes and resting stages, can be found in the Physiology section. Characteristics differentiating the genera of the Methylococcaceae, e.g., Methylococcaceae and the Methylocystaceae, are summarized in Table 2 and Table 3, respectively.

Family Methylococcaceae *Genus Methylococcus* Cells of *Methylococcus capsulatus* are usually spherical and lack flagella. The cell envelope of *Methylococcus* species is typical for Gram-negative bacteria and cells are covered by an exopolysaccharide capsule (Whittenbury et al., 1970a) which often causes cells to clump. Single-bodied spherical cysts similar to those observed in *Azotobacter chroococcum*, but comparatively simpler and less defined in structure, are formed by *Methylococcus capsulatus* and *Methylococcus thermophilus* usually in the stationary growth phase (Whittenbury et al., 1970a; Malashenko et al., 1975b). *Methylococcus* strains also contain granules of poly-β-hydroxybutyrate, which contribute to changes in cell refractility. Cells also may contain polyphosphate (volutin) inclusions. Colonies on NMS agar are circular, convex, and smooth with an entire, even edge and have an off-white to pale tan pigment. *Methylococcus* spp. are thermotolerant or moderately thermophilic with optimal growth temperatures of 40–50°C, with no strains so far found that grow higher than 65°C or below about 25°C. No growth factors are required and the strains are non-halophilic. Some *Methylococcus capsulatus* strains can utilize methylamine, formate and/or formamide as sole carbon and energy sources. However, *Methylococcus thermophilus* strains, the type strain of *Methylococcus capsulatus* ATCC 19069 (strain "Texas"), and the more heavily studied strain ATCC 33009 (strain "Bath") cannot utilize any of these compounds. No carbon-carbon-bonded compounds can be used for growth. Nitrogen sources are usually provided in the form of a nitrate or ammonium salt, though *Methylococcus* strains can use yeast extract, casamino acids and amino acids. In addition, *Methylococcus capsulatus* and most strains of *Methylococcus thermophilus* can fix atmospheric nitrogen using an oxygen-sensitive nitrogenase (Murrell and Dalton, 1983b; Zhivotchenko et al., 1995).

The G+C content of the DNA of *Methylococcus capsulatus* ATCC 19069 has been found (62.5 mol%) using the buoyant density procedure. Analysis of several other strains using the thermal denaturation method found a range of 62 to 65 mol% (T_m). *Methylococcus thermophilus* strains have 59 to 61 mol% G+C (T_m), with the type strain IMV-2Yu possessing 59.1 (T_m; Table 3).

The major fatty acids in *Methylococcus* species have been found to be $16{:}0^{16{:}0}$.

Fatty acid nomenclature: A fatty acid structure can be described as follows. The first digit indicates the number of carbon atoms in the fatty acid. Following the colon, the number indicates the number of double bonds present. Following this a symbol and digit indicate the position of the double bond from the methyl end of the fatty acid and is used to designate bonds from the carboxyl end. Suffixes including "c" and "t" indicate the isomeric form of the fatty acid, with "c" indicating a cis isomer and "t" a trans isomer. Prefixes may also be present to indicate structural modifications of the fatty acid. For example, "i" indicates iso-branching, "a" anteiso-branching, and "cy" that the terminal three methyl units have been cyclized. The prefix "x-OH" indicates the fatty acid is hydoxylated at the x carbon position. Thus, 16:1ω7c is a cis isomer with 16 carbon atoms and a single double bond positioned next to the 7th carbon from the methyl end (for fatty acid nomenclature see footnote). The high levels of 16:0 distinguish this species from psychrophilic and mesophilic Type I methanotrophs. The neutral lipid fraction of *Methylococcus capsulatus* is unusual for a prokaryote, containing squalene, methylated sterols and hopanoids (Bird et al., 1971; Neunlist and Rohmer, 1985). The unusual distribution of cyclic triterpenes and cyclopropane fatty acids (which increase with growth under reduced oxygen tension) are believed to improve the stability of outer- and intracytoplasmic membranes (Jahnke and Nichols, 1986; Jahnke et al., 1992). The major respiratory coenzyme Q in *Methylococcus capsulatus* and *Methylococcus thermophilus* is 18-methylene-ubiquinone-8 (MQ-8; Collins and Green, 1985). Detailed analysis of hydroxy fatty acids from the outer membrane lipopolysaccharide in various *Methylococcus* strains (Bowman et al., 1991a) revealed the major components are 3-OH 10:0, 3-OH 12:0, 3-OH 14:0, and 3-OH 16:0.

Genus Methylocaldum Strains of *Methylocaldum* (Bodrossy et al., 1997) appear as motile, rod-like to coccoidal cells, 0.5–1.2 μm wide and 1.0–1.8 μm long. In logarithmic phase, cells usually appear as short rods; however, in stationary phase, they are quite pleomorphic. Usually, spherical cells are present which possess *Azotobacter*-type cysts typical of other group I methanotrophs such as *Methylococcus*. Cysts

Table 2. Phenotypic characteristics differentiating member genera of the Family Methylococcaceae[a].

	Methylococcaceae[a]					
Characteristics	*Methylococcus*	*Methylocaldum*	*Methylosphaera*	*Methylobacter*	*Methylomicrobium*	*Methylomonas*
Cell morphology	Cocci-rods	Cocci-rods	Cocci	Cocci-ellipses	Rods	Rods
Motility	–[b]	+	–	D	+	+
Cyst formation	+	+	–	+	–	+
Desiccation resistance	–	D	–	+	–	–
Pigmentation:						
Carotenoids	–	–	–	–	–	+
Melanin-like	+/–	+	–	D	–	–
Other	–	–	–	D	–	–
Growth at 0°C	–	–	+	–	–	–
Growth at 25°C	–	–	–	+	+	+
Growth at 45°C	+	+	–	–	–	–
Requires seawater or Na^{+} ions	–	–	+	D	D	–
RuBis CO[c]	+	+	–	–	–	–
Nitrogen fixation	+	–	+	–	–	D
Major fatty acids[d]	16:0, 16:lω7c	16:0, 16:lω7c	16:lω8c	16:lω7c	16:lω8c, 16:lω8c, 16:lω5t	16:lω8c, 14:0
Major quinone[e]	MQ-8	MQ-8	ND	Q-8	Q-8	MQ-8
Mol% G+C (T_m)	62–65	56–60	43–46	48–55	48–60	50–59

[a]Data from Bowman et al. (1993) and Bowman et al. (1997a).
[b]Symbols: +, 90% or more of strains are positive; –, 90% or more of strains are negative; D, result varies between species of the genus.
[c]Rubis CO, ribulose-1,5-bisphosphate carboxylase, key enzyme for the CO_1 fixation Benson-Calvin cycle.
[d]Fatty acids occur at greater than 10% of total phospholipid fatty acid content (Bowman et al., 1993, 1997).
[e]MO-8, 18-methylene-quinone-8; Q-8, quinone-8. Data from Collins and Green (1985).

Table 3. Differentiation of the Type II methanotrophs (Family *Methylocystaceae*) *Methylosinus* and *Methylocystis*.

Characteristics	*Methylosinus*	*Methylocystis*
Morphology	Pyriform or vibrioidal	Reniform to rod-like
Motility	+[a]	–
Exospores	+	–
Lipid cysts, spinae	–	D

[a]Symbols: +, 90% or more of strains are positive; –, 90% or more of strains are negative; D, result varies between species of the genus.

formed may or may not be heat resistant (able to tolerate 80°C for 20 min). In addition, thinner rods may be present in older cultures of *Methylocaldum gracile*. Cells are arranged singly, in pairs and, in the case of *Methylocaldum szegediense* and *Methylocaldum gracile*, in chains. All strains have typical Gram-negative cell walls and some strains may have extensive capsular material, in particular, strains of *Methylocaldum szegediense*. Colonies on NMS agar are circular, convex, smooth or rough, with an entire even edge, often have a cartilaginous consistency, and are pigmented tan to dark brown. At early stages of isolation a diffusible brown pigment is exuded into the agar media; however, this property is lost after repeated subculture.

Methylocaldum strains are obligately methanotrophic, growing best on methane as a sole carbon and energy source. Growth on methanol has not been obtained possibly because cells have a high level of sensitivity to formaldehyde. No growth occurs on other C1 compounds nor any compounds with carbon-carbon bonds. *Methylocaldum* strains can use either nitrate or ammonia as nitrogen sources; however, nitrogen fixation has been found to be absent. The genus includes strains that are mesophilic to thermophilic. *Methylocaldum gracile* and *Methylocaldum tepidum* can grow as low as 20°C and 30°C, respectively, and grow to a maximal temperature of 47°C, with an optimum of about 42°C. *Methylocaldum szegediense* can grow between 37 to 62°C, with an optimal growth temperature of 55°C. In other respects strains are neutrophilic and non-halophilic.

Methylocaldum strains possess mol% G+C values ranging from 56.5 to 57.2. Information on the chemotaxonomic properties is only available for *Methylocaldum gracile*, which overall appears to be similar to *Methylococcus*. The fatty acid profile is dominated by 16:0, 16:1ω7c and, depending on growth conditions, cy17:0. Also, the primary lipoquinone of this species has been found to be 18-methyleneubiquinone-8 (MQ-8; Collins and Green, 1985).

Genus Methylomonas The cells of *Methylomonas* species are regular-shaped rods, which are either straight or slightly curved, and they are also occasionally branched. Cells occur singly, in pairs, and sometimes as chains. All species are motile by a single unsheathed polar flagellum. They possess standard Gram-negative cell walls, well-defined slime capsules, and standard type I intracytoplasmic membranes. Cells contain simple single-bodied cysts similar to but better defined than cysts typically observed in *Azotobacter* and similar to those observed in *Methylococcus* species. In both species, cysts do not confer either desiccation or heat resistance. On NMS agar, colonies are circular, convex, and smooth, possess an entire edge and a creamy consistency and are pigmented either bright pink or orange. The colonies of *Methylomonas aurantiaca* have a more mucoid consistency and often segregate into rough-textured, cartilaginous variants.

Methylomonas spp. are obligate methanotrophs with sole carbon and energy sources restricted to methane and methanol; however, some *Methylomonas aurantiaca* strains also can utilize methylamine and more rarely dimethylamine. Other C_1 compounds that have been tested but are not utilized include formate, trimethylamine and trimethylamine N-oxide. Useable nitrogen sources include nitrate, ammonia, yeast extract, casamino acids, and various amino acids; however, the addition of complex nutrient sources such as yeast extract generally does not lead to growth stimulation and can be growth inhibitory at concentrations over 0.25% (w/v). Most *Methylomonas* strains can form a urease and use urea as a nitrogen source. *Methylomonas fodinarum* and *Methylomonas aurantiaca* are also able to fix atmospheric nitrogen by an oxygen-sensitive nitrogenase, while nitrogenase activity has only been detected in a few *Methylomonas methanica* strains. All *Methylomonas* species have a mesophilic and non-halophilic ecophysiology and grow between 10 and 42°C without the need for growth factors. *Methylomonas fodinarum* strains have temperature optima of about 25–30°C, while *Methylomonas methanica* and *Methylomonas aurantiaca* have temperature optima slightly higher, ranging from 30–35°C. The growth pH range is from 5.5 to 8.5 and best growth occurs at approximately pH 7.0.

The mol% G+C of the DNA of *Methylomonas* ranges from 50 to 59 (T_m). Fatty acid profiles of *Methylomonas* species have been found very similar, with 16:1ω8c, 14:0, 16:1ω7c and 16:1ω5t being the most abundant components. The relatively high levels of 14:0 and 16:1ω8c distinguish *Methylomonas* species from those of *Methylobacter* and *Methylomicrobium*. The fatty acids 16:1ω8c and 16:1ω5t are unusual and, beyond some other Type I methanotrophs, are practically unknown in bacteria and thus make excellent environmental signatures (see Ecology section).

The hydroxy fatty acids from the outer membrane lipopolysaccharide have been analyzed in detail in *Methylomonas methanica*, *Methylomonas fodinarum* and *Methylomonas aurantiaca* (Bowman et al., 1991a). The major component hydroxy fatty acid detected was 3-OH 16:0. Smaller proportions of 3-OH 12:0, 3-OH i13:0, and 2-OH 14:0 were also present, the levels of which vary considerably between the species. The primary respiratory lipoquinone has been identified as MQ-8 (Collins and Green, 1985).

Genus Methylobacter The cells of *Methylobacter* species possess a characteristic elliptical rod-like morphology with a width of 0.8–1.5 μm and a length of 1.2–3.0 μm and occur mostly singly or in pairs; however, chain formation is prevalent in some strains in the late exponential growth phase. *Methylobacter luteus* is nonmotile, while *Methylobacter whittenburyi* strains usually are motile when first isolated but can spontaneously lose the ability after extensive subculture. Motility appears most pronounced in young cultures of *Methylobacter whittenburyi* and *Methylobacter marinus,* with older cultures often devoid of motile cells. Motility is conferred by a single polar flagellum. Cells are surrounded by capsular material detectable by India ink staining, and cell walls are typical of Gram-negative bacteria. *Methylobacter* species form well-defined Azotobacter-type cysts which may give cells a refractile appearance (Whittenbury et al., 1970a). Poly-β-hydroxybutyrate granules tend to form in early log-phase cultures. Colonies on NMS agar are circular, convex, smooth, have an entire edge and possess a creamy consistency. The colonies of *Methylobacter luteus* are pigmented yellow and some strains form a diffusible yellow pigment. Both *Methylobacter whittenburyi* and *Methylobacter marinus* colonies are tanner and slowly exude tan to brown pigments into the agar media.

Methylobacter strains are strictly aerobic obligate methanotrophs with carbon and energy substrates limited to methane and methanol; however, a few strains can also utilize methylamine. Methane appears to be oxidized only by particulate MMO. The presence of soluble MMO (see Physiology section) has not been demonstrated in *Methylobacter* strains so far (Stainthorpe et al., 1991; Stainthorpe et al., 1991; Murrell et al., 1998). *Methylobacter* can utilize nitrate and ammonia salts, yeast extract, casamino acids and various amino acids as nitrogen sources. High levels (>0.5% w/v) of complex organic compounds are inhibitory to their growth. Some strains, particularly those of *Methylobacter luteus*, can produce a urease, but none are known to fix atmospheric nitrogen. *Methylobacter* species are mostly mesophilic, with most strains growing between 15 and 40°C and optimally at about 30°C. However, "*Methylobacter psychrophilus*" (Omelchenko et al., 1996) isolated from tundra is psychrophilic with a growth optimum of about 10°C and growth in the range of 0–20°C. In addition, this species is able to form gas vesicles. Methylobacter species are by nature neutrophilic with the pH range for growth ranging from 5.5 to 9.0 and a pH optimum at about 7.0. An exception is *"Methylobacter alcaliphilus"* (Khmelenina et al., 1997), which was isolated from a soda lake (in the Tuva region of Eastern Siberia) and grows best between 9.0–9.5 and also grows optimally with NaCl levels of 2–4%. Neither *Methylobacter luteus* nor *Methylobacter luteus* strains require growth factors, and they are non-halophilic. The estuarine species *Methylobacter marinus* grows optimally with about 0.1 M NaCl in tap water or with half-strength seawater salts. Some strains of *Methylobacter marinus* require nicotinic acid for growth (Lidstrom, 1988).

The mol% G+C of *Methylobacter* DNA ranges from 46 to 55 (T_m). *Methylobacter* species have very similar fatty acid profiles, with 16:1ω7c predominating and accompanied by lower levels of 14:0, 16:1ω6c, 16:1ω5c, and 16:0. The lack of 16:1ω8c and the relatively low levels of 14:0 and 16:0 distinguish *Methylobacter* from other Type I methanotrophs. The major fatty acids of "*Methylobacter alcaliphilus*" are 16:0, i16:0 and cy16:0, which differs from that of the other *Methylobacter* species (Khmelenina et al., 1997). The fatty-acid and polar-lipid profiles of this species vary considerably when grown under different cultivation conditions including pH and salinity. The outer membrane lipopolysaccharide hydroxy fatty acids have been analyzed in detail in *Methylobacter luteus* and *Methylobacter whittenburyi* (Bowman et al., 1991a). The major components found in *Methylobacter luteus* are 3-OH 10:0 and 3-OH 16:0 plus smaller quantities of 2-OH 12:0, 3-OH 14:0 and 3-OH 15:0, while the major component of *Methylobacter whittenburyi* is 3-OH 16:0, which is almost the only hydroxy fatty acid present. *Methylobacter* spp. contain Q-8 as their major respiratory lipoquinone (Collins and Green, 1985).

Genus Methylomicrobium Cells of *Methylomicrobium* spp. appear as single or paired, regular short rods, 0.5–0.1.5 μm wide and 1.5–2.5 μm long. All species are actively motile, propelled by a single polar flagellum, possess a standard Gram-negative cell wall, and are surrounded by a thin slime capsule. Cells contain type I intracytoplasmic membranes typical of other Methylococcaceae; however, they lack the ability to form cysts. Most strains contain poly-β-hydroxybutyrate and polyphosphate granules. Cells are not

heat- or desiccation resistant and are somewhat sensitive to methane starvation, losing viability in only a few days when exposed to a methane-free atmosphere. Colonies on NMS agar are nonpigmented, circular, convex-flat, and smooth and possess a creamy consistency.

Methylomicrobium strains are strictly aerobic obligate methanotrophs with carbon and energy substrates limited to methane and methanol; however, some strains of *Methylomicrobium agile* can use methylamine, dimethylamine and trimethylamine. Upon isolation most *Methylomicrobium* strains can tolerate and grow quite well on methanol. Methane appears to be oxidized predominantly by a particulate MMO (see Physiology section). The presence of soluble MMO has not been demonstrated in *Methylomicrobium* strains so far. *Methylomicrobium* spp. can utilize nitrate and ammonia salts, yeast extract, casamino acids, and various amino acids as nitrogen sources, though high levels (>0.5% w/v) of complex organic compounds are inhibitory to growth. Urease and nitrigen fixation activity is absent. *Methylomicrobium* species are mesophilic, growing between 10 and 30°C. *Methylomicrobium agile* and *Methylomicrobium album* grow best at 25–30°C, while *Methylomicrobium pelagicum* grows optimally at about 20–25°C. The pH range for growth is 6.0 to 9.0, with optimal growth occurring at about pH 7.0. Neither *Methylomicrobium agile* nor *Methylomicrobium album* requires growth factors, and they are non-halophilic. The marine species *Methylomicrobium pelagicum* grows optimally in media containing either natural or artificial seawater (Sieburth et al., 1987).

The mol% G+C of the DNA of *Methylomicrobium* is relatively broad, ranging from 48 to 60 (T_m). Fatty acid profiles of *Methylomicrobium* species are quite similar and the most abundant components are 16:1ω5c, 16:1ω5t, 16:1ω8c, 16:1ω7c and 16:0 (Table 2). The low levels of 14:0 and abundance of 16:1ω5t and 16:1ω8c distinguish *Methylomicrobium* from *Methylobacter* and *Methylomonas* species. The high levels of the trans fatty acid 16:1ω5t are unusual and do not appear to be due to stressful cultivation conditions. The carbohydrate fraction of the outer membrane lipopolysaccharide in *Methylomicrobium album* includes D-glucose, L-fucose, and D-heptose (Sutherland and Kennedy, 1986). The hydroxy fatty acids from the lipopolysaccharide fraction *Methylomicrobium album* and *Methylomicrobium agile* (Bowman et al., 1991a) are predominantly 3-OH 16:0. The primary respiratory lipoquinone is Q-8, as in Methylobacter spp. (Collins and Green, 1985).

Genus Methylosphaera *Methylosphaera hansonii* strains appear as featureless spherical cells, which exhibit refractility by phase contrast microscopy. Though cells may show signs of uneven binary division, evidence for budding division is still lacking. *Methylosphaera* cells possess standard Gram-negative cell walls and type I ICM when grown under methane; however, cells lack flagella, cysts or other types of resting stages typically found in methanotrophic bacteria. Cells actively accumulate poly-β-hydroxybutyrate, while preliminary electron microscopic examination indicates the presence of gas vesicles (dimensions 0.05–0.1 μm by 0.2 μm; Bowman et al, 1997; Bowman, unpublished data). The species is intolerant to all forms of agar and so far has only been grown successfully in liquid seawater-NMS media.

Methylosphaera hansonii uses methane and methanol as sources of carbon and energy, and no other C_1 or carbon-carbon bonded compound supports growth. Methane monooxygenase activity appears to be restricted to the particulate (pMMO) form with no soluble (sMMO) activity detected by the naphthalene oxidation assay. *Methylosphaera* strains can utilize nitrate, ammonia and L-glutamine for nitrogen; however, yeast extract and casamino acids are less suitable, causing partial inhibition of growth when tested at 0.05–0.1% (w/v) and complete inhibition of growth at concentrations of 0.25–0.5% (w/v). Though strains of *Methylosphaera* cannot form a urease, they can fix atmospheric nitrogen. All strains are psychrophilic with optimum and maximum growth temperatures varying slightly between strains. Optimal growth occurs at 10–15°C, while no growth occurs at 25°C. A doubling time of 20–24 hours was determined for strains growing at or close to their optimum temperature. In NMS-seawater liquid media, growth occurs at pH 6.0–8.0 and a pH of approximately 7.5 is optimal for growth.

The DNA base composition values of *Methylosphaera hansonii* are the lowest among the known methanotrophs, ranging from 43 to 46 mol%. The type strain AM6 (ACAM 549) possesses a mol% G+C of 44.8. The major fatty acids of the genus include 16:1ω8c, 16:1ω7c and 16:1ω6c. The lack of 16:1ω5c and 16:1ω5t is useful in differentiating the species from other Type I methanotrophs.

FAMILY METHYLOCYSTACEAE *Genus Methylocystis Methylocystis* strains typically appear as small, nonmotile kidney bean-shaped or rod-shaped cells (0.8–1.0 μm long, 0.4–0.5 μm wide) which divide by binary division. Several light microscopic photographs of *Methylocystis parvus* can be found in the article by Whittenbury et al. (1970a, 1970b). Cells are in a well-defined polysaccharide capsule. In *Methylocystis parvus* strain OBBP (= ATCC 35066) the capsule is

made up of a viscous exopolysaccharide polymer consisting mainly of D-glucose and L-rhamnose (Hou et al., 1978). *Methylocystis echinoides* strains possess characteristic pericellular prosthecate appendages called spinae (Coombs et al., 1976; Easterbrook, 1989), which have a cylindrical shape, about 300 nm long and 40 nm in diameter, and up to 300 are found on each cell (Suzina and Fikhte, 1977). The rib spacing on the individual spinae has been estimated at 6 to 12 nm (Easterbrook, 1989). The function of the spinae is currently unknown. On NMS agar, *Methylocystis parvus* forms pale pink colonies with a circular shape, convex elevation, smooth creamy consistency, and an entire edge. By comparison *Methylocystis echinoides* grows poorly on agar-solidified media, only producing scant growth and forming white pinpoint colonies, and thus this species should be routinely cultured in NMS liquid.

Methylocystis strains are strict aerobic, obligate methanotrophs that can only use methane and methanol as sole carbon and energy sources and have generation times estimated to be about 4–5 hours when growing in methane (Whittenbury et al., 1970b; Takeda, 1988). *Methylocystis* strains can form catalase, cytochrome c oxidase, and can fix nitrogen using an oxygen-sensitive nitrogenase (Murrell and Dalton, 1983b; Takeda, 1988). *Methylocystis* strains are mesophilic and neutrophilic, with best growth obtained at 25–35°C and at pH ranges of 6.5–7.5. Strains will grow at 10–40°C, with poor to moderate growth occurring at 37°C, and at pH 6.0–9.0. All *Methylocystis* strains are non-halophilic and completely inhibited by 2–3% (w/v) NaCl.

The mol% G+C of the DNA of *Methylocystis parvus* ranges from 63 to 67 (T_m), while the values for *Methylocystis echinoides* are slightly lower, ranging from 61 to 62 (T_m).

Methylocystis species possess a fatty acid profile consisting of mainly 18:1ω8c, 18:1ω7c and 18:0, with smaller amounts of cyclopropane fatty acids (Table 3). The profile is very similar to that of *Methylosinus*, differing only in the greater abundance of 18:0 and lower level of 16:1ω7c. The fatty acid 18:1ω8c is an unusual feature found almost exclusively in the Methylocystaceae, as it is very rarely encountered in other prokaryotes. The major lipopolysaccharide-derived hydroxy fatty acids include 3-OH 14:0 and 3-OH 18:0. In addition, *Methylocystis* species also possess unusual ω-1 hydroxy fatty acids with carbon chain lengths of 26 and 28 (Skerratt et al., 1992) making up about 15% of the total hydroxy fatty acids. These hydroxy acids have been previously detected in freshwater sediments (Mendoza et al., 1987), and *Methylocystis* and *Methylosinus* represent the first recognized biological sources of these particular lipids. *Methylocystis* species contain a suite of polar lipids, which vary slightly between strains due possibly to cultivation conditions (Makula, 1978; Andreev and Galchenko, 1983; Andreev and Galchenko, 1983). Polar lipids are present and include: phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylmethylethanol-amine, phosphatidyl-N, N-dimethylethanolamine and lysophosphatidylglycerol. The major quinone (Collins and Green, 1985) of *Methylocystis* species is Q-8.

Genus Methylosinus Strains of *Methylosinus trichosporium* have a pear-shaped to rod-like morphology with a width of 0.5–1.5 μm and a length of 1.5–3 μm. By comparison, *Methylosinus sporium* strains are of similar size but have a vibrioidal morphology. When grown in liquid media, both species form rosettes consisting of 4–6 cells. Several photomicrographs of the morphology of *Methylosinus* species have been published by Whittenbury et al. (1970a, 1970b). In logarithmic phase, cells divide by standard binary division; however, when cultures enter the stationary growth phase, an increasing proportion of cells reproduce by budding-off exospores as either rosettes or individual cells. Once sporulated, the mother cell ceases to divide or bud, becomes granulated and eventually lyses. Sporulating cells produce an extensive capsule compared to vegetative cells (Whittenbury et al., 1970a) (see Physiology section for more details on exospores). *Methylosinus* strains produce poly-β-hydroxybutyrate as an internal carbon reserve (Weaver et al., 1975; Best and Higgins, 1981) and have standard Gram-negative cell walls. However unlike most other Gram-negative bacteria, *Methylosinus* strains are very resistant to lysis by detergents (such as sodium dodecyl sulfate) and to lytic bacteria (Starostina and Pashkova, 1993). Lysozyme pretreatment (1 mg/ml at 37°C, for 30–60 min) followed by addition of 2% sodium dodecyl sulfate can force cell lysis. Colonies on NMS agar are nonpigmented, circular, and convex and have a smooth creamy texture and even entire edge. Some strains of *Methylosinus sporium* may form a brilliant-red prodigiosin-like pigment.

Methylosinus strains are strictly aerobic and obligately methanotrophic, and the only carbon and energy sources supporting growth are methane and methanol. All strains produce catalase and cytochrome c oxidase. Nitrate and ammonia salts, amino acids, yeast extract and casamino acids can be used as sources of nitrogen (Warner et al., 1983; Toukdarian and Lidstrom, 1984b). In addition, *Methylosinus* spp. are capable of fixing atmospheric nitrogen using an oxygen-sensitive nitrogenase (Murrell and Dalton, 1983b) which appears to be similar to the *Methylocystis* spp. enzyme. *Methylosinus* strains are mesophilic and

neutrophilic and grow at 10–40°C and pH 5.5–9.0. Optimal growth occurs at about 30°C and at pH 6.5–7.0. Most strains will not grow or grow poorly in presence of more than 0.3M NaCl and no strains have been found to require growth factors.

The DNA of *Methylosinus* strains have mol% G+C values ranging from 62 to 67 (T_m). *Methylosinus trichosporium* strains possess values of 62–63 (T_m), while *M. sporium* strains have values of 65–67 (T_m). *Methylosinus* species possess phospholipid fatty acids consisting mainly of 18:1ω8c, 18:1ω7c and 16:1ω7c with smaller amounts of 18:0 and cyclopropane fatty acids. The profile is very similar to that of *Methylocystis* but differs only by having more 16:1ω7c and less 18:0. The major lipopolysaccharide-derived hydroxy fatty acids include 3-OH 14:0, 3-OH 16:0 and 3-OH 18:0 (Bowman et al., 1991a). In addition, about 15% of the total hydroxy fatty acids of *Methylosinus sporium* are unusual ω-1 hydroxy fatty acids with carbon chain lengths of 26 and 28 (Skerratt et al., 1992). The neutral sugar components of the LPS oligosaccharide core in *Methylosinus trichosporium* OB3b include mostly rhamnose, glucose and heptose (Sutherland and Kennedy, 1986). *Methylosinus* spp. contain a suite of phospholipids (Makula, 1978; Andreev and Galchenko, 1983) including phosphatidyldimethylethanolamine, phosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylcholine and lysophosphatidylglycerol. The major quinone of *Methylosinus* is Q-8 (Collins and Green, 1985).

Preservation

Viability of most methanotrophs on plates or in liquid cultures is lost in a week or so, when they are deprived of methane. This is particularly true for cystless methanotrophs such as species of *Methylomicrobium* and *Methylosphaera* and also *Methylomonas* spp., for unknown reasons. However when placed under a 1 : 1 methane : air atmosphere plate or slant cultures, methanotrophs can be stored for several months at 4°C. Takeda (1988) found the shelf-life of type II methanotrophs was enhanced to over 12 months if cultures were kept in a 100% nitrogen atmosphere. *Methylocystis* and *Methylosinus* strains are amenable to cryopreservation (using 20% v/v dimethylsulfoxide or 20–30% v/v glycerol as a cryoprotectant) and to freeze drying (using 20% w/v skim milk or 10% v/v horse serum as cryoprotectant). Most Type I methanotrophs unfortunately do not survive freezing well (Nesterov et al., 1986). Cryopreservation with 20–30% glycerol may be used in most cases; however, vials must be replaced quite regularly (once every 1–2 months), which limits this method's usefulness. Type I methanotrophs can be kept more successfully in liquid nitrogen; however, recoverability usually becomes problematic after 6 months.

Physiology and Genetics

Detailed aspects of the genetics of methanotrophs as well as the biochemistry of the oxidation of methanol (and other C1 compounds) are covered in the chapter "Aerobic Methylotrophic Prokaryotes" in Volume 2. This section details the major physiological characteristics of obligate methanotrophs.

Metabolism

Methanotrophs possess a strictly aerobic metabolism, which uses C1 compounds. Most methanotrophs are limited to methane and methanol as substrates; however, a few species can utilize other C1 compounds including formate and methylamine. In some strains, complex carbon sources can be used as a source of carbon by methanotrophs grown in the presence of methane. In some cases this results in significant growth stimulation (Whittenbury and Dalton, 1981).

Methanotrophic strains can grow over a wide range of oxygen concentrations (<0.5% to 60% v/v) and are not microaerophilic, as has been suggested in some previous studies. Only when oxygen levels drop below 0.5% does growth become limiting (Ren et al., 1997) and in some species, significant growth still takes place at <0.1% oxygen. The work of Takeda (1988) and more recent research (Roslev and King, 1994; Roslev and King, 1995) have shown methanotrophs can survive anoxia for several months (presumably due to resting cell formation) and can rapidly respond when methane and oxygen once again become available.

Resting Stages

Cyst Formation within the Methylococcaceae. Single- or multibodied spherical cysts, similar to those in *Azotobacter* species, develop in stationary growth phase cultures of *Methylobacter* and *Methylobacter*-like species (Whittenbury et al., 1970a; Malashenko et al., 1975b; Hazeu et al., 1980). Increased cyst formation is usually associated with increasing cell refractility and increased (tan) pigmentation of colonies. The cysts appear to confer some advantages to cells experiencing deprived conditions (such as lack of methane availability and drying), and thus the cysts are able to survive desiccation for several weeks. However the cysts do not confer heat resistance such as that provided by the exospores of *Methylosinus* species. In other Type I methanotrophs (*Methylomonas*, *Methylococcus* and *Methylocaldum* species), cyst formation is not as profuse as has been found in species of *Methylobacter* nor are cysts (appearing as single bodies) as well defined or as resistant to

dessication. Moreover, cysts contain glucan-type polysaccharides, which may act as an endogenous source of energy (Sutherland and MacKenzie, 1977). Cysts can be visualized by light microscopy by using a stain developed for Azotobacter- type cysts (Vela and Wyss, 1964).

Lipid Cysts of *Methylocystis.* In stationary growth phase, a proportion of *Methylocystis parvus* cells (but not its sister species *Methylocystis echinoides*) becomes refractile and more coccoid, eventually forming lipoidal cysts. These cysts are different from glucan-rich Type I methanotroph cysts by containing mostly poly-β-hydroxybutyrate and also possess a degree of desiccation resistance but no significant heat resistance.

Exospores of *Methylosinus.* As cultures enter the stationary growth phase, an increasing proportion of cells reproduce by budding-off exospore resting stages either when the cells are arranged in rosettes or by individual cells. When spores appear they are initially nonrefractile, Gram-negative, coccoidal bodies at the end of sporulating cells and as spores mature, they become increasing refractile and acid-fast. The malachite green spore stain (Doetsch, 1981) can be used to visualize mature spores. Thin sections show that exospores consist of an electron-dense outer coat (exosporium) surrounded by a cell wall derived from the parent cell. *Methylosinus trichosporium* exospores possess a capsular coat, attached to the exosporium but distinct from the parent cell's capsular layer (Reed et al., 1980). Within the exospore, there is a laminated inner coat and a poorly defined cortex (Reed et al., 1980; Dugan et al., 1982) lacking dipicolinic acid (a chemical present in the spores of Gram-positive bacteria) and no detectable respiratory activity. Exospores are resistant to desiccation, surviving 18 months of drying, and can also withstand heating at 85°C for 10 min and 10 min of ultrasonication.

Intracytoplasmic Membranes Methane oxidation usually takes place in methanotrophs within membrane systems referred to as intracytoplasmic membranes (ICM), which appear as a series of intracellular elaborate membrane folds that can be readily observed by electron microscopy. Intracytoplasmic membranes occur in two major ultrastructural forms. In Type I methanotrophs, ICM appear as a series of laminations or vesicular arrangements of the cytoplasmic membrane crossing the cell horizontally. In Type II methanotrophs, the ICM occur along the periphery of the cell wall and encloses a distinct lumen. Intracytoplasmic membranes are formed best when methanotrophs grow on methane, but ICM form to a lesser extent when methanotrophs grow on methanol (Best and Higgins, 1981). The amount of ICM increases in proportion to methane oxidation rates, acting to increase available surface area for the oxidation of methane. The affinity of methanotrophs to methane appears to vary with growth conditions, changing by greater than an order of magnitude (K_m 0.05→1 μM). The shifts in affinity are linked to the concentration of MMO within cells and changes in the relative levels of ICM (Dunfield et al., 1999). Methanotrophs in natural habitats experiencing low fluxes of methane are thus able to cope by maximizing methane-oxidizing efficiency. Methanotrophs experiencing oxygen limitation exhibit a reduction in ICM (Scott et al., 1981), while ICM synthesis is inhibited by removing copper from the medium (Prior and Dalton, 1985), which is linked to the concomitant repression in the synthesis of pMMO (see below).

Dissimilatory Methane Oxidation This pathway results in the oxidation of methane to CO_2 and is used by methanotrophs to generate energy and obtain carbon for biosynthesis. The first step of the pathway involves the oxidation of methane to methanol. Methanol is then oxidized to formaldehyde, the compound through which methanotrophs obtain the majority of their cellular carbon. A proportion of the formaldehyde is oxidized to formate and then to CO_2. In these latter steps, reducing power in the form of $NAD(P)H_2$ is generated and is used by methanotrophs to power electron transport and ATP synthesis. Only the first step of this pathway is dealt with in detail in this section, as the oxidation of methane to methanol is central to the growth of methanotrophs. The biochemistry and genetics of the remaining steps of the pathways are described in the chapter "Aerobic Methylotrophic Prokaryotes" in Volume 2.

Methane Monooxygenase (E.C.1.14.13.25) Methane monooxygenase (MMO) is the enzyme responsible for the oxidation of methane to methanol. It does this by incorporating a single atom of oxygen and the reaction has the following stoichiometry (Dworkin and Foster, 1956):

$$CH_4 + NAD(P)H_2 + O_2 \rightarrow CH_3OH + NAD(P^+) + H_2O$$

Particulate Methane Monooxygenase (pMMO). Two types of MMO have been found in methanotrophs; however, all methanotrophs possess pMMO—a copper-containing enzyme which is tightly bound within the ICM. Particulate MMO has an active site which includes copper and in the presence of copper limitation, pMMO synthesis is repressed, resulting in reduced growth yields on methane (unless the methanotroph possesses sMMO) and in

reduced ICM development. Previously it was found that the *amoA* gene, which codes for the active subunit of ammonia monooxygenase, hybridizes to methanotroph DNA (Semrau et al., 1995). Using *amoA* as a probe, the genes for pMMO were isolated and sequenced (Semrau et al., 1995) in three methanotrophs (*Methylococcus capsulatus*, *Methylomicrobium album* and *Methylosinus trichosporium)*. In each of these species pMMO contains two subunits, coded by *pmoA* and *pmoB* (45- and 27-kDa, respectively), which are present in duplicate gene copies in methanotrophs. The *pmoA* gene product has considerable amino acid sequence similarity to *amoA* product and the genes are believed evolutionarily related (Holmes et al., 1995a).

Soluble Methane Monooxygenase (sMMO) Some methanotrophs can form a soluble, membrane-free, form of MMO, sMMO. Soluble MMO is distributed irregularly amongst the methanotrophs and has been detected so far in most *Methylosinus* strains (Bowman et al., 1993a), some strains of *Methylocystis* (McDonald et al., 1997), *Methylococcus capsulatus* (i.e., strain ATCC 33009; Brusseau et al., 1990), and *Methylomonas methanica* (Koh et al., 1993). This enzyme has engendered considerable research interest owing to its enormous lack of substrate specificity. More than 250 known compounds of many different structural types (ranging from alkanes to heterocyclic compounds) can be oxidized by sMMO. This versatility has suggested a potential role for sMMO in bioremediation and industrial applications (see Applications section). Soluble MMO consists of three proteins including a non-heme hydroxylase, a ferredoxin-like reductase, and a regulatory coupling enzyme, with the separate proteins coded by a single gene cluster. The *mmo* operon has been characterized and sequenced in both *Methylococcus capsulatus* (Bath) (Stainthorpe et al., 1990) and *Methylosinus trichosporium* (OB3b) (Cardy et al., 1991). The hydroxylase component is a dimer of three separate subunits and has a non-heme di-iron active site (Nordlund et al., 1992). It is the unusual nature of this active site that is responsible for the potent oxidative nature of sMMO. The individual subunits are coded by *mmoX*, *mmoY* and *mmoZ*, respectively. The 3-dimensional structure of the non-heme hydroxylase enzyme has been resolved (Rosenzweig et al., 1993). The ferrodoxin-like reductase enzyme (coded by *mmoA* and *mmoC*) transfers electrons to the hydroxylase for the catalysis of methane oxidation. The coupling protein, coded by *mmoB*, links the reductase and the hydroxylase. It is thought this enzyme has a regulatory role, decoupling the hydroxylase and reductase possibly when formaldehyde reaches a critical level. Soluble MMO synthesis in methanotrophs is repressed in the presence of copper (at levels as low as 50 nM) with concurrent increased synthesis of pMMO, and thus it is thought sMMO may have evolved in conditions of copper limitation (Hanson and Hanson, 1996). Limitation in copper may arise from chelation, adsorption and complexation processes, especially with various organic compounds. Copper-limited environments such as groundwater are often dominated by sMMO-producing methanotrophs (Bowman et al., 1993a). When growing methanotrophs for the purpose of making sMMO, all glassware and media should be free of trace copper ions. Soluble MMO can be readily detected by the naphthalene oxidation assay (Brusseau et al., 1990; Graham et al., 1992), by gene probe (for example using regulatory gene, mmoB; Stainthorpe et al., 1991), or by PCR using specific primers (McDonald et al., 1995; Murrell et al., 1998). The genes for both pMMO and sMMO are genetically distinct (Martin and Murrell, 1995); however, they have a common copper-inducible regulatory pathway (Neilsen et al., 1997).

Carbon Assimilation Pathways Methanotrophs fix carbon in the form of formaldehyde, which is rapidly cycled owing to its high toxicity. Formaldehyde is fixed by two different pathways in methanotrophs: the ribulose monophosphate (RuMP) pathway used by Type I methanotrophs and the serine pathway used by Type II methanotrophs (Anthony, 1982; Hanson and Hanson, 1996). By assaying the key enzymes of these pathways, Type I and II methanotrophs may be distinguished. In the case of the RuMP pathway, the key enzyme is hexulose phosphate synthase, whereas in the serine pathway the key enzyme is hydroxypyruvate reductase. More details on these pathways can be found in the chapter "Aerobic Methylotrophic Prokaryotes" in Volume 2.

Nitrogen Metabolism Most methanotrophs assimilate ammonia and nitrate by the glutamine synthetase-glutamine 2-oxoglutarate aminotransferase system (Shishkina and Trotsenko, 1979; Murrell and Dalton, 1983a; Toukdarian and Lidstrom, 1984b). Methanotrophs also can assimilate nitrogen from amino acids and other complex mixtures such as yeast extract. In addition, many methanotrophs are able to fix atmospheric nitrogen and include all Type II methanotrophs and various members of the Type I methanotrophs, including *Methylomonas* spp., *Methylococcus capsulatus*, and *Methylosphaera hansonii*. In most cases the nitrogenase formed is oxygen-sensitive (Murrell and Dalton, 1983b;

Takeda, 1988; Zhivotchenko et al., 1995), except for *Methylosphaera hansonii*, which appears to possess a more oxygen-tolerant enzyme (Bowman et al., 1997). In the case of methanotrophs with oxygen-sensitive nitrogenase, reducing the oxygen partial pressure in the headspace enhances growth on agar under nitrogen-free conditions; however, oxygen concentrations over 10% will abolish nitrogenase activity almost completely (Zhivotchenko et al., 1995). A number of homologs of *nifH* have been shown in strains such as *Methylomonas methanica*, which appeared unable to fix nitrogen in vitro (Oakley and Murrell, 1988). Southern blotting indicates the *nif* genes of various methanotrophs are homologous with each other and genes of *Klebsiella pneumoniae* (Toukdarian and Lidstrom, 1984a; Oakley and Murrell, 1988).

Applications

Methanotrophs have been considered as cheap sources of single-cell protein (Anthony, 1982) and poly-beta-hydroxybutyrate (a natural polymer; Hou, 1984; Wendlandt et al., 1998). However most biotechnologically directed studies of methanotrophs have focused on the capability of sMMO from *Methylosinus trichosporium* OB3b to co-oxidize a wide range of carbon substrates. The compounds oxidized are too many to list but include a wide range of aliphatic, heterocyclic and aromatic compounds (Burrow et al., 1984). Several studies have focused on the industrial applications of this biocatalytic ability, in particular in the production of epoxides for plastics manufacture (Hou, 1984), but the primary focus of research has been in the bioremediation field, and this aspect has been reviewed extensively (Sullivan et al., 1998). *Methylosinus trichosporium* OB3b and other strains forming sMMO can co-metabolize a range of chlorinated aliphatic compounds including major groundwater pollutants such as trichloroethylene, chloroform and tetrachloroethylene (Oldenhuis et al., 1989; Tsien et al., 1989; Castro et al., 1996; Hamamura et al., 1997; Moran and Hickey, 1997; Tartakovsky et al., 1998). However, the industrial application of methanotrophs has been hampered by their relatively slow growth and requirement for methane, a potentially explosive substrate which also competitively inhibits co-metabolic reactions. Another problem is that trace copper levels can suppress sMMO activity, thus eliminating or reducing transformation rates (Oldenhuis et al., 1989; Lontoh and Semrau, 1998). To overcome this, constitutive mutants lacking sMMO activity suppression [RG7] have been created by chemical mutagenesis and by marker exchange (Phelps et al., 1992; Fitch et al., 1993; Martin and Murrell, 1995; Tellez et al., 1998). These mutants have been shown to be able to co-metabolize trichloroethylene in the presence of high levels of copper. Some of these copper-tolerant mutants have been utilized in treatments of chlorinated aliphatic pollutants (Tschantz et al., 1995). In addition, the sMMO gene cluster has been successfully cloned into *Pseudomonas putida* F1, which is not only able to degrade trichloroethylene but can grow much faster and lacks the problem associated with methane competitive inhibition (Jahng and Wood, 1994; Jahng et al., 1996).

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Prokaryotes (2006) 5:290–314
DOI: 10.1007/0-387-30745-1_16

CHAPTER 3.1.15

The Genus *Xanthobacter*

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Introduction

Strains of *Xanthobacter autotrophicus*, the type species, were originally described as *Corynebacterium autotrophicum* (Baumgarten et al., 1974) and as "Gram-positive Knallgas bacteria." They became of interest when Gogotov and Schlegel (1974) found that a strain named "7c" could grow chemolithoautotrophically and use molecular nitrogen (N_2) as nitrogen source, a property otherwise not known to exist in other bacteria. Ooyama (1971) had, however, published on N_2-fixing chemolithoautotrophic new isolates from Japan, but his publication in a non-mainstream journal did not receive much attention and his strains were never formally described. However, on the basis of the described properties, these isolates were probably *Xanthobacter* strains. De Bont and Leijten (1976b) observed similar properties with their isolates from The Netherlands. This initiated the isolation of more than 50 strains (Wiegel and Schlegel, 1976) later recognized as members of the novel genus *Xanthobacter* (with the type species *X. autotrophicus*; Wiegel et al., 1978b) on the basis of numerical taxonomy, Gramnegative staining reaction, and Gram-type negative classification (Wiegel and Mayer, 1978a). (For a definition of the term "Gram type," see Wiegel [1981] and Wiegel and Quandt [1982b].) The second recognized *Xanthobacter* species was also reported previously as an N_2-fixing heterotroph, *Mycobacterium flavum* strain 301 (Biggins and Postgate, 1969), which was subsequently renamed as the type strain of *X. flavus* (Malik and Claus, 1979).

Presently the following species have been recognized and validly published (Euzéby, 2004): *Xanthobacter autotrophicus* (type species; Wiegel et al., 1978b), *X. flavus* (Malik and Claus, 1979), *X. agilis* (Jenni and Aragno, 1988), *X. tagetidis* (Padden et al., 1997), *X. aminoxidans* (Doronina and Trotsenko, 2003), and *X. viscosus* (Doronina and Trotsenko, 2003; Table 1). In addition, "*X. polyaromaticivorans*" (Hirano, 2004) and "*X. methylooxidans*" strain 32P (Doronina et al., 1996) have been published but not validated.

All validly published members of the genus *Xanthobacter* are yellow Gram staining reaction negative and Gram-type negative pleomorphic rods containing the water insoluble yellow zeaxanthin dirhamnoside. However, "*X. polyaromaticivorans*" is slightly pinkish orange, which (based on the absorption peak at 473 nm) is probably due to a zeaxanthin but with different (yet to be confirmed) terminal substitutions. Owing to high concentrations of polyphosphate granula (see below), some species and strains give the appearance of staining Gram-positive.

Xanthobacter belongs phylogenetically to the family Hyphomicrobiaceae in the class Alphaproteobacteria. The relationship to the genera *Aquabacter* and *Azorhizobium* is not clear at this time (see below). The genomic G+C content is 60–70 mol%. Cells are pleomorphic rods that can become highly irregular, multibranched or twisted (e.g., *X. tagetidis*). The degree of pleomorphism depends on the species and the substrates on which they are grown (Figs. 1–4).

All known strains of *Xanthobacter* can obtain energy from the reaction $2H_2 + O_2 \rightarrow 2H_2O$ and fix CO_2 for carbon sequestering. Therefore, they belong to the group called "hydrogen-oxidizers" or "Knallgas bacteria" (see The H_2-Metabolizing Prokaryotes in Volume 2). *Xanthobacter* species are generally also methylotrophic. They grow well organoheterotrophically under aerobic or microaerophilic conditions with acids, alcohols, and selectively with carbohydrates as energy and carbon source. Carbohydrate utilization varies but is frequently restricted to the utilization of not more than three of the following: fructose, galactose, mannose and sucrose; some do not use any hexoses and pentoses (e.g., *X. autotrophicus* strain 14g, all strains of *X. agilis* and "*X. polyaromaticivorans*"). The ability to utilize mannose depends on growth conditions and is not always reproducible in taxonomic tests. For most strains, glucose is not a preferred substrate, but many strains of the type species tested could be adapted to utilize glucose as growth substrate through extended incubation in glucose-containing medium; however, the ability to grow

Table 1. Differential characteristics of the species of the genus *Xanthobacter*.[a]

Characteristics	1.	2.	3.	4.	5.	6.	7.	8.
Cell morphology: pointed ends, twisted	–	–	–	+	+	+	+	–
Highly pleomorphic on nutrient broth agar + succinate (TCA-cycle intermediates)	+	+	–	+	nd	nd	nd	nd
Asymmetric cell division ("budding")	–	–	–	–	+	+	–	–
Excessive slime production	+	+	–	(+)	–[b]/+	+	+	(–)
Zeaxanthine dirhamnoside (yellow)	+	+	+[c]	+	+	+	–	+
Zeaxanthine (orange, pinkish)	–	–	–	–	–	–	+	–
Motility under autotrophic growth conditions	–[d]	+	+	+	–	–	–	nd
Vitamins required for growth (biotin, riboflavin)	(+)	+	–	–	–	–	–	–
Sensitivity to chloramphenicol	–	–	+	+	nd	nd	nd	nd
Autotrophic growth at 37°C	+	+	–	+	nd	–	–	nd
Utilization of hexoses	+	+	(+)	+	+	+	–	+
Growth on nutrient broth	+	+	–	+	nd	nd	nd	+
Growth on glutamine as carbon source	+	–	–	nd	+	nd	nd	nd
Growth on citrate	+	nd	–	+	nd	nd	+	nd
Degradation of various aromatic compounds	–	(+)[e]	–	–	–	–	+	–
Degradation of cyclohexene (incl. derivatives)	+	–	nd	nd	nd	nd	nd	nd
Utilization of methanol	+	+	+	+	+	+	–	+
Utilization of hydrocarbons	–	+/–	–	+	–	–	+	nd

Symbols and abbreviations: +, positive; (+), positive except for some unusual strains; –, negative; (–), negative except for some unusual strains; +/–, ; nd, not determined; and TCA, tricarboxylic acid.

[a]1., *X. autotrophicus*; 2., *X. flavus*; 3., *X. agilis*; 4., *X. tagetidis*; 5., *X. aminoxidans*; 6., *X. viscosus*; 7., "*X. polyaromaticivorans*"; and 8., "*X. methylo-oxidans*."

[b]Slime production in glucose.

[c]Pale yellow indicating low concentration.

[d]Under some conditions, tumbling is observed using phase contrast microscopy, but when grown on propanol, cells are motile (strains 7C and 301).

[e]Strain 14p1 does not degrade various aromatic compounds.

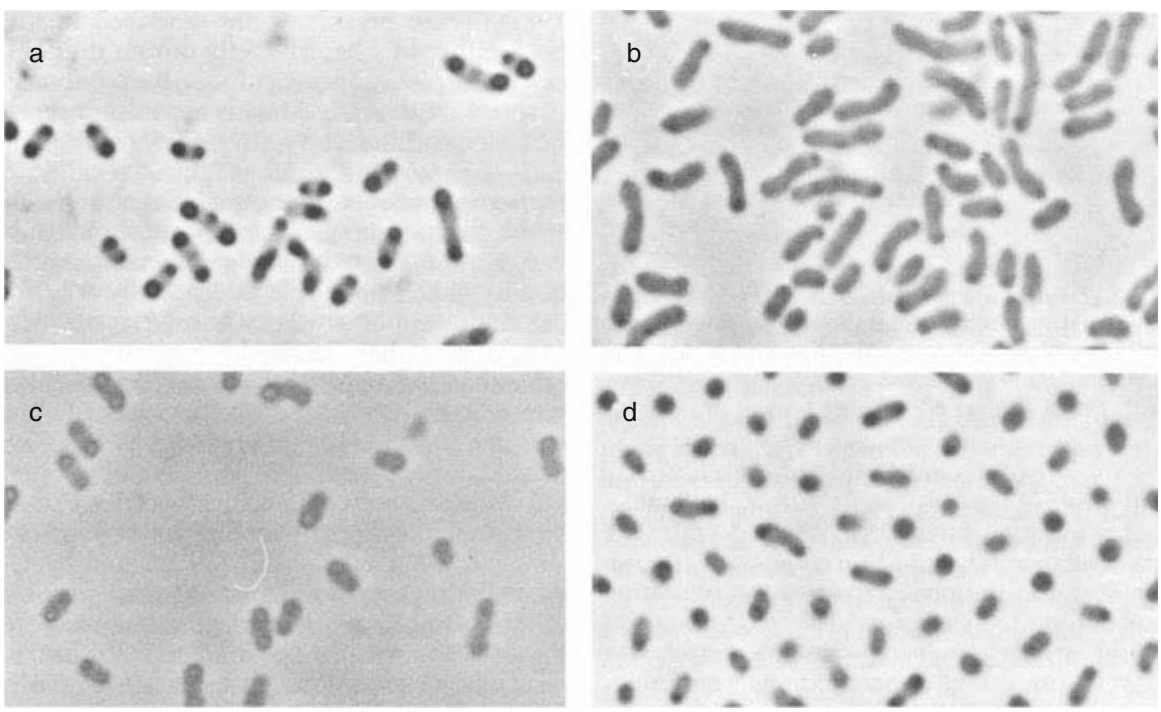

Fig. 1. Phase contrast photomicrographs of *X. autotrophicus*. Reference strain JW 33 a) to c) grown on various substrates in mineral medium plus 0.1% ammonium chloride. Growth conditions were: a) 0.5% fructose; b) autotrophically, 80% H_2 + 10% CO_2 + 10% O_2; and c) 0.2% propanol. d) Strain JW-KR-R10/47 isolated from rice roots, grown on rice roots in sterile sand and half-strength Hoagland's medium in the presence of nitrate. However, no significant changes where observed when grown under similar conditions but in the absence of nitrate. Phase contrast light microscopy. (All photos courtesy of K. Reding.)

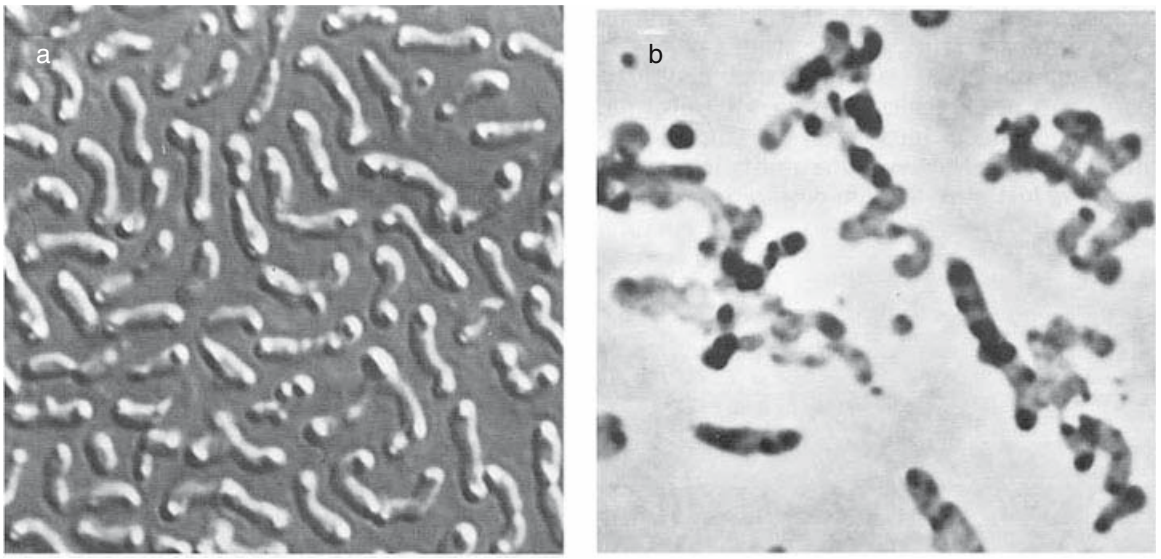

Fig. 2. *Xanthobacter autotrophicus* reference strain JW 33: a) grown on mineral salt medium plus 0.3% succinate, late exponential phase; b) grown on nutrient broth agar plates containing 0.3% succinate, 3- to 4-day-old colony. Nomarski technique. (Both photos courtesy of K. Reding.)

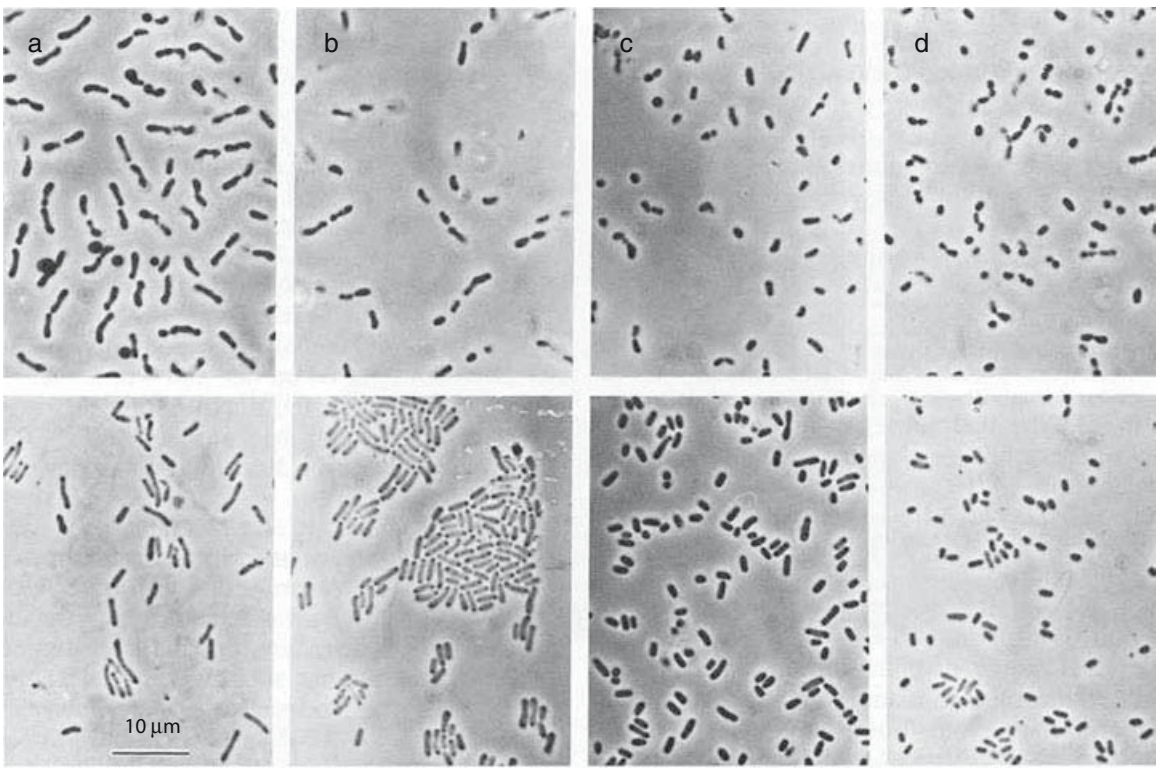

Fig. 3. Change in appearance of (top) *X. autotrophicus* reference strain JW 33 and (bottom) *X. agilis* type strain SA35 in the course of growth of a batch culture employing mineral medium plus 0.2% succinate at pH 7.0. The growth periods were: a) 24 h; b) 2 days; c) 4 days; and d) 8 days. (Photos courtesy of B. Jenni and M. Aragno.)

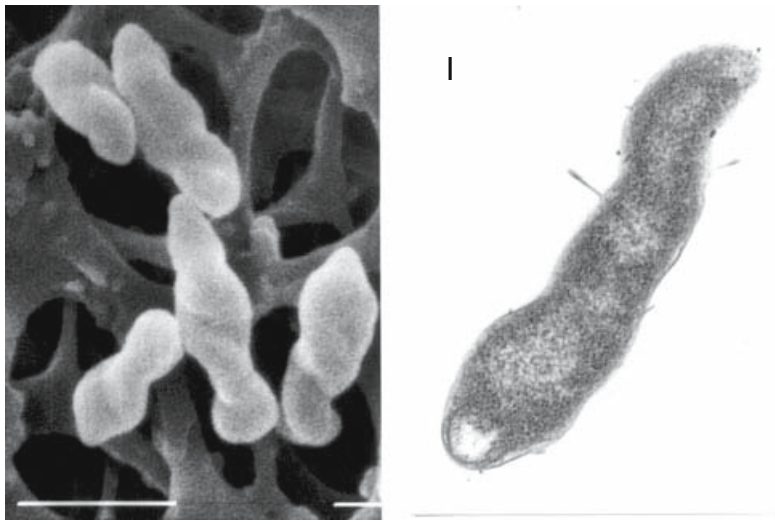

Fig. 4. (A) Scanning electron micrograph of *X. tagetidis*. Bar = 1 µm. (B) Transmission electron micrograph of a twisted cell and showing the insertion of flagella. Bar = 0.1 µm. (Courtesy of A. P. Wood; modified from Padden et al., 1997.)

on glucose is lost after a batch culture is grown in the absence of glucose. Several species and strains can utilize a variety of special substrates such as thiophenes (*X. tagetidis*; Padden et al., 1997) or polycyclic or heterocyclic aromatics (*X.* "*polyaromaticivorans*"; Hirano et al., 2004), on which they had been isolated.

All recognized *Xanthobacter* species can fix dinitrogen under chemoheterotrophic and chemolitho-autotrophic conditions (e.g., growing with H_2 plus O_2 or H_2 plus thiosulfate as energy source and with CO_2 as carbon source) but only at reduced oxygen tension and in the absence of organic nitrogen sources or ammonia. On the basis of their numbers, *Xanthobacter* should be regarded as an associative diazotroph (although entering roots to some extent, they do not form nodules like the symbiotic N_2-fixing bacteria). Historically, the special position of *Xanthobacter* among the chemolithoautotrophs and among the N_2-fixing aerobic microorganisms was due to their ability to fix N_2 while growing chemolithoautotrophically. At present, they share this property with other proteobacteria such as strains of *Hydrogenophaga pseudoflava* (synonym: *Pseudomonas pseudoflava*; Jenni et al., 1989; Willems et al., 1989), *Alcaligenes latus* (Malik et al., 1981b), *Azorhizobium* (K. Reding and J. Wiegel, unpublished observations), some strains of *Azospirillum lipoferum*, *Derxia gummosa*, *Bradyrhizobium japonicum* (synonym: *Rhizobium japonicum*), and possibly some others for which the N_2-fixation under chemolithoautotrophic growth has not been demonstrated unequivocally (Malik and Schlegel, 1981a). These organisms are described in the chapters dealing with hydrogen utilizers or the dinitrogen-fixing bacteria. However, if suitable conditions are provided, including the correct H_2 concentration and frequently a critical, low-O_2 partial pressure (below 5% [v/v] O_2 in the gas phase), it is possible that more diazotrophs may grow chemolithoautotrophically and may also fix N_2 under chemolithoautotrophic conditions. This is especially true for the strains known to grow with $H_2/CO_2/O_2$ or to have strong hydrogenase activity (other than the hydrogenase activity resulting from a side reaction of nitrogenase). Examples include strains of *Azotobacter*, *Derxia*, *Bradyrhizobium* and *Rhizobium* (Malik and Schlegel, 1981a) and *Azorhizobium* (R. Robson, personal communication).

Originally, one key taxonomic property for discriminating *Xanthobacter* from other yellow-pigmented bacteria, including diazotrophs, is the presence of the water-insoluble yellow pigment zeaxanthin dirhamnoside. However, for several of the newly described species, this property has not been tested unequivocally using a thin layer chromatographic assay (see below).

Xanthobacter strains can be enriched specifically and be isolated easily if certain conditions are applied: no other (or very limiting) sources of nitrogen other than N_2 or $H_2/CO_2/O_2/N_2$ (10 : 10 : 1 : 79) as gas phase providing an electron donor, a carbon source, electron acceptors, and nitrogen source, respectively, in liquid media; yellow colonies containing branched cells are selected from nutrient broth (with 1% [w/v] succinate) agar plates. Because of their metabolic diversity, *Xanthobacter* species are widespread. For this reason, and because there are some unusual strains which do not fit into the present scheme, it is expected that more species will be described beyond those presently known (Table 1). Note also that several strains described before the general use of 16S rRNA sequencing also appear to be *Xanthobacter* strains but have

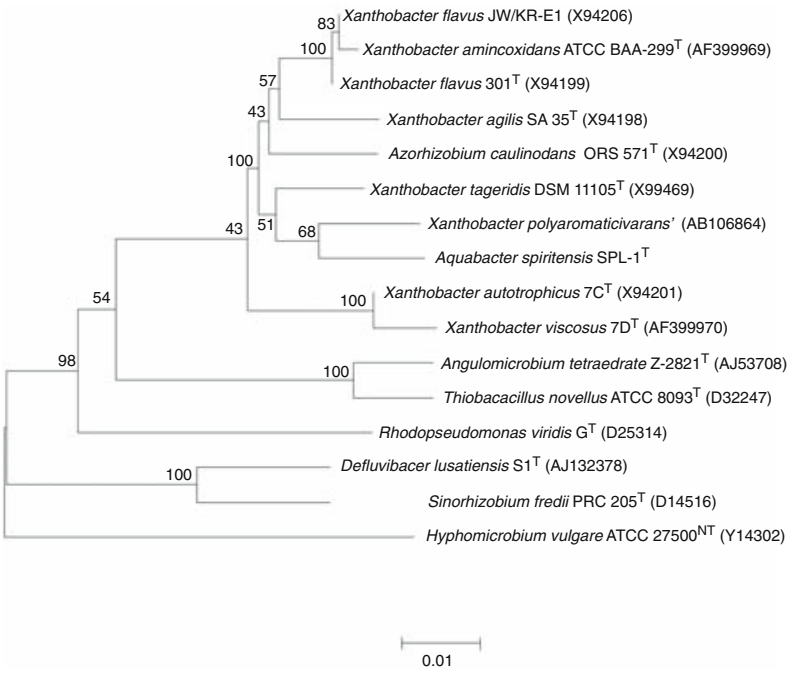

Fig. 5. 16S rRNA gene sequence-based phylogenetic tree for the *Xanthobacter-Aquabacter-Azorhizobium* clade using as outgroup *Hyphomicrobium vulgare*, which is the type genus and species for the family Hyphomicrobiaceae. We used the sequence of the falsely introduced "neotype strain" (http://www.bacterio.cict.fr.//h/hyphomicrobium.htm). (Courtesy of R. Onyenwoke.)

not yet been tested and thus are not validly described.

Phylogeny and Taxonomy

Phylogeny

The phylogenetic position of *Xanthobacter* based on 16S rRNA sequence analysis is as given in the *Bergey's Manual of Systematic Bacteriology*. According to the outline for *Bergey's Manual of Systematic Bacteriology*, the genus *Xanthobacter* (genus XX) belongs to the family Hyphomicrobiaceae, order Rhizobiales (order VI), class Alphaproteobacteria (class I), and phylum Proteobacteria (phylum BXII). Previously, *Xanthobacter* was described to belong to the *Rhodopseudomonas palustris* rRNA branch of the fourth rRNA superfamily of the Gram-type negative bacteria (De Smedt et al., 1980; Dreyfus et al., 1988).

All described species (in addition to the physiologically atypical type strain 7c and the reference strain JW 33) form a relatively narrow cluster around the type species *X. autotrophicus*. However, using phylogenetic trees constructed on the basis of 16S rRNA sequence comparisons, the type strains of *Aquabacter spiritensis* and *Azorhizobium caulinodans* appear intermingled with the otherwise well-defined genus cluster *Xanthobacter* (Dreyfus et al., 1988; Rainey and Wiegel, 1996; Fig. 5). *Aquabacter* and *Azorhizobium* (both single species genera) are presently recognized in the above-mentioned scheme as separate genera (V and VI) within the same family Hyphomicrobiaceae. Unfortunately, some of the key properties described in this chapter for the type species *X. autotrophicus* and all other recognized *Xanthobacter* species have not been tested or found for these two bacteria. Until this has been done and more isolates of this clade have been obtained and described, it is suggested to keep the separate genera names despite the 16S rRNA sequence similarity. Two nonvalidly published species of *Blastobacter* (genus IV of family VIMethylocystaceae) were recently renamed as *X. viscosus* and *X. aminooxidans*. The 16S rRNA sequence of the latter is more than 98% similar to those of *X. flavus* and *X. autotrophicus* strains (Fig. 5). However, the morphology and some of the physiological characteristics are different enough to retain a separate species, which is also supported by the low (below 50%) DNA-DNA hybridization data.

Taxonomy

Although most identifications of isolates are done via 16S rRNA sequence analysis, in a first traditional identification step, helpful diagnostic taxonomic properties are: 1) yellow, "fried egg" shaped colonies with various amounts of slime production when plated; 2) highly polymorphic, branched, twisted cell morphology while growing on nutrient broth agar containing succinate

(a tricarboxylic acid [TCA] cycle intermediate); 3) formation of the water insoluble zeaxanthin dirhamnoside, giving the colonies their characteristic yellow appearance (the yellow *Xanthobacter* colonies appear a little paler than the brilliant and darker yellow colonies of most Flavobacteria); 4) ability to grow chemolithoautotrophically; and 5) ability to fix dinitrogen under microaerophilic chemolithoautotrophic or organoheterotrophic conditions. Other characteristics are given in Table 1.

Azorhizobium, which appears to have a different habitat (stem nodulation) than that of *Xanthobacter* (free-living in soil and water as well as root-associated), exhibits acetylene reduction under chemolithoautotrophic growth conditions (K. Reding and J. Wiegel, unpublished observations). However, *Azorhizobium*, lacking the characteristic yellow *Xanthobacter* color, has not been tested for the presence of zeaxanthin dirhamnoside; colonies of *Azorhizobium* are cream-colored, which could be due to low amounts of zeaxanthin dirhamnoside or a difference in the zeaxanthin end group. Furthermore, colonies of *Azorhizobium* as well as of *Aquabacter* differ from *Xanthobacter* in lacking strong pleomorphism, having different colony morphology and physiological characteristics (such as being unable to utilize methanol and ethanol).

Note that many other features of *Xanthobacter* and of the high G+C-flavobacteria (as well as some flavobacteria sensu stricto and some *Cytophaga* spp.) are similar, including: 1) antibiotic sensitivity; 2) positive reaction for catalase, oxidase and phosphatase; 3) negative reaction for methyl red, gas from carbohydrates, and the Voges-Proskauer test; and 4) the presence of main ubiquinones Q_{10} and Q_8 (Urakami et al., 1995). However, a similar branching cell pattern is also seen with the yellow and non-yellow Gram-type positive *Corynebacterium* sensu stricto and other coryneform bacteria (Wiegel et al., 1978b), *Rhizobium* (Urban and Dazzo, 1982; Kaneshiro et al., 1983; Reding and Lepo, 1989), *Beijerinckia* (Thompson and Skerman, 1979; Vincent [1981] and literature cited therein), and *Azotobacter* (Löhnis and Smith, 1923). Succinate, which gives the strongest reaction for *Xanthobacter*, can be replaced by other acids from the TCA cycle (except malate for "*X. polyaromaticivorans*"). However, the motile species *X. agilis* exhibits only a slight pleomorphism, and only a few cells in a population show some branching after prolonged incubation. Thus, for definite identification of this species, demonstration of the presence of the pigment zeaxanthin dirhamnoside and acquisition of the 16S rDNA sequence are necessary (see below for the performance of the biochemical assay).

The frequently occurring larger amounts of polyphosphate granula (up to 15 mg per g dry weight of cells) can lead to the false impression of a Gram-positive staining reaction; however, experienced researchers will note that all *Xanthobacter* stain truly Gram negative when using a counterstain. Interestingly, polyphosphate-free cells of *X. autotrophicus* (only strains 7c and JW33 tested) could not be obtained by limiting phosphate concentrations in the media (Wiegel et al., 1978b).

Habitats

The known habitats of *Xanthobacter* reflect its physiological properties, especially its catabolic versatility. The sources for isolated strains include oil-contaminated soil and sludge from Japan (Ooyama, 1971; Hirano et al., 2004), marine sediments (Lidstrom-O'Connor et al., 1983), water and sediment samples from freshwater lakes (Aragno, 1975), soil of flooded rice fields, rhizosphere of wetland rice (Chistyakova, 1985; Oyaizu-Masuchi and Komagata, 1988; Reding et al., 1991a) and of marigold plants (Padden et al., 1997), street ditches and wet meadow soil and garden soil from Europe, South Africa, North America, and Asia (De Bont and Leijten, 1976b; Wiegel and Schlegel, 1976; Wiegel and Schlegel, 1984; Oyaizu-Masuchi and Komagata, 1988), sewage samples (Jenni and Aragno, 1987a; White et al., 1987; Doronina and Trotsenko [2003] and literature cited therein), and tree leaves (Samanta et al., 1986b). The author believes that *Xanthobacter* is ubiquitous in microaerophilic environments with decaying organic material or matter containing sufficient concentrations of H_2 and CO_2 and other products of anaerobic microbial activity, such as organic acids and alcohols. Thus, the metabolically versatile *Xanthobacter* species should be important in the microaerophilic interface between the anaerobic and aerobic zones. This is also indicated by their documented chemotaxis to aliphatic alcohols (Reding and Wiegel, 1993). Therefore, it is very likely that *Xanthobacter*, and possibly also other N_2-fixing Knallgas bacteria, can be found in habitats other than the ones mentioned above. So far, no thermophilic, psychrophilic, or halophilic strains have been isolated. Furthermore, it is not clear whether *Xanthobacter* contributes significantly as an associative azotroph to the nitrogen cycle in the environment including wetland rice or marigold flowers, even though in greenhouse experiments, *X. flavus* strains isolated from rice roots have been shown to increase growth rate and growth yields of rice plants. Despite the ease with which *Xanthobacter*, especially *X.*

autotrophicus and *X. flavus* strains, can be isolated from the many environments mentioned above, *Xanthobacter* 16S rRNA is not frequently detected by sequence studies of the various environments. This could be an artifact since *Xanthobacter* cells can sometimes be difficult to lyse, thus not releasing their 16S rRNA. The author is not surprised that *Xanthobacter* isolates are not that often found in studies that look for aerobic dinitrogen-fixing bacteria, since most strains are only azotrophic under conditions where the oxygen tension is low (below 2% [v/v] in the gas phase), and most of the studies use either anaerobic (negative E_h) or aerobic conditions but not microaerophilic conditions.

Enumeration Studies

Only a few specific enumerations for *Xanthobacter* have been carried out. De Bont and Leijten (1976b) reported most-probable-number (MPN) counts for yellow chemolithoautotrophic N_2-fixing bacteria (assumed to be *Xanthobacter*) of 35 and 1700 per ml of sample (water from a ditch) and 4.9–11 $\times$ 10^5 per g of wet weight of sediment. Reding et al. (1991b) found 500–20,000 colony-forming *Xanthobacter* cells per g dry weight of soil (excluding the rhizoplane and rhizosphere) from wetland rice fields and about 5×10^5 per g dry weight of washed, soil-free roots.

Interactions with Plants

Oyaizu-Masuchi and Komagata (1988) reported a detailed survey of N_2-fixing bacteria from roots of rice in Japan. They proposed that strains (which they called "group 2") were *X. autotrophicus* and found *Xanthobacter*-like strains (group 5; possibly a new genus). They employed not fully specified anaerobic conditions for the first enrichment step on plates. However, no enumeration or further detail on the microbe-plant interaction was given. Chistyakova (1985) found between 2.5×10^3 and 4.5×10^6 N_2-fixing methylotrophic bacteria per g of soil under rice. Some of these bacteria were identified as strains of *X. flavus* on the basis of morphological and physiological properties. Up to 25% of the nitrogen fixed by soil bacteria was incorporated into rice plants within three growing seasons. In one soil (takyr-like soils of Kasakh, Armenia) *Xanthobacter* made up 40–70% of the methanol-utilizing N_2-fixing population. Thus, Chistyakova and Kalininskaya (1984) and Chistyakova (1985) concluded that the methanol-utilizing N_2-fixing bacteria contribute significantly to N_2 balance in the soil of paddy rice. Reding et al. (1991b) demonstrated that strains close to *X. autotrophicus* and *X. flavus*, beside being present in the sediment of patty rice fields in Arkansas, United States, are more abundant (more than 10^5 cells per g dry weight of roots) in the rhizosphere of rice and were clearly associated with the roots. This was evident from enumerations of *Xanthobacter* after stepwise washings (for up to 10 min) of rice roots from the field and by electron microscopy of rice grown in sterile sand that had been inoculated with a *Xanthobacter* strain isolated previously from the rice roots (Fig. 6A). So far, no strains were isolated from inside of the roots. However, a plant-microbe interaction is indicated by the finding

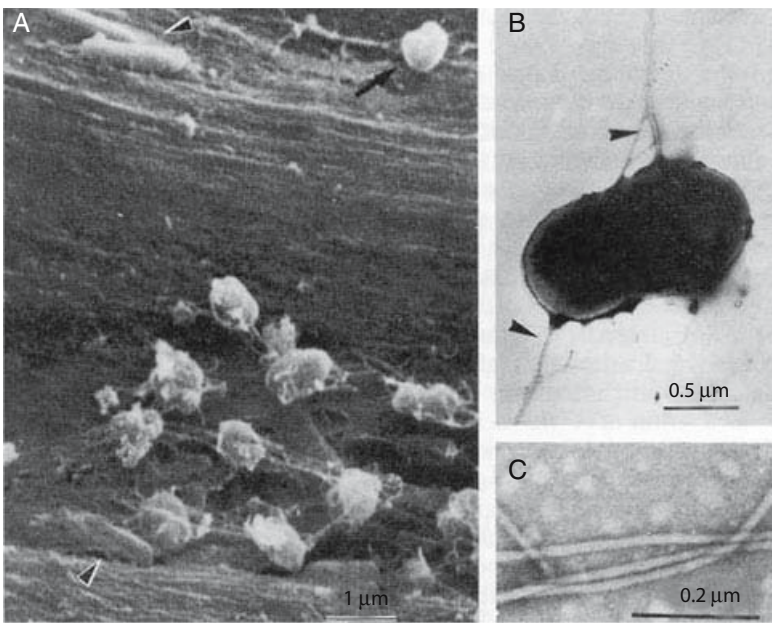

Fig. 6. A) Scanning electron micrograph of *Xanthobacter* strain JW-KR1 colonizing the surface of gnotobiotically (Hartel et al., 1990) grown rice. Notice the different shapes of the *Xanthobacter* cells, including typical rod-shaped cells (arrowhead), curled rods (long arrow), and coccoid cells. Electron micrograph (negative staining; 2% uranyl acetate) of: B) a flagellated cell of *X. flavus* type strain 301 grown on 0.1% propanol and C) flagella from *Xanthobacter* strain JW-KR1. The flagella are 13.7 nm in diameter. (All photos are courtesy of K. Reding.)

that a rice isolate, JW-KR1 of *Xanthobacter*, increased plant growth in both nitrogen-containing (15% greater top dry weight and 19% greater number of leaves per plant in comparison to the uninoculated control) and nitrogen-free plant growth medium (Reding et al., 1991a). Therefore, *Xanthobacter* can be classified as an associative diazotroph.

Samanta and Sen (1986a) reported that *X. flavus*, as well as other N_2-fixing microorganisms, contributed nitrogen to the plants through the leaves (doubling the nitrogen contents of the plants) when sprayed on rice and wheat plants. They suggested that the microbes use leaf wax components and leaf leachates to produce the energy required for N_2-fixation (Samanta et al., 1986b).

The possible role of *Xanthobacter* as a contributor of fixed N_2, a growth factor stimulant of the rice plants (as either a water or sediment organism), and an associative N_2 fixer (through either the phyllosphere or even stem nodules if in the future *Azorhizobium* is incorporated into the genus *Xanthobacter*) needs to be studied further. These studies should examine: 1) the role of the slime produced by *Xanthobacter* in its adherence to the rhizosphere and phyllosphere (an involvement of slime in adherence processes was shown for various anaerobic organisms; Brook, 1986); 2) the possible role of the polyglutamine polymer produced under high-nitrogen conditions (e.g., directly after fertilization); and 3) the role of plant growth stimulant formation by root and leaf-associated *Xanthobacter* cells. We have detected in cultures of *X. flavus* KR2 formation of indoleacetic acid when grown in medium containing tryptophan (Reding, 1991a; H. K. Reding and J. Wiegel, unpublished results).

So far, no *Xanthobacter*-like strains have been associated with any plant disease. However, the work of Kawai et al. (1989) indicates the possibility that because of the properties of the slime produced, *Xanthobacter* could indirectly mediate plant diseases by fostering the adherence of pathogenic microorganisms to plant cells.

Isolation

Selective Enrichments

CHEMOLITHOAUTOTROPHIC ENRICHMENT CULTURES. Inoculation could be done with soil, upper layers of marine or freshwater sediments, lake water, rice or marigold plant (*Tagetes*; yielding mainly *X. tagetidis* strains) roots or their washings, or samples of wet and decaying organic material (such as rotten leaves in ditches). A simple and proven method to isolate *X. autotrophicus* and *X. flavus* strains is as follows, but this procedure can be modified to isolate other species and strains with different properties and substrate specificity:

Incubate with shaking 1–2.5 g of material from freshwater lakes, rivers, or ditches or soil from wet meadows (rice fields) in 25–100 ml of 10 mM phosphate buffer (pH 7.0) and in an atmosphere of 5–10% air, 10% CO_2, 10% H_2, and 70–75% N_2 in the headspace. To supply the enrichment with the gas atmosphere, place the 100- or the 250-ml Erlenmeyer flasks containing the enrichment in an anaerobic jar (or any type of desiccator), evacuate it with the help of an aspirator, and then fill the jar with the above-mentioned gas mixture using a three-way valve. To obtain the required ratio of the gases, insert a manometer in the combined gas lines feeding the desiccator (for details, see The H_2-Metabolizing Prokaryotes in Volume 2). Exchange gas after 5, 8 and 10 days; after 12–14 days, transfer the culture to new medium (1–5% inoculum) and incubate the same way for 7 more days. The subculturing is repeated after another 5–7 days. The culture should have a slimy consistency and a yellowish, egg yolk color. Use this enrichment for further purification as described below.

DIRECT PLATING (FILTER METHOD) OF AQUEOUS SAMPLES. Another method involves direct plating using the filter method and incubation under chemolithoautotrophic growth conditions in the absence of ammonium salts. This method is especially recommended for the isolation of strains from water columns and for slower growing strains, such as strains of *X. agilis*. In liquid enrichments, strains of *X. agilis* tend to be overgrown by strains of *X. autotrophicus* and *X. flavus*. The following description of the filter method is adapted from that of Aragno and Schlegel (1981):

Filter 10 ml to 1 liter (depending on the filter size and source of the sample) of water through sterile 45- or 90-mm membrane filters with 0.2-µm pore diameter. To remove ammonium salts, low-molecular-weight organic nitrogen sources, and soluble carbon sources present in the samples, wash the membrane filters with 10–20 ml of 0.9% (w/v) saline solution to minimize the growth of oligocarbophilic and non-N_2-fixing organisms. Place the membrane filters on well-dried agar plates made with ammonium-free mineral medium solidified with 1.2% agar. Incubate the plates under an atmosphere of 80% N_2, 10% H_2, and 10% CO_2 (lithoautotrophic conditions) as described above until no new colonies appear. To discriminate between oligotrophs and H_2-utilizers (hydrogenase-positive strains), remove filters from the agar plates and place on filter paper soaked with a freshly prepared solution of 0.1% triphenyltetrazolium chloride. After 10 min of incubation under air, incubate the filters for another 10 min under 100% H_2. Except for the colonies that become red during the first incubation under air (i.e., colonies that reduce the dye via other reactions such as utilizing endogenous substrate or impurities from the agar), the red colonies represent hydrogenase-positive cells. Since the staining procedure is not lethal, the colonies can be transferred to succinate-nutrient broth agar plates (see below) for further purification and identification.

VARIOUS HETEROTROPHIC ENRICHMENTS. Using special substrates (non-N_2-fixing), the liquid enrichment procedure

described above will yield *X. autotrophicus* and *X. flavus* strains with high probability (Wiegel and Schlegel, 1976). To obtain new *Xanthobacter* strains with properties not present in the strains isolated using the above methods, other isolation procedures need to be and have been employed. For example: 1) Malik and Schlegel (1980) used normal aerobic, N_2-fixing conditions and, in addition to various other organisms, isolated the less oxygen-sensitive *Xanthobacter* strain CB6. Nakamura et al. (1984) isolated *X. autotrophicus* strain Y38 on plates as an oxygen-insensitive segregant of *X. autotrophicus* strain N34. Strain Y38 can grow on autotrophic plates in the presence of 40% O_2, although more slowly than under 10% O_2 (Ooyama, 1971; Nakamura et al., 1985a; Nakamura et al., 1985b). However, more oxygen-tolerant strains and segregants must be isolated and further taxonomic studies carried out to see whether this is a general property. 2) Enrichment with methanol and an inoculum of marine origin (Lidstrom-O'Connor et al., 1983) yielded the somewhat atypical strain H4-14 (Lehmicke and Lidstrom, 1985; Weaver and Lidstrom, 1985) now recognized as a strain of *X. flavus* (Meijer et al., 1990c). 3) "*Xanthobacter polyaramaticivorans*" was isolated from sludge of crude oil tanks under microaerophilic to anoxic conditions using dibenzothiophene-supplemented carbon and sulfur-free mineral medium (Doronina et al., 1996). 4) *Xanthobacter tagetidis* was isolated from roots of *Tagetes* (marigold) plants using 2.5 mM thiophene-2-carboxylate or thiophene-2-acetate supplemented mineral media (pH 7.3). Aerobic conditions in batch or continuous culture inoculated with material from the *Tagetes* root system yielded cultures where more than 90% of the cultures were *Xanthobacter*-like cells (Padden et al., 1997). 5) *Xanthobacter* strain E5a, which contains an interesting esterase, was isolated from sewage sludge on ethyl sulfate (White et al., 1987). And finally, 6) other promising heterotrophic substrates include various methyl(alkyl)amines (J. Wiegel, unpublished observations), propanol, propane, propene, ethane and butene (other gaseous and volatile hydrocarbons should also be tested), yielding strains with industrially interesting properties (see below; Siebert, 1969; De Bont, 1976a; Van Ginkel et al., 1986b; Van Ginkel et al., 1987; Van den Tweel et al., 1986). Other unusual strains which cannot be affiliated unambiguously into the present species have been isolated as Knallgas bacteria: strains such as 23A, RH9, 14g (tentatively, *X. autotrophicus*; Wiegel et al., 1978b), MA2 (tentatively, *X. agilis*; Jenni et al., 1987b), and strain 25a (Meijer et al., 1990c), which is similar to *X. autotrophicus* but exhibits a biotin requirement in continuous culture and greater motility than the type strain 7c, reference strains (JW33 and *X. flavus* 301), and several isolates from rice roots (including strain JW-KR1).

Unfortunately, many of the above-mentioned special strains have not been included in recent taxonomic studies and thus may represent other species or require the emendation of the present species description.

Isolation of Pure Strains

Because of slime formation (especially by *X. autotrophicus* and *X. flavus* strains), the use of agar plates containing special substrates frequently does not yield axenic cultures. This is especially true of chemolithoautotrophic agar plates without ammonium or organic N sources. Frequently, other oligotrophic organisms grow as contaminants in the slimy colonies of *Xanthobacter*. The nitrogen-fixing conditions enhance the formation of copious amounts of slime, which tend to keep the cells clumped together. Therefore, frequently it is difficult to obtain colonies from single cells using the common methods of plating cell material taken from liquid cultures or with a loop from colonies on plates. To overcome these difficulties, the following procedure, which involves treatment with NaOH, is recommended:

Preparation of Single Cells and Solubilization of Slime

Dilute an aliquot of a culture or suspended colony 1 : 3 with sterile medium, and adjust to pH 10–11 by adding drops of sterile 2 N NaOH. Mix the cell suspension vigorously for at least 1 min (on a vortex-mixer or similar equipment) to solubilize the slime and generate a homogenous suspension of single cells. Pellet the cells by centrifugation (5 min at 5000 *g*) and resuspend in fresh medium (pH 7.0), or, preferably, plate the cells directly on succinate-containing nutrient agar plates. Diagnostically characteristic branched cells grow on this medium.

Nutrient Broth Succinate Medium

Nutrient broth (Difco or Oxoid)	5 g
Yeast extract (Difco)	4 g
NaCl	3 g
Sodium succinate	5 g
Agar	10 g
Distilled water	1 liter

Adjust the pH to 6.8–7.0.

Besides leading to a suspension of single cells, the alkaline treatment of primary enrichment cultures markedly reduces the viability of non-*Xanthobacter* cells, resulting in a ratio of close to one *Xanthobacter* cell per cell of another organ-

ism. *Xanthobacter* strains can be recognized by the characteristic yellow color of the colonies and microscopically (if grown on the succinate-nutrient broth medium) by their irregularly branched cells (Wiegel and Schlegel, 1976; Wiegel, 2004; Fig. 2). The method described above has proven to be a reliable, rapid means of isolating pure strains of *X. autotrophicus* and *X. flavus* from various sources.

Cultivation and Storage

Media

Most *Xanthobacter* strains can be grown on various mineral media. However, since a low ionic strength is favorable for most *Xanthobacter* strains, a 5–10 mM sodium-potassium phosphate buffer with a pH around 7.0 is suggested. Phosphate buffer is preferred since storage at 4–25°C on nutrient broth plates leads within 2 weeks to nonviable cells. The following basal medium can be used for autotrophic as well as heterotrophic growth (Wiegel and Schlegel, 1976):

Basic Mineral Medium

K_2HPO_4	1 g
KH_2PO_4	0.5 g
$NaHCO_3$	2 g
$MgCl_2 \cdot 6H_2O$	0.1 g
$CaCl_2 \cdot 2H_2O$	0.04 g
$FeSO_4 \cdot 5H_2O$	0.01 g
Trace element solution	1 ml
Distilled water to	1 liter

A recipe for the trace element solution is given in Ormerod et al. (1961). Similar recipes containing 0.1–1 μM nickel ion can also be used. The addition of 0.1 μM nickel sulfate to previously published media recipes containing no nickel enhances growth, since nickel is required for hydrogenase in all tested strains. However, nickel ion concentrations of 1 mM and above appear to be inhibitory. Addition of nickel is not required for heterotrophic growth except when urea is used as nitrogen source, since *Xanthobacter* urease is a nickel-containing enzyme (e.g., as shown for strain Y38; Nakamura et al., 1985a). Traces of nickel are frequent impurities of other ingredients, leading to a limited growth in the absence of added nickel ions. No vitamins or additions of yeast extract are required for most *Xanthobacter* isolates, except for *X. flavus* and some special strains mentioned above which need the addition of biotin. However, the addition of 10–100 mg of yeast extract per liter to the mineral medium can reduce or avoid an extended lag time for autotrophic growth (both under nitrogen-fixing and non-nitrogen-fixing conditions) or when switching from one type of substrate to another.

During isolation, a vitamin solution (any mixture containing biotin can be used) is added to avoid the exclusion of strains that require biotin as well as other vitamins. For *X. flavus* and for fast growth of other strains, 10 ml of Pfennig's vitamin solution is satisfactory (Pfennig, 1965). For chemolithoautotrophic growth, the air is exchanged with an autotrophic gas mixture: 10% CO_2, 10% O_2, and 80% H_2 (v/v) when ammonium salts are present or 10% CO_2, 5% air, 10% H_2, and 75% N_2 when N_2-fixation is sought in the absence of ammonium or any organic nitrogen source. For heterotrophic growth, various carbon sources are added: 0.5% sugars, 0.3% (v/v) alcohols (except butanol, 0.1–0.2%), or 0.4–0.8% organic acids. For growth under non-N_2-fixing conditions, 0.1% of ammonium chloride or sulfate is added; a concentration of ammonium salts above 0.15% is inhibitory for most strains. For nitrogen-fixing conditions under heterotrophic growth conditions, delete ammonium salts and any organic nitrogen source above trace concentration (e.g., 10 mg yeast extract per liter medium) and use N_2 gas with 2–5% (v/v) air as the gas phase.

Complex Medium

For characterization or isolation purposes, the use of a succinate-nutrient broth-agar medium as selective medium, which leads to the formation of the characteristic branched cells, is recommended. Most strains have their shortest doubling time (as low as 2 h) in this medium. The exact composition of this medium is not critical, and good results have been obtained with the nutrient broth-succinate medium described earlier as well as nutrient broth containing other substrates such as methanol or glutarate rather than succinate. However, neither the liquid nor the agar-solidified complex medium, especially when supplemented with organic acids, is recommended for storage of cultures beyond 10 days; many strains lose their viability during storage on this medium even at 4°C.

pH and Temperature Ranges

Detailed studies of pH and temperature ranges and optima have not been performed for the type strains of the type species or for most other strains of *X. autotrophicus* and *X. flavus*. Most of the strains tested grow at pH 5.0–8.5 (Wiegel et al., 1978b). Except for *X. agilis*, *X. aminoxidans* and *X. viscosus*, which grow between 5°C and 34°C, the other species grow at 37°C and above (Jenni et al., 1987b). *Xanthobacter autotrophicus* strain 14g, studied in more detail, has its pH optimum for growth at 6.8–7.2 and its temperature optimum around 30°C (Schneider et al., 1973). For strain *X. autotrophicus* GZ29, only

the pH optimum for acetylene reduction has been determined; it exhibits the highest activity at a slightly lower pH of 6.7–6.8 (Berndt et al., 1976). *Xanthobacter aminoxidans* will grow between 6.5 and 8.5 (optimum 7.2–7.9), whereas *X. viscosus* and *X. tagetidis* have an optimum of pH 6.8–7.2 and 7.6–7.8, respectively. *Xanthobacter tagetidis* grows at temperatures of up to 43°C (Padden et al., 1997).

Storage of Cultures

Xanthobacter cultures grown on chemolithoautotrophic agar slants can be stored 1.5 years at 4°C after sealing the tubes tightly with parafilm (H. G. Schlegel and J. Wiegel, unpublished observations). Also, liquid cultures grown under chemolithoautotrophic conditions (mineral medium supplemented with 0.02% [w/v] yeast extract) have been kept for more than 15 months at 4°C and, if glycerol was added (40–60% [v/v] final concentration), at –20°C and –75°C for more than 8 years. For long-term storage, cultures should be lyophilized in the presence of skim milk and honey (10%), as originally described by Malik (1975) or later improved by Malik (Malik, 1988; Malik, 1990). Using these methods, cultures have been preserved at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)~~G~~erman Collection of Microorganisms and Cell Cultures~~an~~d the author's laboratory for more than 20 years without a drastic loss of viability.

Harvesting

The copious amounts of slime produced under many growth conditions can lead to difficulties in harvesting cells since the cells form a paste whether harvested by centrifugation or filtration. Except for *X. autotrophicus* and *X. flavus* cells grown in the presence of ammonium salts with propanol or butanol as carbon and energy source or under chemolithoautotrophic conditions, it is recommended that the pH be increased to about 10–11 or decreased to pH 4.5–4.8 before cells are collected by centrifugation. Otherwise, when no alkali treatment is possible, high-speed centrifugation (20,000–35,000 *g*) at 10–15°C is the method of choice to harvest the cells (at 0°C, the slime can become more viscous while being pelleted). Amicon hollow fiber cartridges (or less easily, the Pellicon Cassette System, Millipore) can be used to concentrate cultures. However, some of the slime is also concentrated so that, depending on the slime and the pH of the broth, only a minimal concentration of less than 1 : 5 can be obtained. For further concentration, an alkaline or acidic treatment for temporary solubilization of the slime must be employed.

Identification

Colony Morphology and Pigmentation

The morphological features of *Xanthobacter* can be used initially for identification. Colony morphology depends on the substrate and growth conditions. On most carbohydrates, the colonies of all three species are large (1–5 mm in diameter), smooth, convex, circular, filiform, opaque, and of a characteristic egg-yolk yellow color due to zeaxanthin dirhamnoside. The colonies become less yellow and less opaque as the amount of slime increases. Strains of *X. agilis* generally produce less slime than strains of the other species produce. A few strains have been isolated which grow as whitish colonies under autotrophic conditions because they produce not only small amounts of zeaxanthin dirhamnoside but also copious amounts of slime. The production of slime on nutrient broth agar plates frequently results in colonies resembling fried eggs. For the isolation of *Xanthobacter* strains, it is recommended that the color and colony morphology be compared with that of one or two authentic strains (e.g., the reference strain JW 33 = DSM 1618) grown on the same agar plates. A variety of other organisms produce yellow pigments. Flavobacteria produce the carotenoid pigment zeaxanthin, but not zeaxanthin dirhamnoside; thus the shade of yellow differs (greenish-yellow, sulfur-yellow, or yellow containing a slight pink or brown). Zeaxanthin dirhamnoside is water-insoluble, in contrast to the reddish/pinkish/brown pigment or to the yellow-green diffusing pigments (also exhibiting fluorescence) of *Beijerinckia* and *Derxia* (the other yellowish diazotroph which can be obtained using the enrichment procedures described). *Xanthobacter* colonies do not noticeably change color with age, although the yellow darkens with an increase in cell density, partly due to shrinkage of the slime as water is lost. The latter fact also makes it easy to distinguish *Xanthobacter* from *Derxia* colonies, which turn brown with age (Becking, 1981). The exception among *Xanthobacter* spp. is "*X. polyaromaticivorans*," which has an orange water insoluble pigment that is proposed to be a zeaxanthin on the basis of its absorption spectrum (i.e., peak at 473 nm instead of the normal peak of around 450 nm; Hirano et al., 2004).

Cell Morphology

SIZE. The morphology of *Xanthobacter* (especially strains of *X. autotrophicus* and *X. flavus*) changes with growth conditions and carbon source (Figs. 1–4). Cells grown under chemolithoautotrophic conditions or grown het-

erotrophically with sugars or organic acids are straight or slightly irregular rods (0.4–0.7 μm × 2–4 μm; Figs. 1a, 1b, and 3). Most strains grown on methanol and ethanol are similar in morphology to cells grown autotrophically; cells grown on agar plates containing propanol as the sole carbon source can be coccoid or short rods (0.8–2 μm long) with little slime formation, and cells grown on agar plates containing 0.1% butanol can be coccoid or up to 10-μm-long rods (Van Ginkel and De Bont, 1986a; Wiegel, 2004; Fig. 1c). In contrast to the carbon source, the nitrogen source does not have a pronounced effect on the morphology. Cells grown under N_2-fixation conditions tend to be slightly longer than those grown in the presence of ammonium salts or other organic nitrogen sources. Cells grown on complex medium with 0.2–0.3% succinate or in the presence of other TCA cycle intermediates are multibranched and vary greatly in size (3–10 μm; Fig. 2).

The highly irregular branching pattern of *Xanthobacter* cells on nutrient broth agar plates is a characteristic morphological feature as well as the zeaxanthin dirhamnoside pigment. The formation of highly irregular, branched cells separates *Xanthobacter* from yellow flavobacteria and the so-called "high G+C" (65–70 mol%) flavobacteria such as *Flavobacterium capsulatum*, which are not related to *Xanthobacter*.

Suspensions of chemolithoautotrophically grown cells and cells heterotrophically grown with sugars frequently contain cell aggregates like those (due to snapping-type division) typical of *Corynebacteria* and some *Arthrobacter* spp. However, the cell aggregates formed by *Xanthobacter* are due to slime and not to the one-sided rupture of the cell wall during the cell division process. *Xanthobacter viscosus* and *X. aminoxidans* have a different cell type. Their cells are also pleomorphic and exhibit under various growth conditions branching cells that divide asymmetrically via a process resembling budding. *Xanthobacter aminoxidans* cells can occur in a V-formation, and *X. tagetidis* cells usually occur in a twisted form (Fig. 4).

CELL INCLUSION BODIES. Under most growth conditions, for all *Xanthobacter* species typical, large polar inclusion bodies (black cell poles in phase contrast microscopy) filling the cell tip are found at one or both cell ends. The multibranched cells can have inclusion (polyphosphate or poly-β-hydroxybutyrate [PHB]) bodies at each branch tip. PHB can constitute up to 600 mg per g dry weight of cells (Wiegel, 2004). Lipid inclusion bodies at both cell ends are also characteristic of *Beijerinckia* (Becking [1981] and literature cited therein). Nevertheless, *Beijerinckia* can be easily distinguished from *Xanthobacter* species by differences in 16S rRNA sequence, pigmentation, acid tolerance (most *Beijerinckia* species can grow and fix nitrogen at pH 3–4), growth on nutrient broth (which is poor for *Beijerinckia*), and ability to fix N_2 under air, i.e., under normal oxygen concentrations (*Beijerinckia* has this capability).

FLAGELLATION. *Xanthobacter agilis* exhibits the greatest motility of all *Xanthobacter* species under all growth conditions. Using light microscopy, strains of *X. autotrophicus* and *X. flavus* usually do not appear to be motile, but when grown chemolithoautotrophically on sugars or under N_2-fixing conditions, motility greater than the Brownian movement can be observed occasionally. This is due to reduced peritrichous flagellation found on many strains of these two species (Reding et al., 1992; Reding and Wiegel, 1993; Fig. 6). The flagella have the Type 1 morphology (Aragno et al., 1977), are 14.2 μm in diameter and 9–14 μm long, and have a wavelength of 2.0–2.5 μm. However, the flagella are fragile and break easily during preparations for electron microscopy (Aragno et al., 1977). The extent of flagellation appears to depend on growth conditions, including substrate and growth stage. Strains of *X. autotrophicus* (including type strain 7c), *X. flavus* (including type strain 301), strain JW/KR2, and many isolates from rice roots are motile when grown on methanol, ethanol, isopropanol (0.3%), and butanol (0.1%)~~w~~hich includes the longer chain alcohols that chemotactically attract *Xanthobacter*—or on gluconate, glutamate and diglutamate (Reding, 1991a; Reding et al., 1992; K. Reding and J. Wiegel, unpublished results). Thus, in contrast to the findings in prior reports (Jenni et al., 1987b), the differentiation of *X. agilis* from other *Xanthobacter* species has to be based on its pronounced motility under chemolithoautotrophic growth conditions, its reduced pleomorphology, and differences in substrate utilization (Table 1). For the other species, whether cells are motile under some growth conditions remains unclear since motility was probably tested under heterotrophic growth conditions (though not specified) and not checked when cells were grown on other compounds including alcohols during substrate spectrum determinations.

CHEMOTAXIS. *Xanthobacter* exhibits a positive chemotaxis toward aliphatic alcohols such as isopropanol and butanol but not towards methanol, ethanol, sugars or organic acids (Reding, 1991a; Reding and Wiegel, 1993).

BIOCHEMICAL PROPERTIES USEFUL FOR IDENTIFICATION Though 16S rRNA sequence analysis has become the standard for identification of novel isolates, other properties are still useful for identification of unusual strains at the species level, since significant differences can

occur despite a high 16S rRNA similarity (case in point, *X. flavus* and *X. aminooxidans*).

GRAM-STAIN REACTION AND CELL WALL TYPE AND COMPOSITION. Interpreting the Gram stain for *Xanthobacter* is sometimes misleading, as is evident from the various previous assignments of strains~~no~~w identified as *Xanthobacter~~to~~* "Gram-positive" *Corynebacterium* and *Mycobacterium*. False reading of the Gram-stain was due to large inclusion bodies that appear as dark zones in the otherwise not well stained cells. As was unequivocally shown (Wiegel, 1981; Wiegel and Quandt, 1982b), *Xanthobacter* species are Gram-type negative organisms, as defined by the presence of lipopolysaccharide (LPS; Wiegel and Mayer, 1978a). The presence of LPS, although at lower concentrations than found with other typical Gram-type negative organisms, can be demonstrated specifically and rapidly with the lipopolysaccharide-polymyxin B test of Wiegel and Quandt (Wiegel and Quandt, 1982b; Fig. 7). The cell wall type is the *meso*-diaminopimelic acid direct type (tested for *X. autotrophicus* and *X. flavus*), typical for Gram-type negative bacteria. Teichoic and teichuronic acids are absent. The peptidoglycan content for the tested strains of *X. autotrophicus* (including reference strain JW 33) is 15–25%, and thus is intermediate between typical Gram-type positive and Gram-type negative cells. Micrographs of ultrathin sections for this and the other species reveal a multilayer cell wall similar to that of typical Gram-type negative organisms. This is also supported by the presence of a citrate synthase in *Xanthobacter* strains GZ29 and JW 33, typically found in Gram-type negative bacteria, which have molecular weights above 250,000 and are inhibited by reduced nicotinamide adenine dinucleotide (NADH; Weizman and Jones, 1975; Berndt et al., 1976). Presently, there are no detailed data on cell wall composition of the other *Xanthobacter* species available.

One special effect was observed for *X. autotrophicus* strains (not tested for other *Xanthobacter* spp.), in that under heterotrophic growth conditions polyglutamine can substitute for the peptidoglycan in the cell wall. Kandler et al. (1982) demonstrated that all 20 strains tested from *X. autotrophicus*, *X. flavus* and *X. agilis* form an extracellular α-polyglutaminyl polymer. This polymer is isolated in association with the cell wall but not the slime from cells growing heterotrophically in the presence of sufficient ammonium or organic nitrogen, and the polymer is not dissolved along with the slime when cells are subjected to alkaline treatment. Cells shifted to nitrogen-limiting conditions apparently re-utilize the polymer (J. Wiegel, unpublished observations). The only other organism known to produce this polymer is *Flexithrix* (Kandler et al., 1982).

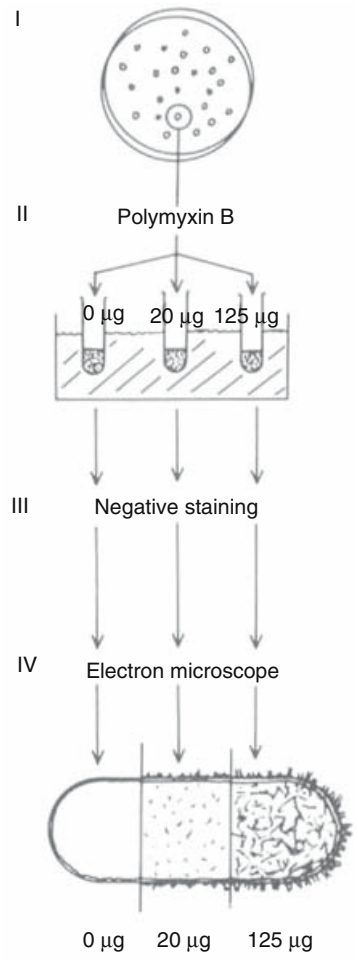

Fig. 7. Diagram for the LPS-polymyxin B test to determine the Gram type.

GLYCINE TREATMENT. The cell wall of *Xanthobacter* species (mainly observed with *X. autotrophicus* and *X. flavus*) can be difficult to break by French press or ultrasound disruption and the usual cell lysis procedure for DNA or cell wall analysis. Cells can be rendered easy to break and lyse by growing them for one to two doublings in the presence of glycine. The required concentration (0.1–0.4% [w/v] glycine) to be used varies with the strain. The glycine is added at the mid-to-late exponential growth phase, and then the cells are harvested after growth ceases within two doublings.

LPS-POLYMYXIN B TEST FOR THE PRESENCE OF LPS AND THE DETERMINATION OF THE GRAM-TYPE. The presence of LPS can be quickly determined by demonstrating specific bleb formation (representing LPS-polymyxin B complexes) in electron micrographs of negatively stained cells (Wiegel and Quandt, 1982b) as diagrammed in Fig. 7. The procedure is as follows:

Suspend cells, preferably grown on plates, in 1 ml of 10 mM Tris hydrochloride, pH 7.2. Incubate the cells for 5 min in the presence of 0 (control), 10 and 125 µg of polymyxin B per ml of cell suspension at 35°C. Fix the incubated cells directly to carbon-covered, copper grids and negatively stain them. In electron micrographs, Gram-type positive cells and the control cells (no polymyxin B treatment) have smooth cell walls, whereas Gram-type negative cells exhibit blebs of LPS-polymyxin B complex (for a detailed discussion of the method, see Wiegel and Quandt, 1982b).

Xanthobacter will exhibit only a few isolated blebs, in contrast to species of *Flavobacterium*, pseudomonads, or enterobacteria. Cells from an agar plate give cleaner preparations than washed cells grown in liquid cultures. This test is the most precise test for identifying the Gram type (Wiegel, 1981), since, in contrast to some other sensitive biochemical tests (e.g., the limulus lysate test for the presence of LPS), many individual cells are visually examined and this procedure avoids the error of recording a positive reaction due to a few contaminating dead LPS-containing cells. Presently most researchers use 16S rRNA sequence analysis to determine the phylogenetic position.

ANALYSIS FOR ZEAXANTHIN DIRHAMNOSIDE. As mentioned earlier, a specific biochemical check for *Xanthobacter* is to demonstrate the presence of zeaxanthin dirhamnoside, which is (on the basis of absorption spectra) otherwise found only in *Corynebacterium flavescens* and *Hydrogenophaga* species (Urakami et al., 1995). The following procedure is a modification of K. Schmidt's procedure, which is based on the one by Hertzberg et al. (Herzberg et al., 1976; K. Schmidt, personal communication).

Identify by thin layer chromatography after converting the pigment to the peracetylated form using the method described below. Exclude light and oxygen as much as possible during all the steps, since the carotenoid compounds are light and oxygen sensitive. Perform all steps under a black velvet cloth or in a room with low-level lighting. Furthermore, after freeze-drying the cells, keep all material in an atmosphere of N_2 or argon. Argon is heavier than air and thus can more easily replace the air in the reaction vessel.

Extract about 0.5–1 g of freeze-dried cells in a tightly stoppered separation funnel or bottle overnight with an argon-flushed 1 : 1 mixture of acetone and methanol. Remove the solvent and dry the residue over silica gel in a desiccator flushed with argon. After 12–18 h, dissolve the dry residue in 1 ml of water-free pyridine flushed with argon. To this solution, add 0.1 ml of acetic anhydride. Allow the mixture to react in the dark under argon at about 25°C for 20 h. Then add 2–3 ml of diethyl ether to stop the reaction, and add about 5 ml of argon-flushed, aqueous 3% NaCl solution to drive the acetylated product into the diethyl ether. Remove the pyridine quantitatively from the ether fraction by repeated washings with the argon-flushed aqueous NaCl solution. Evaporate the ether extract to dryness in a rotary evaporator; break the vacuum with argon. Then dry the sample further using an oil vacuum pump to remove traces of pyridine. If the sample needs to be stored, do so under argon in a desiccator kept in the dark. Dissolve the dry residue in about 100 µl of argon-flushed acetone. Use this solution immediately for thin layer chromatographic analysis on silica gel (e.g., no. 5721, Merck, Darmstadt, Germany, or a similar product) and an acetone-benzene ether (petro-ether; boiling point, 40–60°C) 3:7 (v/v) as solvent. R_F-values are around 0.8 for zeaxanthin, 0.63 for zeaxanthin monorhamnoside-peracetate, and 0.4 for zeaxanthin dirhamnoside-peracetate. Some minor other spots of breakdown products and intermediates of pigment synthesis are found. Use an authentic sample derived from a type strain (e.g., *X. autotrophicus* 7c or the reference strain JW 33) as a standard to ensure that the procedure was carried out correctly and to compensate for differences in specifications of solvents or silica gel plates. Spectral data (maxima) in acetone are: for the major (natural) *trans* form (428 nm), 453 and 480 nm; and for the minor form (at least partly derived as artifact 342 [425 nm]), 452 and 478 nm (Herzberg et al., 1976; K. Schmidt, personal communication).

OTHER PROPERTIES *Xanthobacter* strains are sensitive to various antibiotics, but the response depends on the method employed (e.g., liquid cultures or the use of Difco Dispense-O-Disk minifilters). With the latter method, *X. autotrophicus* and *X. flavus* were insensitive to ampicillin, chloramphenicol, erythromycin and penicillin. The four tested strains of *X. agilis* were sensitive to erythromycin but exhibited different responses with the other three antibiotics (Jenni et al., 1987b). In liquid culture, *X. autotrophicus* strains are usually sensitive to 100 µg/ml penicillin, novobiocin, and polymyxin B per ml of culture; they are resistant to 200 µg/ml erythromycin and bacitracin per ml of culture. The available data do not suggest antibiotic typing as a valid method for identification of *Xanthobacter*.

Several other indicative reactions give mixed results depending on the strains. For instance, only some strains can grow on violet red-bile medium (Oxoid), deoxycholate medium (Oxoid), tellurate agar (Difco), and mineral medium supplemented with crystal violet (red colonies; Wiegel, 2004). Also the effect of phosphoenolpyruvate on the easy-to-determine activity of glucose-6-phosphate dehydrogenase (Opitz and Schlegel, 1978) or the effect of the branched-chain amino acids on α-isopropylmalate synthase (Wiegel and Schlegel, 1977) can be used as a taxonomic test.

Xanthobacter autotrophicus strain 7c and CB2 grown at 37°C contain 8–13 µmol of putrescine and 16–22 µmol of *sym*-homospermidine per g dry weight of cells (Kneifel et al., 1986).

The distribution of the lipid fatty acids is presented in Table 2 as far as they have been analyzed. The main fatty acid (constituting up to 93% of the lipid) is the monounsaturated C-18:1 (*cis* and *trans*). In "*X. methylooxidans*" strain 32P (Doronina et al., 1996), the *cis*-vaccenic acid (C-

Table 2. Fatty acid analysis of *Xanthobacter* strains.[a]

	1.		2.			3.	4.	5.	6.	7.	8.
Fatty acid	7c[T]	7cSF	301[T] KR1	4-14H	JW-KR2 JW-JWKR6 R1-2	SA35[T]	TagT2C[T]	14a[T]	7d[T]	127W[T]	32P
	16:0	4.83	3.48	2.67	3.84	1.19–2.68	1.19	10.32	n.d.	n.d.	22.7
16:0 3OH	1.32	0.98	0.73	1.45	0.0–1.69	1.45	0.74	n.d.	n.d.	n.d.	n.d.
16:1 *cis* 9	5.04	6.24	0	1.86	0–0.52	0	3.45	n.d.	n.d.	n.d.	n.d.
17:0	0	0	0	0	0	0.76	0	n.d.	n.d.	n.d.	n.d.
17:0 *cyclo*	2.45	1.23	0	0	0	0	0	n.d.	n.d.	n.d.	n.d.
18:0	1.98	1.53	2.65	1.19	1.79–2.69	1.99	3.25	n.d.	n.d.	10.6	n.d.
18:1[b]	76.81	81.64	91.90	91.66	93.1–96.03	93.01	57.44	n.d.	76.0	n.d.	70–92
18:2 *cis*, 9,12 and 18:0	0	0	0	0	0	0	1.13	n.d.	n.d.	n.d.	n.d.
19:0 *cyclo* C11-12	5.99	3.02	1.84	0	0–0.86–0.74	0.86	19.68	n.d.	n.d.	n.d.	n.d.
19:1 *trans* 7	0	0	0	0	0	0	1.40	n.d.	n.d.	n.d.	n.d.
20:1 *trans* 11	1.55	1.90	0	0	0	0.74	n.d.	n.d.	1.35	n.d.	n.d.

Abbreviations: [T], type strain; and n.d., no data.

[a]1., *Xanthobacter autotrophicus*; 2., *X. flavus*; 3., *X. agilis*; 4., *X. tagetidis*; 5., *X. aminoxidans*; 6., *X. viscosus*; 7., "*X. polyaromaticivorans*"; and 8., "*X. methylo-oxidans*."

[b]Value given is the sum of 18:1 *cis* 11, 18:1 *trans* 9, and 18:1 *trans* 6.

Data for 1.–3. from Henry Keith Reding. Ecological, physiological, and taxonomical studies of *Xanthobacter* strains isolated from the roots of wetland rice. Dissertation (1991): University of Georgia, Athens, GA (United States). Samples were prepared and analyzed by Microbial ID Inc. (Newark, DE).

Data for 4. from Padden et al. (1997), for 5. and 6. from Dorina and Trotsenko (2003), for 7. from Hirano et al. (2004), and for 8. from Doronina et al. (1996).

18:1-*cis* 11) is 55–75%. "*Xanthobacter polyaromaticivorans*" has a higher concentration of the saturated C-16:0. The main hydroxy fatty acid is the 3-OH C-16:0 acid.

Physiological Properties

Chemolithoautotrophy One of the characteristic properties of all strains of *Xanthobacter* spp. is the Knallgas reaction. All strains grow easily under chemolithoautotrophic conditions with doubling times from 3 to more than 12 h (Wiegel and Schlegel, 1976; Berndt et al., 1978). They differ in this respect from *Aquabacter* and *Azorhizobium*, which are intermingled with *Xanthobacter* species in the 16S rRNA sequence-based phylogenetic tree, but no chemolithoautotrophic growth has been demonstrated for these two. For detailed tests and methods of autotrophic growth, see The H_2-Metabolizing Prokaryotes in Volume 2. Thus far, none of the tested strains oxidize carbon monoxide to CO_2 or grow with carbon monoxide in either the presence or absence of H_2 (O. Meyer and J Wiegel, unpublished observations). However, H_2 can be replaced by thiosulfate to provide energy for CO_2 fixation (Friedrich and Mitrenga, 1981), and this has been observed in several strains of *X. autotrophicus* and *X. flavus* strain 301 by J. Wiegel (unpublished observations). When cells of *X. autotrophicus* type strain 7c were grown heterotrophically with high heterotrophic substrate concentrations and in the presence of an autotrophic gas mixture, no mixotrophy was observed; H_2 inhibited the induction of enzymes for heterotrophic growth in cells grown autotrophically and inhibited the utilization of sugars (fructose; Tunail and Schlegel, 1976). Using *X. flavus* strain H4-14 and *Xanthobacter* strain 25a in batch and continuous cultures, Meijer (1990a) could show that mixotrophy occurred when heterotrophic substrate concentrations were reduced or when pseudoheterotrophic substrates are used for growth. (Pseudoheterotrophic substrates such as methanol and formate [metabolized via oxidation to CO_2] are utilized at least partly via the autotrophic pathway.) Cells grown on acetate had no ribulose-1,5-biphosphate carboxylase (Rubisco) activity, and no activity was found when the gene was fused to the *lacZ* gene. However, at lower acetate concentrations, the addition of methanol or formate resulted in the induction of this enzyme and consequently utilization of both substrates, leading to additive growth yields. Thus, Rubisco is apparently regulated by repression caused by heterotrophic compounds and derepressed by autotrophic energy sources including the cryptic autotrophic substrate methanol (Opitz, 1977; Meijer, 1990a; Meijer et al., 1990b; Meijer et al., 1990c; Meijer et al., 1990d).

In *X. autotrophicus* strain 14g, H_2 exerts catabolite repression on the utilization of the organic

substrates gluconate, succinate and citrate in autotrophically grown cells, as well as in citrate grown cells. However, this was not observed in cells grown on gluconate or succinate. The uptake hydrogenase is inducible and its activity is increased up to 2500 µl of H_2 per mg of protein per h by incubation under autotrophic growth conditions with a gas mixture. In strain 14g, this occurs also in heterotrophic (succinate) grown cells in the presence of the organic substrate, although only to a lesser extent. In such induced cells both hydrogen and succinate are simultaneously utilized (mixotrophy). The gluconate-dehydratase, 6-phosphogluconate-dehydratase, and the Entner-Doudoroff enzyme system are induced in gluconate grown cells of strain 14g but not in autotrophically grown cells (Schneider et al., 1973).

Steinbüchel et al. (1983) have shown that anaerobically fermentative enzymes are induced in *X. autotrophicus* strains, and Berndt et al. (1978) reported the occurrence of anaerobic N_2 fixation by *Xanthobacter* strains. Oyaizu-Masuchi and Komagata (1988) used anaerobic conditions (with an unspecified technique) during enrichment. *Xanthobacter* strains are not killed during incubation under anaerobic conditions over several days, even when H_2S evolves (J. Wiegel, unpublished observations). However, *Xanthobacter* strains have not yet been shown to grow and multiply under strictly anaerobic conditions.

Rubisco, the key enzyme for autotrophic growth, is inducible but has been studied only to a small extent. *Xanthobacter* species fix CO_2 mainly via the ribulose-biphosphate pathway (Meijer et al., 1990b; Meijer et al., 1990c; Meijer et al., 1990d), but phosphoenolpyruvate carboxylase activity also has been demonstrated (Bowien and Schlegel, 1981). For *Xanthobacter flavus* strain H4-14, it has been shown that the genes encoding the key enzymes of the Calvin cycle are organized in two operons (Meijer, 1990a).

Ensign et al. (1998) have shown that the fixation of CO_2 plays an important role in the degradation of aliphatic epoxides and ketones by novel carboxylases (Ensign and Allen [2003] and literature cited therein).

The other key enzyme, hydrogenase, is membrane-bound and constitutive (in *Xanthobacter autotrophicus* type strain 7c, GZ29, and Y38) or inducible (in strain 14g). This enzyme cannot directly reduce NAD; no soluble hydrogenase has been found (Schneider et al., 1973; Tunail and Schlegel, 1974; Schneider and Schlegel, 1977; Berndt and Wölfle, 1979; Pinkwart et al., 1979; Schink and Schlegel, 1980; Nakamura et al., 1985a). Especially for strain Y38, it has been shown that nickel is required for hydrogenase activity; if nickel availability is restricted by adding metal-chelating agents, no hydrogenase activity is obtained. Addition of higher nickel concentrations restores autotrophic growth and hydrogenase activity. It is of taxonomic interest that the enzyme from *X. autotrophicus* strain GZ29 did not crossreact immunologically with the hydrogenase from *Alcaligenes eutrophus* H16 or with membrane extracts of *X. autotrophicus* strain 7c, 14g, and 12/60/x (Schink and Schlegel, 1980).

Heterotrophic Respiration Rates Using radiorespirometry on strain *Xanthobacter autotrophicus* 10/–/x, half maximal respiration rates were obtained with 0.26 mM fructose. With the addition of glucose at equimolar concentrations of fructose, no inhibition was observed. Glucose, used as sole carbon and energy source, leads only to low respiration rates, and increased concentrations were inhibitory (Opitz, 1977). When *X. autotrophicus* strain 19/–/x was plated on minimal medium containing glucose as carbon source, several colonies of glucose utilizing variants appeared after 2–6 weeks of incubation. In these cells, half maximal saturation for glucose respiration was 22 mM. Contrary to fructose, the glucose uptake seemed to be passive. Fructose, in contrast to sucrose, was respired in these cells without a lag phase (Opitz, 1977).

Methylotrophy (and Use of Other Alcohols) All strains of *Xanthobacter* species (except strain CB6 and two other strains out of over 60 tested strains of *X. autotrophicus* and *flavus*) can utilize methanol. Most strains also can grow on ethanol, *n*-propanol, *n*-butanol, 2-butanol and amyl alcohol (Wiegel et al., 1978b; Jenni et al., 1987b; K. Reding and J. Wiegel, unpublished observations). The longer alcohols have not been tested sufficiently as sole sources of carbon and energy.

Xanthobacter autotrophicus oxidizes methanol to CO_2 via a pyrrolo-quinoline quinone (PQQ)-containing methanol dehydrogenase that is stimulated by ammonium (Opitz, 1977; J. A. Duine, personal communication). Methanol utilization has been studied in detail in *X. flavus* strain H4-14 (Lehmicke and Lidstrom, 1985; Weaver and Lidstrom, 1985; Weaver and Lidstrom, 1987; Meijer, 1990a; Meijer et al., 1990c), in *Xanthobacter* strain 25a (Meijer, 1990a), in connection with the degradation of 2-chloroethanol in strain GJ10, which involves the PQQ-dependent methanol dehydrogenase (Janssen et al., 1987), and in "*X. methylooxidans*" (Doronina et al., 1996). Methanol is oxidized to CO_2 via methanol, formaldehyde, and formate (NAD) dehydrogenases, and carbon dioxide is then assimilated via the Calvin cycle, as in nearly all "Knallgas" (H_2/O_2/

CO_2-utilizing) bacteria. The mutant obtained by Weaver and Lidstrom (1987) indicated that the methanol dissimilation is regulated by a repressor mechanism. The PQQ-dependent methanol dehydrogenase was purified (Janssen et al., 1987). In *X. flavus*, the maximal induction of the *cbb* and *gap-pgk* operons (regulated by LysR-type regulator) encoding enzymes of the Calvin cycle occurs in the absence of multicarbon substrates and the presence of methanol, formate, hydrogen or thiosulfate. The operon is proposed to be regulated by the intracellular concentration of NADPH (Shively et al., 1998; Van Keulen et al., 2000; Van Keulen et al., 2003).

Nitrogen Fixation *Xanthobacter autotrophicus* fixes typically dinitrogen under heterotrophic growth conditions at rates of about 20–25 mg N per g sucrose; however, extreme values of up to 65 mg N per g have been found for cells from the early exponential growth phase (Wiegel and Schlegel, 1976). N_2-fixation under autotrophic growth conditions is the most important feature of *Xanthobacter*, but little work has been published in this respect. N_2 fixation was demonstrated unequivocally for several strains of *X. autotrophicus* using $^{15}N_2$ incorporation into cell protein (Wiegel and Schlegel, 1976). The biochemical studies on the enzyme and its relationship to oxygen have been restricted to *X. autotrophicus* strain GZ29 (Berndt et al., 1976; Berndt et al., 1978) and to *X. flavus* strain 301 (Biggins and Postgate, 1969; Biggins and Postgate, 1971). The nitrogenase in these two strains is similar to that in other aerobic diazotrophs. There is strong variation among the strains in respect to the optimal O_2 concentration for growth under N_2-fixing conditions (J. Wiegel, unpublished observations). In *X. flavus* 301 and *X. autotrophicus* GZ29, the response of the nitrogenase to exposure to air is quite different. The optimal partial pressures of O_2 for acetylene reduction are 5 and 2.5 kPa (whole cells) for strains *X. flavus* 301 and H4-14, respectively, compared to 0.36 kPa for *X. autotrophicus* strain GZ29 (Berndt et al., 1976; Murrell and Lidstrom, 1983). The nitrogenase activity in a cell-free extract of strain 301 decays exponentially with a half-life of about 5 min when stirred under an atmosphere of air. Growth of strain 301 in the presence of ammonium is not influenced by an oxygen partial pressure between 1 and 20 kPa, whereas strain GZ29 had a clear optimum around 15 kPa. This difference in the response to the oxygen partial pressure is even more pronounced for growth under N_2-fixing conditions. The O_2 optimum is around 10 kPa for strain 301 but only 1.5 kPa for strain GZ29.

Miller et al. (1988) demonstrated the presence of a strong vanadium uptake system in some *X. autotrophicus* strains. However, the presence of the alternative vanadium nitrogenase system could not yet be shown through substantial ethane production or through a substantial increase in growth above background when vanadium was added to molybdenum deprived medium (K. Reding and J. Wiegel, unpublished observation). Further research is needed to clarify this question.

Genetics Wilke (1980) described conjugational gene transfer in *X. autotrophicus* strain GZ29, obtaining a low-frequency recombination system with various isolated mutants. Several genes involved in carbon dioxide fixation and carbon metabolism were studied in strain H4-14 (Meijer, 1990a; Meijer et al., 1990b; Meijer et al., 1990c). The authors identified and sequenced the genes for the large (cfx1) and small (cfxS) subunit of Rubisco. Both genes are cotranscribed, and the cfxSL promotor appears to be similar to the *Rhodospirillum rubrum* cfxSL promotor. Downstream are the genes encoding for fructose-biphosphatase (cfxF) and phosphoribulokinase (cfxP). The sequence is similar to those from other sources (Meijer et al., 1990d).

Janssen et al. (1989) used the broad host-range cosmid vector pLAFR11 in a triparental mating system to prepare a gene bank of strain GJ10. Four clones containing genes involved in 1,2-dichloroethane metabolism by strain GJ10 were isolated, and consequently the gene for a haloalkane dehalogenase was cloned and sequenced. The cloned dehalogenase was expressed at a high level (up to 30% of the soluble intracellular protein) in *E. coli*, two strains of *Pseudomonas*, *P. oleovorans* TF41L, *X. autotrophicus* strains 7c, GZ29, XD, and in a halogenase-negative mutant of strain GJ10. The efficiency of conjugation with strong slime-producing strains (e.g, with strain 7c) was generally low (Wilke, 1980; Janssen et al., 1989). Interestingly, *Pseudomonas* strain GJ1 was able to degrade 1,2-dichloroethane after the genes for the dehalogenase were introduced. Also, vectors derived from *Xanthobacter* after introducing the broad-host-range plasmid IncP1 (a RK3 derivative) are suitable for gene cloning in *Xanthobacter* hosts. Whether the methods used by Janssen et al. (1989) provide general and efficient genetic systems for introducing genes into *Xanthobacter* species or cloning genes derived from various *Xanthobacter* species into various other organisms needs to be demonstrated.

Svaving et al. (1996) described an optimized electroporation procedure for *X. autotrophicus* GJ10 and some other strains with high transformation efficiencies (2×10^6 cells transformed per μg DNA). An efficient and useful transposon mutagenesis system (using hyperactive Tn*5*

transposase) has been recently developed and used for the genetic analysis of the pigment biosysnthesis in *X. autotrophicus* Py2 yielding frequencies of approximately 1×10^3 per recipient cell. Since this method worked also with other bacteria, it should work with most *Xanthobacter* strains (Larsen et al., 2002).

Applications Several strains of *Xanthobacter* are of interest for applied research and industrial applications. During the last few years the versatility of *Xanthobacter* strains has become evident. Some of these strains are deposited in nonaccessible deposits as patent strains or as nondisclosed industrial strains. It is anticipated that many novel strains (species) will emerge when further anthropogenic waste compounds are used as carbon and energy source in enrichments. A few strains with features of special interest for applications are described below.

Dehalogenation of Chlorinated Aliphatic and Chloroaromatic Compounds Strain *X. autotrophicus* GJ10 (Janssen et al., 1985) is able to utilize halogenated alkanes as its sole carbon and energy source. The utilization of 1,2-dichloroethane has been studied in detail because the United States Environmental Protection Agency's List of Hazardous Waste Compounds includes 1,2-dichloroethane, along with chloroethane and chloromethane. Strain GJ10 contains a novel haloalkane dehalogenase, which dehalogenates chlorinated, brominated, and iodinated one- to four-carbon *n*-alkanes (Keuning et al., 1985; Inguva and Shreve, 1999). The 36-kDa monomeric enzyme (the cloned gene appears to be a 310-amino acid polypeptide, corresponding to a molecular weight of 35,143; Rozeboom et al., 1988). Janssen et al. assume (Janssen et al., 1985; Janssen et al., 1987), based on physiological data and studies with a mutant, that 1,2-dichloroethane is dehalogenated to 2-chloroethanol in the cytoplasm; 2-chloroethanol, after being exported to the periplasm, is converted by a PQQ-containing 2-chloroethanol dehydrogenase (identified as a dehydrogenase) to chloroacetaldehyde (Bergeron et al., 1998); 2-chloroethanol can appear intermittently in the culture medium. The involvement of a cytoplasmic 2-chloroacetaldehyde dehydrogenase for further transformation to chloroacetate is indicated by the finding that the mutant lacking this enzyme was not able to grow on 1,2-dichloroethane. The final step is a chloroacetate dehalogenase reaction. The 1.7Å crystal structure of the L-2-haloacid dehalogenase (EC 3.8.1.2) is available on the web (http://www.rcsb.org/pdb/1qq7; Ridder et al., 1999). A comparison with other strains of *Xanthobacter* revealed that only the dehalogenases were unique to the 1,2-chloromethane utilizer, whereas the dehydrogenases are common enzymes with broad substrate specificity present in other alcohol-utilizing strains with similar specific activities. Spiess et al. (1995) published strain 14p1, which degrades readily 1,4 dichlorbenzene, whereas strain MAB2 (published as *Arthrobacter* sp.; Villarreal, 1991) degrades a variety of heteroaromatic compounds such as 2-chloracetanilides (2-chloro-*n*-ethylacetamide and propachlor). The substituted anilides are partly only converted to aniline, which is released into the medium. However in coculture with *Sphingomonas* sp. (DSM 7356), propachlor is totally degraded.

Ditzelmüller et al. (1989) isolated *Xanthobacter* strain CP, which could degrade 2,4-dichlorophenoxyacetic acid (2,4-D) with a growth rate of 0.13 per h and a growth yield of 0.1 g biomass per g of 2,4-D, as well as 2,4-dichlorophenoxybutyric acid, 4-chloro- and 2-methyl-4-chlorophenoxyacetic acid, and 4-chloro- and 2,4-dichlorophenol. However, activity for the required enzyme of the ortho-cleavage pathway, catechol 2,3-dioxygenase, could not be demonstrated in cell-free extract. The removal of 1,2-dichloroethane was shown on a laboratory scale using a packed-bed (immobilized microbes on porous diatomaceous earth) bioreactor that was continuously fed with contaminated groundwater from a site adjacent to a petrochemical facility (Friday and Portier, 1989). Feed concentrations of 160 and 230 mg/liter of ethylene dichloride were mineralized to less than 1 mg/liter employing a 12- and 18-h retention time, respectively (Portier and Friday, 1989). In another field study a similar 75-liter fermentor was tested, which was started with an adapted culture of strain GJ10; using a 20.5-h retention time and an approximate dissolved oxygen level of 2.5 mg/liter in the feed stream (leaving the aerator), 86% of 226 mg of ethylene dichloride per liter, 64.7% of 10.9 mg trichloroethylene per liter, and 57% of 2.5 mg tetrachloroethylene per liter were removed. After 42 days, in addition to *Xanthobacter*, four indigenous bacteria from the groundwater including *Comomonas acidovorans* had successfully acclimated to the reactor bed (Miller et al., 1990). Using oxygen gas for oxygenation, shorter retention times and minimization of stripping losses are achieved. Further scale-up for industrial use is under development (R. J. Portier and G. P. Miller, personal communication).

Aliphatic Alkane and Alkene Degradation and Epoxide Formation Adding 5% alkene in air to the gas headspace, Van Ginkel and De Bont (1986a) isolated six propene utilizers and one

butene utilizer (strain By2) from various soil and water samples. The alkenes were converted stereospecifically to the corresponding epoxides. The propene utilizers PY2 and PY10 were studied in more detail. The substrate utilization test indicated that the isolates are related closely to *X. autotrophicus*, although the type strain 7c and the reference strain JW 33 do not utilize either of the alkenes. None of the new isolates grew on alkanes, although alkane-utilizing *Xanthobacter* have been isolated previously on butane (Coty, 1967) and cyclohexane (Trower et al., 1985). DNA-DNA hybridization studies have not yet been published for these strains. The use of 1% alkene in air led only to the isolation of various strains belonging to *Mycobacterium* and *Nocardia*. These strains have a lower Km value for the alkene than do the *Xanthobacter* strains, but at higher concentrations, the *Xanthobacter* strains grow considerably faster. Thus, these strains of *Xanthobacter* can be enriched specifically using 5% alkenes as the sole carbon source. The conversion rates found were 65–70 nmol alkenes per min and mg of protein. The industrial interest is based on the high stereospecificity of the transformation. For example, strain PY10 utilizes ethene, propene, 1-butene, and 1,3-butadiene, but it contains two different 1,2-epoxyalkene-utilizing enzyme systems when grown on ethene or propene (Van Ginkel et al., 1986b). In addition, strain PY2 converts 3-chloro-1-propene to 1-chloro-2,3-epoxypropane with 80% as the S-enantiomer. Propene is converted to the R-1,2-epoxypropane and butene to R-1, 2-epoxybutane at 97% and 94%, respectively (Habets-Crützen et al., 1985). Weijers et al. (1988) used strain PY2 grown on propene for the chiral resolution of 2,3-epoxyalkanes. Since only the 2S-form is metabolized, a chiral resolution was obtained for *trans*-2,3-epoxybutane, *trans*-2,3-epoxypentane, and *cis*-2,3-epoxypentane. However, none of the *Xanthobacter* strains could be used for obtaining the enantiomers of 1,2-epoxyalkanes since all tested strains utilized both enantiomeric forms.

The group of Scott Ensign, using mainly strain Py2 over the last 10 years to work out the biochemistry of CO_2 fixation, aliphatic alkene degradation, epoxide formation, and degradation in *Xanthobacter*, has accumulated a wealth of data too numerous to report here in detail. The reader is referred to his excellent reviews on aliphatic epoxide carboxylation (Ensign and Allen, 2003) and on microbial metabolism of aliphatic alkenes (Ensign, 2001) as well as to his website (http://www. chem.usu.edu/~ensigns/index.htm) for updates on recent publications. Interestingly, the enzymes for aliphatic alkene and epoxide metabolism, which includes also the biosynthetic enzymes for the coenzyme M (2-mercaptoethanesulfonate), are apparently located on a linear megaplasmid (Krum and Ensign, 2001).

The finding that *Xanthobacter* contains coenzyme M was a great surprise since until then it was believed that coenzyme M was unique to methanogens. Subsequently, Ensign and his coworkers have elucidated at least some (5 catalytic activities) of coenzyme M in *Xanthobacter*, i.e., the involvement in the epoxyalkane degradation, including an epoxyalkane:CoM transferase (Zn-enzyme), a 2-ketopropyl-coenzyme M oxidoreductase and carboxylase, and a dehydrogenase (Allen et al., 1999; Krum et al., 2002; Nocek et al. [2002] and literature cited therein).

Magor et al. (1986) showed that the ability to utilize cyclohexane and its derivatives differs in different *Xanthobacter* species (*X. flavus* and *X. autotrophicus* type strains were not able to utilize cyclohexane, but *X. autotrophicus* could degrade cyclohexanol). However, on the basis of recent descriptions of new species and strains, the author believes it is more a property of strains than a discriminator between species per se. The degradation in the cyclohexane degrading strain (closely related to *X. autotrophicus*, but no final taxonomic position has been published) proceeded via cyclohexanol → cyclohexanone → ε-caprolactone → adipidic acid. The cyclohexanone monoxygenases in *X. autotrophicus* and the new strain differed significantly. The authors stated that there was no indication that the degrading strain contained plasmids. In light of the more recently found megaplasmid in strain Py2, the presence of a missed megaplasmid cannot be excluded at this time.

Degradation of Various Hazardous Waste Compounds *Xanthobacter* strain 124X can utilize 4-hydroxyphenylacetate, styrene oxide, and 2-phenylethanol (Van den Tweel et al., 1986; Hartmans et al., 1989). According to the authors, this organism can be used in biofilters to remove these compounds from industrial waste gases. In particular, the malodor of styrene, an important starting material for synthetic polymers and a possible cause of environmental problems, could be removed. A new degradation path was proposed for the metabolism of styrene oxide in strain 124X (Hartmans et al., 1989). Strain GJ10 (or mutants thereof) apparently has some potential for use in bioremediation processes of halocarbon-contaminated groundwaters.

The group of S. Ensign studied the biochemistry of acetone degradation, especially the inducible, ATP-dependent acetone carboxylase (Sluis and Ensign, 1997; Clark and Ensign, 1999; Sluis et al., 2002). Furthermore, Small et al. (1995) described a novel pathway for the degradation of epichlorohydrin by *Xanthobacter* strain Py2. *Xanthobacter tagetidis* (type strain Tag and

strain A2T2C) degrades thiophene-2-carboxylate, thiophene-2-acetate, pyrrole-2-carboxylate, furan-2-carboxylate, and dibenzothiophene while utilizing H_2, sulfide and thiosulfate, respectively, as energy and e-donor sources. The presence of glucose did not prevent the utilization of these substrates (Padden et al., 1997). *Xanthobacter autotrophicus* strain "J. Child, NCIB 11171" was isolated from oil contaminated soil and was shown to utilize a variety of alcohols such as ethylene glycol and propane-1,2-diol.

Applications for Slime Produced by Various *Xanthobacter* Strains Nearly all isolated strains of *X. autotrophicus* and *X. flavus* produce copious amounts of slime. Under chemolithoautotrophic growth conditions and with ammonium as the N source, strain 7c produces about 15–20% (w/w) of its biomass as slime, but grown with 2% fructose and ammonium, about 75–80% of the produced biomass was slime (Andreesen and Schlegel, 1974; Tunail and Schlegel, 1974). Generally, the highest slime production is under nitrogen-fixing conditions, and some strains can produce copious amounts of slime with almost no visible growth; upon cooling with ice, such a culture becomes a solid gel (J. Wiegel, unpublished observations). In this connection it should be mentioned that slime-free mutants have been isolated from *X. autotrophicus* strain 7c (Tunail and Schlegel, 1974) and from some of the alkene utilizers (Van Ginkel and De Bont, 1986a) using carbon-limiting chemostat cultures. We also isolated slime-less variants of the rice isolate JW/KR-1 during a greenhouse experiment when the strain was grown on root exudates from rice seedlings in sterilized sand without added carbon and nitrogen sources (K. Reding et al., unpublished observations).

Kern (1985) and Schubert et al. (Schubert et al., 1986; W. W. Schubert, personal communication) have shown that the slime is an excellent drag-reducing substance (68% reduction using a 1 : 10 dilution of a culture) for minimizing friction in turbulent flows in pipelines and water turbines. The properties of the slime favor its industrial application: apparently slime is resistant to microbial degradation (Andreesen and Schlegel, 1974) and has good shear stability.

Another suggested application of slime is as a viscosifier in oil fields. Wan et al. (1988) isolated a *Xanthobacter* strain producing slime with promising properties (e.g., excellent suspending ability, shear and thermal stability, high viscosity at low concentrations, and salt tolerance). The slime of this strain is composed of 25% glucuronic acid, 50% glucose, and 25% mannose. The largest amounts of slime were produced when this strain was grown using glucose as the carbon source and corn steep liquor as the nitrogen and mineral source. The concentration of both, as well as aeration and pH, played a major role in optimal slime production. In contrast to the new strain, the slime from strain 7c contains about 30% (mol/mol sugar) uronic acids, glucose, galactose, and mannose. The viscosity of this slime was not affected much by the presence of 0.5 M inorganic salts. A 0.4% (w/v) solution of the isolated slime had a viscosity 30 times greater than that of water (Andreesen and Schlegel, 1974).

Kawai et al. (1989) suggest that the slime from *Xanthobacter* could be used as an enhancer in gene recombination experiments of plants, especially when using *A. tumefaciens* as vector. This is based on their finding that the isolated slime (designated "PS-1") from the soil isolate *Xanthobacter* KB-3001 enhanced the attachment of and consequently the frequency of transformation by *Agrobacterium tumefaciens* in the agglutination test using a potato tuber disc assay to screen transformants and by measuring the attachment to tobacco protoplasts and cells. The partially purified (DEAE-cellulofine, AM column chromatography) slime has an apparent affinity for both the bacterial and the plant cells.

Transformations *Xanthobacter autotrophicus* strain 14g, isolated as a Knallgas bacterium, was shown to transform bicyclo [3.2.0]hept-2-3n-6-one and 7-endo propylbicyclo[3.2.0]hept-2-3n-6-one.

Cultures Deposited at Culture Collections For detailed description of the species, see the chapter "Genus *Xanthobacter*" by Wiegel (2004) in *Bergey's Manual of Systematic Bacteriology*, second edition, and in the cited original literature.

The following *Xanthobacter* strains are deposited in culture collections. The culture collections are: American Type Culture Collection (http://www.atcc.org/), Rockville, MD, United States; Deutsche Sammlung für Mikroorganismen (www.dsmz.de/species/gn202260.htm), Mascheroder Weg 1b, Braunschweig, Germany; Culture Collection Institute for Microbiology; (NEU), University Neuchatel, Switzerland; Culture Collection Fermentation Research Institute (FERM), Tsukuba Science City, Japan; and National Collection of Industrial Bacteria (www.ncimb.co.uk/), Torrey Research Station, Aberdeen, Scotland, United Kingdom. Other strains mentioned in this chapter can be obtained from the authors of the various publications, e.g., the author has an extensive collection of *X. autotrophicus*- and *X. flavus*-like strains.

Xanthobacter autotrophicus: Type species. Type strain 7c: DSM 432, ATCC 35674; NCIB 10809 slimeless mutant of 7c: DSM 2267; reference strain JW 33: DSM 1618; IFO 14758; strain GZ29: DSM 1393; strain 23A: DSM 685; strain 14g (oxidation of bicyclo[3.2.0]hept-2-3n-6-one and 7-endo propylbicyclo[320]hept-2-3n-6-one): DSM431, NCIB 10811; strain 19/–/x DSM 2009; strain GJ10 (degradation of chlorinated aliphatic compounds): ATCC 43050, DSM 3874; strain N34: FERM-P1098; strain Y38: FERM-P6724; strain T101: ATCC 700551; strain T102: TCCA 700552; and strain from J. Child (1,2-propandiol metabolism): NCIB 11171.

Xanthobacter flavus: Type strain 301: NCIB 10071, DSM 338, ATCC 35867; strain JW-KR-2 (associative N-fixation on rice roots): ATCC 51492; strain SA12: NEU 2003; strain SA14: NEU 2005; strain CB2: NEU 2151; strain CB5: NEU 2152; and strain 14p1 (1,4-dichlorobenzene utilization): DSM 10330.

Xanthobacter agilis: Type strain SA35: DSM 3770, NEU 2015, ATCC 43847, NCIB 12683; strain MA2: NEU 2038; strain MA37: ATCC 43848; and strain MA40: NEU 2069, ATCC 43849.

Xanthobacter tagetidis: Type strain TagT2C: DSM 11105, ATCC 7000314, NCIB 13547; and strain A2 (thiophenol utilizer): ATCC 700315.

Xanthobacter aminoxidans (basonym *Blastobacter*): Type strain 14a: VKM-B-2254, ATCC BAA-299, DSM 15009.

Xanthobacter spp.: Some of the strains are industrial strains whereas others are just isolates with no special features and which were isolated before 16S rRNA sequences were readily available. Strain CB2: DSM 14517; strain CB 5: DSM 14519; strain CB6: NEU 2153, DSM 14520; strain R-10: DSM 14521; strain MAB2 (degradation of propachlor): DSM 7325, ATCC 49876; strain Texaco NW-11 (patent involved strain for polysaccharide production): ATCC 53272, DSM 6448; and strain 124X (stryrene and toluene degrader): DSM 6696, ATCC 49450.

Acknowledgments. I thank all the colleagues who provided me with preprints, photos, and unpublished data to include in this chapter and K. Reding for providing most of the photographs. This chapter was written with the support of a Department of Energy (DOE) Grant (DE-FG02-01ER15260).

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Prokaryotes (2006) 5:315–456
DOI: 10.1007/0-387-30745-1_17

CHAPTER 3.1.16

The Genus *Brucella*

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Members of the genus *Brucella* are pathogenic bacteria of humans and animals exceedingly well adapted to their host and not surviving for extended periods of time in open conditions. Although the classical definition of *Brucella* species describe these bacteria as facultative intracellular parasites, this definition does not honor their true nature which is better understood as a facultatively extracellular intracellular parasite. That means that the *Brucella* preferred niche is the intracellular environment of host cells. This environment sustains extensive replication, allowing bacterial expansion and the subsequent transmission to new host cells, frequently achieved through the heavily infected aborted fetus. In contrast to in other pathogenic bacteria, no classical virulence factors, such as exotoxins, cytolysins, capsules, fimbria, flagella, plasmids, lysogenic phages, resistant forms, antigenic variation, endotoxic lipopolysaccharide (LPS) or apoptotic inducers have been described in *Brucella* organisms. Instead, the true virulence elements of *Brucella* are those molecular determinants that allow them to invade, resist intracellular killing and reach their replicating niche in professional and non-professional phagocytes.

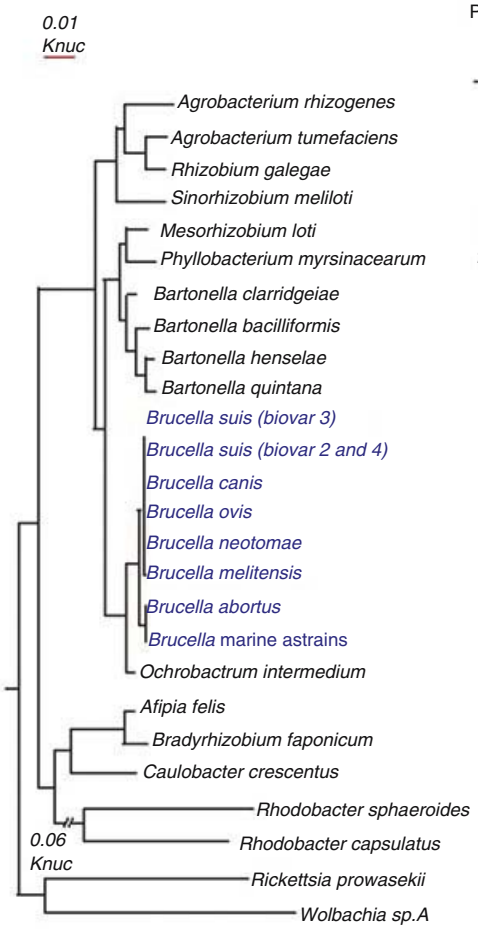
Brucella organisms are α-*Proteobacteria* phylogenetically closely related to plant pathogens and symbionts such as *Rhizobium*, *Agrobacterium* and *Wolbachia*, intracellular animal parasites such as *Bartonella* and *Rickettsia* and to opportunistic and free living bacteria like *Ochrobactrum* and *Caulobacter*. The demonstration of the phylogenetic closeness between the brucellae and other eukaryotic cell-associated bacteria, no matter whether they are of animal or plant origin, has had a profound impact in the way we envisage the virulence mechanisms of these bacteria. In course, this circumstance has resulted in new research strategies and biological interpretations that have greatly contributed to understand the biology and parasitism of *Brucella* organisms.

Phylogeny

Brucella species constitute a closely related monophyletic group with DNA-DNA reassociation values near 100% (Verger et al., 1985). Ribosomal cistron similarities and 16S RNA sequence comparisons demonstrate the phylogenetic position of *Brucella* species in the α-2 of the class Proteobacteria (De Ley et al., 1987; Moreno et al., 1990). The genus clusters with plant and animal pathogens, as well as with free-living heterotrophic and photosynthetic organisms of water and soil (Fig. 1). Phylogenetic analysis using GroEL heat shock proteins reveals similar branching dispersion to that obtained with ribosomal genes (Viale et al., 1994; Fig. 2). Comparison of lipid As, ubiquinones, membrane lipids, cyclic glucans and several cytoplasmic and membrane proteins also reveals close relationship of *Brucella* with other members of the α-2 Proteobacteria (Gupta, 1997; Moreno, 1992; Moreno et al., 1990; Velasco et al., 2000). On the basis of these genotypic and phenotypic characteristics, a model for explaining the evolution of *Brucella* parasitism from a common free-living ancestor has been proposed (Moreno, 1992; Moreno et al., 1990). This scheme has been upgraded and validated in successive works according to recent findings (Moreno, 1997; Moreno, 1998; Pizarro-Cerdá et al., 1999b; Sola-Landa et al., 1998; Velasco et al., 1998a; Velasco et al., 2000).

Ancestor-Descendent Relationships

The closest *Brucella* relatives are members of the genus *Ochrobactrum* (De Ley et al., 1987; Holmes et al., 1988; Lebhun et al., 2000; Velasco et al., 1998b). This genus contains five known species. *Ochrobactrum intermedium* is the most phylogenetically and taxonomically related to the *Brucella*, with 16S rDNA similarity values close to 97%. Both *Brucella* spp. and *O. intermedium* diverge from the same branch, outside of the other *Ochrobactrum* spp. (Fig. 1). In spite



Plasmid size in kb	Major chromosome in kb	Minor chromosome in kb	Chronmosomal G+C %	Preferred habitat and host
200-600	4000	2700[a]	62	Soil and pericellular pathogen of dicotyledons[b]
200-500	2841	2076[c]	60	Soil and pericellular pathogen of dicotyledons[b]
400-600	5850	1200[a]	63	Soil and intracellular symbiont of legumes
1354	3654	1683	63	Soil and intracellular symbiont of legumes
208-352	7596	none	63	Soil and intracellular symbiont of legumes
250-700[d]	5330	none	60	Soil and peri- and intracellular plant pathogen
none	1700	none	41	Intracellular pathogen of cat, human and lice
none	1540	none	40	Intracellular pathogen of humans and sand flies
none	1900	none	41	Intracellular pathogen of cat, humans and lice
none	1600	none	39	Intracellular pathogen of humans and lice
none	3100	none	57	Intracellular pathogen of swine
none	1850	1350	57	Intracellular pathogen of swine and reindeer
none	1850	1350	57	Intracellular pathogen of dogs
none	2100	1150	57	Intracellular pathogen of ovines
none	2100	1150	57	Intracellular pathogen of wood rats
none	2100	1150	57	Intracellular pathogen of caprines
none	2100	1150	57	Intracellular pathogen of bovines
none	2100	1150	57	Intracellular pathogen of marine mammals
50-200	2700	1900	59	Soil-rhizoplane and opportunistic for humans
40-100	Unknown	Unknown	63	Opportunistic for cats and humans
200-800	8700	none	65	Soil and intracellular symbiont of legumes
none[e]	4017	none	67	Water and free living prosthecate
40-200	3046	914	65	Water and free living photoheteretroph
100-200	3800	none	66	Water and free living photoheteretroph
none	1112	none	29	Intracellular pathogen of rodents and fleas
none[f]	1500	none	31	Endosymbiont of insects

Fig. 1. Phylogenetic tree showing the relationship among representative plant-associated, free-living, opportunistic and animal-associated α Proteobacteria of the subdivision. The genomic characteristics and the preferred host(s) for each bacterium are indicated in the table. The tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) from Knuc values derived from 16S rRNA sequences available at the European Molecular Biology Laboratory (EMBL), GeneBank and DNA Databank of Japan (DDBJ) Nucleotide Sequence Database.[a] The presence of essential genes has not been confirmed.[b] These bacteria may behave as animal-opportunistic organisms.[c] Linear chromosome.[d] Non-curable essential replicon.[e] Plasmids have been detected in Caulobacter strains.[f] Lysogenic phages and recombination have been documented in *Wolbachia*. References for representative strains in the tree are as follows: *Agrobacterium* spp. (Allardet-Servent et al., 1988; Jumas-Bilak et al., 1998b; Goodner et al., 2001; Hulse et al., 1993; Southern, 1996; Yanagi and Yamasato, 1993); *R. galegae* (Huber and Selenskapobell, 1994; Jordan, 1984; Yanagi and Yamasato, 1993); *S. meliloti* (Galibert et al., 2001; Honeycutt et al., 1993; Kolsto, 1997; Yanagi and Yamasato, 1993); *M. loti* (Jordan, 1984; Kaneko et al., 2000; Yanagi and Yamasato, 1993); *P. myrisinacearum* (Jordan, 1984; Jumas-Bilak et al., 1998b; Lambert et al., 1990); *Bartonella* sp. (Birtles et al., 1995; Brenner et al., 1993; Dehio and Meyer, 1997; Jumas-Bilak et al., 1998b; Krueger et al., 1993; Maruyama et al., 2001; Maurin et al., 1994); *Brucella* spp. (Bricker et al., 2000; DelVecchio et al., 2002; Jahans et al., 1997; Jensen et al., 1999; Jumas-Bilak et al., 1998a; 1998b; Meyer, 1990; Michaux-charachon et al., 1993, 1997); *O. intermedium* (Holmes et al., 1998; Jumas-Bilak et al., 1998b; Lebhun et al., 2000; Velasco, 1996; 1998a; Yanagi and Yamasato, 1993); *Afipia felis* (Brenner et al., 1991; Fumarola et al., 1994); *Rhodobacter* spp. (Choudhary et al., 1995; Fonstein et al., 1992; Imhoff and Trüper, 1984; Jumas-Bilak et al., 1998b; Mackenzie et al., 1995; Nikolskaya et al., 1995; Suwanto and Kaplan 1992); *B. japonicum* (Jordan, 1984; Kündig et al., 1994; Masterson et al., 1985; Wong et al., 1994); *C. crescentus* (Nierman et al., 2001; Schoenlein and Ely, 1983); *R. prowaseki* (Andersson et al., 1998; Roux and Raoult, 1993; Weisburg et al., 1989); and *Wolbachia* sp. A. (Sun et al., 2001; Werren and Bartos, 2001). The *Brucella* marine strains possess two chromosomes with molecular weights similar to those of *B. abortus* (D. O'Callaghan, personal communication).

of the strong similarities between these two genera, their biological properties are strikingly different. While *Brucella* organisms are intracellular primary parasites, cycling from animal to animal, not requiring host predisposing conditions, *Ochrobactrum* organisms are opportunistic bacteria that cycle from soil-rhizoplane to immunocompromised humans.

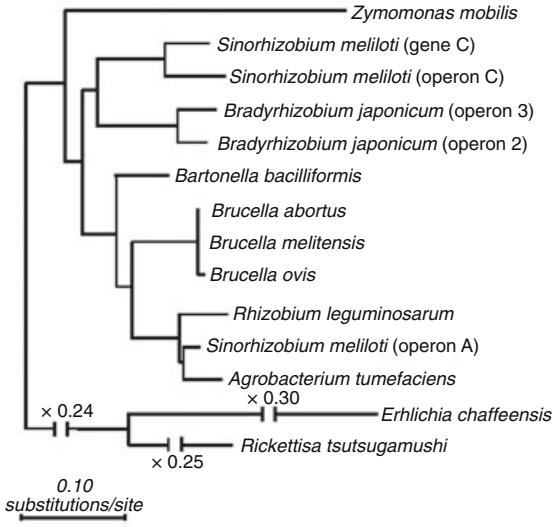


Fig. 2. Evolutionary relationships between α-2 Proteobacteria based on GroEL sequence comparisons. The tree was constructed by the neighbor-joining distance method (Saitou and Nei, 1987). *Escherichia coli* was used as an outgroup reference for rooting the tree. GroEL sequences were obtained from EMBL, GeneBank and DDBJ Nucleotide Sequence Database. Notice that the general topology of the tree agrees with that of (Fig. 1).

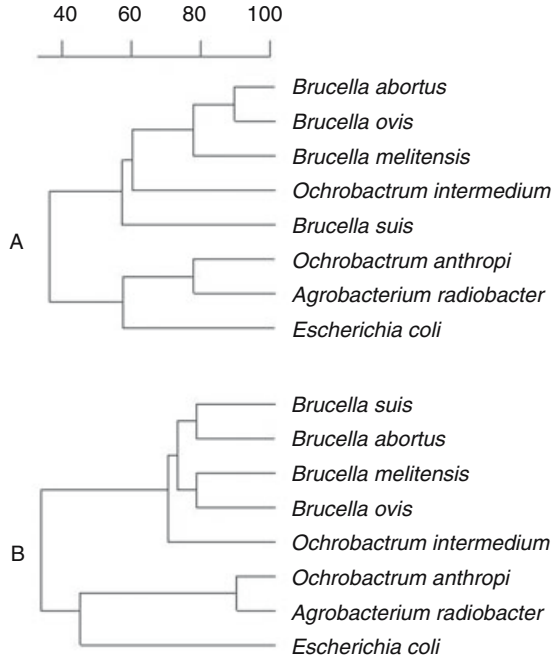


Fig. 3. Dendrograms derived from unweighted pair group average linkage of correlation for the corresponding banding patterns constructed by comparison of the whole cell protein antigens detected by Western blotting with antiserum against *O. intermedium* 3301 (A) and antiserum against *B. suis* 1330 (B). Notice that *O. intermedium* clusters together with *Brucella* species, in agreement with the phylogenetic tree constructed by 16S rRNA sequence comparison (Fig. 1). Modified from Velasco et al. (1998a), with permission.

Although *Brucella* demonstrate low DNA reassociation values with other members of the α-2 subdivision (20–30% for *Ochrobactrum*; Holmes et al., 1988), extensive immunochemical crossreaction between the proteins of *Brucella* and proteins of other α-2 Proteobacteria are evident (Cloeckaert et al., 1999; Velasco et al., 1998b). Dendrograms constructed on this basis reveal intermixing of *Ochrobactrum* and *Brucella* species and close relationship with members of the family Rhizobiaceae (Fig. 3). For this group of bacteria proteomic analysis seems to be a more reliable taxonomical tool than DNA hybridization (Cloeckaert et al., 1999; Velasco et al., 1998b).

Several of the characteristics present in *Brucella* and shared by various members of the α-2 subdivision are considered ancestral (Table 1). Among them are the RopA and RopB outer-membrane protein family, the long-chain fatty acids of the lipopolysaccharides (LPSs) and other membrane lipids, quinovosamine sugar in the LPS core (in smooth strains), the presence of phosphatidylcholine, ornithine-containing lipids and various lipoproteins, as well as the periplasmic cyclic glucans, catalase and superoxide dismutase (Sod), the inner membrane ubiquinones and various cytochrome types (Table 1). In metabolic activities, absence of a functional hexose diphosphate pathway, the oxygen and carbon dioxide requirements, a hexose monophosphate shunt, and the erythritol pathway seem to be ancestral features. The presence of two chromosomes is also considered to be ancestral, since several members of the α-2 subdivision, including *Ochrobactrum*, possess two chromosomes (Jumas-Bilak et al., 1998a; Fig. 1). The tendency of their members to associate with eukaryotic cells may be one of the most striking ancestral properties of the group (Dorsh et al., 1989; Moreno et al., 1990).

In addition to the metabolic and biochemical properties that distinguish the *Brucella* genus from other bacteria, there are some important derived characteristics (Tables 1–4). They include certain features affecting the cell surface, such as the presence of genes coding for *N*-formylperosamine polysaccharides and their expression in smooth strains, as well as the absence of capsules, flagella, pili and fimbriae (Table 1). Although genes coding for some of these structures have been identified in the *Brucella* genome, (DelVecchio et al., 2002), their expression in the *Brucella* surface has not been

Table 1. Characteristics of eukaryotic cell-associated Proteobacteria.

Characteristic	*Brucella*	*Ochrobactrum*	*Bartonella*	*Sinorhizobium*	*Agrobacterium*	*Mesorhizobium*	*Rickettsia*	*Caulobacter*
GENOTYPIC:								
16SrRNA or GroEL analysis	α-2	α-2	α-2	α-2	α-2	α-2	α-1	α-3
Tm(e) with *B. abortus* RNA	80	78	?	72	73	68	?	?
DNA-DNA (%) with *B. abortus*	98–100	26–40	?	7	7	7	?	?
Chromosome number	1–2[a]	2	1	2	2[b]	1	1	1
Mol % G+C	57	56–59	39–40	61–63	59–61	60–63	29–33	62–67
Potential coding regions (%)	87	?	?	90	90	90	74	91
Lysogenic bacteriophages	absent	?	absent	present	present	present	absent	?
Plasmids	absent	present	absent	present	present	present	absent	present/absent[c]
Transformation efficiency	+	+++	+	+++	+++	+++	+	+++
Genetic variability	+	+++	++	+++	+++	+++	+	+++
Number of known strains	22–25	≅100	18–20	>100	>100	>100	18–25	≅100
Generation time in h	2.5–3	1–2	4–7	1–2	1–3	1–3	9–12	1
Genome size (base pair average)	3200	4800	1500	5500	5200	9000	1200	4000
CELL ENVELOPE:								
Flagella	absent[d]	present	present/absent	present	present	present	absent	present
Pili/fimbria	absent	absent	present/absent	present	present	present	absent	present
Type IV secretion system	present	?	present	present[e]	present	present[e]	present[e]	present[e]
Extracellular polysaccharides	absent	absent	absent	present	present	present	absent	present
Cycloglucan	present	present	present	present	present	present	?	?
Cytochrome c size	medium	?	?	medium	medium	medium	medium	medium
$Cu^{++}Zn^{++}$SOD	present	?	?	present	present	present	absent	present
Catalase	present	present	absent	present	present	present	absent	present
Ubiquinone	Q10	Q10	Q10	Q10	Q10	Q10	?	?
Ornithine-containing lipid	present	present	?	present	present	present	?	?
LPS O chain perosmanine	present	absent	absent	absent	absent	absent	absent	present
LPS core heptose	absent	absent	present	absent/present[f]	absent/present[f]	present	absent	present
LPS core uronic acids	absent	absent	present	present	present	present	absent	absent
LPS core quinovosamine	present	present	?	present	present	absent	present	present[g]
Lipid A backbone sugars	DAG	DAG	?	GlcN	GlcN	DAG	GlcN[h]	?
OH-$C_{28:0}$ to OH-$C_{30:0}$	present	present	?	present	present	present	?	absent
LPS endotoxicity	low	low	low	low	low	low	low	?
Phosphatidylcholine	present	present	present	present	present	present	present	absent
Resistance to polycations	resistant	susceptible	resistant	susceptible	susceptible	susceptible	?	?
Hydrophobic molecule permeability	+++	–	+++	–	–	–	?	?
Sensitivity to EDTA	–	+++	?	+++	+++	?	?	?
Resistant to acid fast stains	++	–	–	–	–	–	?	?
METABOLISM:								
O_2 requirements[i]	+++	+++	+++	+++	+++	+++	+++	+++
CO_2 requirements	+/–	–	+	+	+	+	?	–
Acid produced from ASS	+/–	+++	–	+++	+++	+++	–	–

Gas production	–	–	–	–	–	–	–	–
Utilization of amino acids	+/–	+++	+/–	+++	+++	+++	+++	+++
Utilization of organic acids	+	+++	+	+++	+++	+++	+++	+++
Utilization of alditols	+	+++	–	+++	+++	+++	+++	+++
Utilization of sugars	+	+++	–	+++	+++	+++	+++	+++
Vitamins required	++	+	+++	+	+	+	+++	–
Urease activity	present	present	absent	present	present	present	absent	present
Nitrate reduction	present	present	absent	present	present	present	–	present
Nitrogen fixation	absent	absent	absent	present	absent	present	absent	absent
Functional glycolisis	not detected[j]	not detected[j]	not detected[j]	low[j]	low[j]	low[j]	absent	not detected[j]
Hexose monophosphate shunt	present	present	present[k]	present	present	present	absent	present
Functional Entner-Doudoroff	not detected[l]	present	not detected[l]	present	present	present	absent	present
Functional Erithrytol pathway	present	present	?	present	present	present	absent	not detected[m]
TCA cycle	present	present	present	present	present	present	present	present
Optimal growth range in°C	36–38	25–28	28–37	25–28	25–28	20–37	32–34	20–25
HABITAT								
Range	mammals	rhizoplane-soil-humans	arthropods/ mammals	legumes-soil	dicotyledones-soil	legumes-soil	arthropods/ vertebrates	free-living water
Cell association	intracellular	pericellular?	pericellular/ intracellular	intracellular	pericellular	intracellular	intracellular	none
Parasitic status	strict	opportunistic	facultative	endosymbiont	opportunistic	endosymbiont	strict	none

Symbols and abbreviations: ?, unknown; **– to +++**, detected in variable quantities; **SOD**, superoxide dismutase; **EDTA**, ethylene diamine tetraacetate; **ASS**, Ammonium salt sugars, **GlcN**, glucosamine; **DAG**, diaminoglucose; **TCA**, tricarboxylic acid cycle.

Specific footnotes: a. With the except for *B. suis* biovar 3, which possesses one chromosome, all other known *Brucella* possess two chromosomes; **b.** The smaller chromosome is linear; **c.** Some strains like *C crescentus* CB15 do not have plasmids; **d.** Incomplete set of genes coding for putative proteins for flagellum basal body and cryptic genes for flagellin have been detected; **e.** The function of putative type IV secretion system has not been demonstrated; **f.** Heptose sugars have been detected in the LPS of a few species; **g.** Putative enzymes for synthesis of quinovosamine have been detected and quinovosamine containing glycolipids have been detected; **h.** Extensively detected in LPS preparations but not confirmed in lipid A; **i.** Oxigen is as terminal electron acceptor. With the exception of *Rickettsia*, nitrate potentially could be used as electron acceptor; **j.** Genes coding for glycolitic enzymes have been detected, but functional activity is absent or low; **k.** Functional activity is unknown, but genes coding for enzymes participating in the hexose monophosphate shunt have been detected; **l.** Genes coding for enzymes participating in the Entner-Doudoroff patway have been detected, but functional activity is absent; **m.** Functional activity is unknown, but genes coding for enzymes participating in the Erithrytol pathway shunt have been detected.

References: *Brucella* spp. (Beck et al., 1990; Bhat et al., 1991; Bricker et al., 2000; Chester and Cooper, 1979; Cloeckaert et al., 1999; Corbel and Brinley-Morgan, 1984; DelVecchio et al., 2002; Dickerson, 1980; Cherwonogrodzky et al., 1990; De-Ley et al., 1987; Jahans et al., 1996; Moore and Schnurrenberger, 1981; Moreno et al., 1990; Moreno, 1998; Velasco et al., 1998a, 1998b, 2000); *Ochrobactrum* spp. (Chester and Cooper, 1979; Holmes et al., 1988; Lebhun et al., 2000; Moreno et al., 1990; Velasco et al., 1996; Velasco et al., 1998a, 1998b, 2000); *Bartonella* spp. (Andersson and Dehio, 2000; Canback et al., 2002; Hollingdale et al., 1980; Maeno et al., 1999; Maeno et al., 2002; Maruyama et al., 2000; Merrel et al., 1978; Minnick and Anderson, 2002; Moreno et al., 1990; Reschke et al., 1990; Schmiederer et al., 2001; Weiss and Moulder, 1984); *Sinhorizobium* spp. (Bhat et al., 1991; Campbell at el., 2002; Canback et al., 2002; De Lajudie et al., 1994; Dickerson, 1980; Galibert et al., 2001; Jordan, 1984; Long, 1989; Moreno et al., 1990; Tighe et al., 2000); *Agrobacterium* spp. (Bhat et al., 1991; Canback et al., 2002; Dickerson, 1980; Goodner et al., 2001; Jordan, 1984; Matthysse, 2002; Moreno et al., 1990; Tempst and Van Beeumen, 1983; Zambryski, 1988); *Mesorhizobium* spp. (Bhat et al., 1991; Canback et al., 2002; Choma et al., 2000; Correa et al., 1999; Jordan, 1984; Kaneko et al., 2000; Long, 1989; Russa et al., 1995; Tighe et al., 2000; Urbanik-Sypniewska et al., 2000); *Rickettsia* spp. (Amano et al., 1998; Amano et al., 1993; Andersson et al., 1998; Andersson and Dehio, 2000; Canback et al., 2002; Rava et al., 1999; Weisburg et al., 1991; Weiss and Moulder, 1984; Zezerov et al., 1985); *Caulobacter* spp. (Abraham et al., 1999; Anast and Smit, 1988; Awram and Smit., 2001; Batrakov et al., 1997; Canback et al., 2002; De Siervo and Homola, 1980; Nierman et al., 2001; Poindexter, 1989; Poindexter, 2001; Ravenscroft et al., 1992; Schoenlein and Ely, 1983; Sly et al., 1999; Stackebrandt et al., 1998; Steinman et al., 1990).

revealed, in spite of several attempts to detect them (J. Gir6 et al., unpublished observations). Features that seem to have been derived independently in the *Brucella* lineage are the absence of a functional Entner-Doudoroff pathway, the more restricted metabolic alternatives, the absence of plasmids, the reduction of chromosome size and the reduction of G+C content with respect to the ancestor (Table 1; Fig. 1). Several of these properties are also present in various of the *Brucella* animal pathogen's close relatives (Moreno, 1992), but it is likely that they are the result of convergent evolution rather than of ancestral origin. The basis for this relies on the fact that *Brucella*, *Bartonella* and *Rickettsia* are intracellular parasites, which may have been exposed to equivalent evolutionary constraints within the intracellular environment of cells. For instance, reduction of chromosomal size, concomitant reduction of G+C content and the absence of plasmids (Fig. 1) probably emerged independently in these three genera. Similar to this may be the slow generation time displayed by intracellular bacteria. This characteristic, which correlates with the reduced number of *rrn* operons in the genomes of these organisms, may be the consequence of heterotrophic metabolism required for growth. This feature may have derived independently in several intracellular bacteria, such as *Mycobacterium*, *Rickettsia* and *Bradyrhizobium*, and therefore may be the result of coevolution rather than the consequence of an ancestor-descendent relationship (Moreno, 1998).

Brucella Speciation

A striking feature that distinguishes the *Brucella* genus is the clonal structure of its species as compared to the reticular arrangement of its soil-plant-associated bacterial relatives (Moreno, 1998; Yanagi and Yamasoto, 1993; Young and Wexler, 1988; Population Genetic Composition of the *Brucella* Species). This feature is probably the result of a founder effect during parasitism, and in fact, there are a considerable number of intracellular Proteobacteria with no recognized plasmids (species of *Brucella*, *Bartonella*, *Rickettsia* and *Anaplasma*), as well as some endosymbionts that must be considered clonal rather than reticulate bacteria (Fig. 1).

In *Brucella* species, restriction maps at insertion sequences and maps based on outer membrane proteins, fatty acids and numerical analysis generate dendrograms that are commensurate with the preference of *Brucella* species for their animal host (Fig. 2). On the one hand, the *Brucella* ancestor may have originated by a "brief" burst of change between 75 and 40 million years ago (Moreno, 1992; Ochman and Wilson, 1987), according to the mode of evolution proposed by punctuated equilibrium (Eldredge and Gould, 1972). On the other hand, dispersion of *Brucella* species and strains may be the result of more gradual genetic changes (i.e., point mutations, transpositions, recombinations, inversions and deletions) within the isolated environment of their host (Moreno, 1998). The fact that the *Brucella* genus stands as a clearly distinct assemblage among Proteobacteria, but at the same time constitutes a close monophyletic group of species, supports this path of evolution. Therefore, it is likely that speciation of intracellular pathogens and endosymbionts is commensurate with the evolutionary history of the bacterial host (Ochman and Wilson, 1987), since most of the environmental constraints that direct natural selection seem to be present in the host (Brunham et al., 1993; Laguerre et al., 1993). This also means that extinction of the natural host may cause extinction of the specific parasitic *Brucella* species or strain (Biological Attributes in the Context of *Brucella* Species Definition). In this respect, the evolutionary history of *Brucella* may reflect the outcome of a combination of these two modes of change, punctuated equilibrium followed by gradualism.

By comparing the various dendrograms (Fig. 4), it seems feasible to hypothesize that *B. abortus* and *B. melitensis* shared the same ancestor, and this ancestor probably shared a relationship with the common ancestor of the marine strains. Likewise, *B. canis* and *B. suis* shared a common ancestor. Indeed, the size of the two chromosomes of *B. canis* and biovars 2 and 4 of *B. suis* correspond; similarly, the size of the two chromosomes of *B. abortus* and *B. melitensis* also correspond, supporting the topologies of the trees. It has been hypothesized that *B. suis* is the closest species to the *Brucella* ancestor (Plommet, 1991). This postulate is sustained by several observations. For instance, *B. suis* is the most diverse brucellae in genomic structure (Number and Species Differences) and host preferences (Table 2), and because the species has a wider range of biochemical alternatives, its metabolic capability comes closest to prototrophy (Nutritional Requirements). Moreover, trees constructed on the basis of biochemical characteristics or protein crossreactivity, on which the heterotrophic *O. intermedium* is included as an outgroup, show that *B. suis* strains are the brucellae most closely related to *Ochrobactrum* (Fig. 3). If we accept that speciation in some clonal bacterial populations, with no horizontal transfer of genes, may be gradual and commensurate with time, then *B. suis* is the most diverse among the different *Brucella* species, and the most closely related to the heterotrophic ancestor.

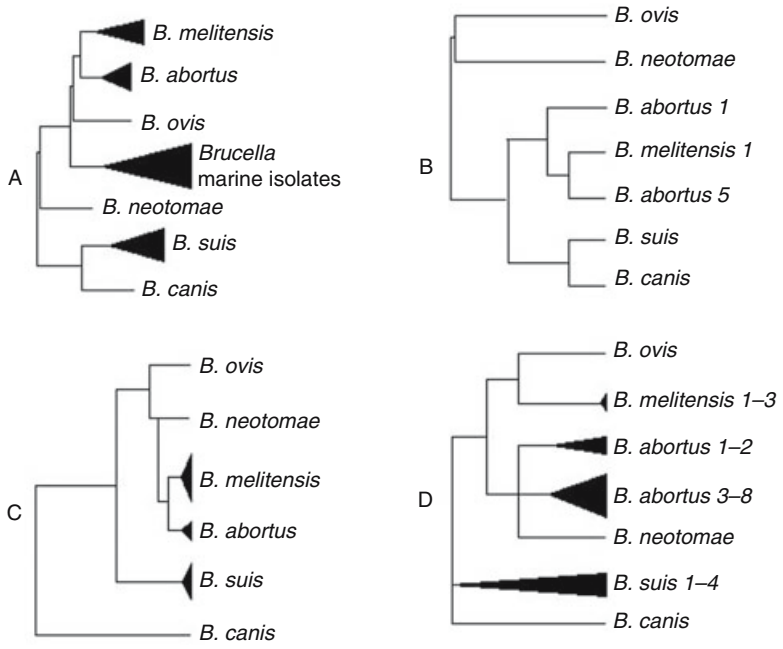


Fig. 4. Dispersion of *Brucella* species in dendrograms constructed by various methods. Modified dendrogram constructed on the basis of fingerprints with restriction endonucleases (A: Jensen et al., 1999); modified dendrogram constructed on the basis of sequence analysis of *omp*2 locus (B: Ficht et al., 1996); modified dendrogram constructed on the basis of fatty acid analysis (C: Tanaka et al., 1977); modified dendrogram constructed on the basis of numerical analysis (D: Gargani, 1977). Notice that although the species are resolved as independent units in the trees, the topology and the relative distances among the extant organisms in the different trees vary. The cones represent the dispersion of the various *Brucella* strains.

Genome Evolution

Brucella organisms are closely related to and phylogenetically entwined with heterotrophic bacteria that carry a single chromosome but have no plasmids and are associated with animal cells (Fig. 1). They are also entwined with opportunistic, plant-associated, chemoautotrophic and photosynthetic α Proteobacteria possessing one or more chromosomes and plasmids (Fig. 1). The genome organization and phylogenetic ancestor-descendent relationships between *Brucella* containing two chromosomes and *Brucella suis* biovar 3 harboring one single chromosome (*Brucella* Chromosomes) suggest that this biovar has undergone transition from two chromosomes to a single replicon (Moreno, 1987). The time at which this event took place can be estimated by comparing specific sequences from *B. suis* strains 1, 2 or 4 with two chromosomes with sequences in the chromosome of *B. suis* biovar 3 (Jumas-Bilak et al., 1998a). From phylogenetic analysis, it is likely that transition from two chromosomes to a single replicon in *B. suis* biovar 3 occurred after speciation. This is supported by the inclusion of *B. suis* biovar 3 within the same tree topology (Figs. 1 and 4) and by the maintenance of the biochemical characteristics and host preferences in relation to the other *B. suis* biovars (Tables 2 and 3). Therefore, the presence of one or more chromosomes within the same *Brucella* species seems to be the result of a contingency with no apparent consequences, as long as the essential information is correctly expressed.

The "local adaptation" hypothesis provides a framework for explaining the selective forces that generated phylogenetically related bacteria, some with larger and other with smaller genomes and some with plasmids and others without these genetic elements (Eberhard, 1990). This hypothesis states that "many of the characters that tend to occur on plasmids are adaptations to local variations in environmental conditions that occur only sporadically in time or space." In some instances, such as local adaptations to antibiotics, which are generally restricted to the immediate vicinity of antibiotic-producing organisms like fungi and actinomycetes in soil, "this kind of sporadic selection makes the maintenance of local adaptations more likely when genes are on plasmids than when they are on chromosomes." This circumstance also applies to the production of virulence factors, medical usage of antibiotics, heavy metal resistance, inactivation of poisons or degradation of unusual substrates, among others. Therefore, local variations in open environments, such as soil, water, manure, gut systems and the external surfaces of plants and animals, probably selected α Proteobacteria with extensive metabolic alternatives, broad genetic diversity, and more flexible and larger genomes with ability for horizontal gene flux. This would be the case for plant-associated *Rhizobium*, *Sinorhizobium*, *Agrobacterium*, *Phyllobacterium* and *Ochrobactrum* species. On the contrary, the constant and isolated animal cellular milieu selected heterotrophic *Brucella* species with smaller genomes, no plasmids, and a reduced genetic diversity as compared to their

Table 2. Differential characteristics of the classical *Brucella* species.

Species	Preferred host	LPS and colonial morphology	Sensitivity to phage(s)[b]	Nitrate-nitrite reduction	Oxidase	Urease	Oxidative metabolic pattern[b]	
							Amino acids	Sugars
B. melitensis	Sheep, goats	Smooth	Bk2	+	+	+	L-Glutamic, L-asparagine, L-alanine, L-asparagine	*meso*-Erythritol
B. abortus	Cattle	Smooth	Tb, Wb, Bk2	+[c]	+	+[c]	L-Glutamic, L-asparagine, L-alanine	L-Arabinose, D-galactose, D-ribose*, *meso*-erythritol*
B. suis	Pigs (biovars 1, 2, 3) Hares (biovar 2) Reindeer (biovar 4) Wild rodents (biovar 5)	Smooth	Tb[d], Wb, Bk2	+	+	+		biovar 1
							L-Glutamic**, L-asparagine, L-arginine, DL-ornithine, L-lysine	L-Arabinose, D-galactose, D-ribose*, D-xylose, *meso*-erythritol*
								biovar 2
							L-Glutamic, L-asparagine, L-arginine, DL-ornithine, L-lysine**	L-Arabinose, D-galactose, D-ribose*, *meso*-erythritol
								biovars 3, 4, 5
							L-Glutamic, L-asparagine, L-arginine, DL-ornithine	D-Ribose*, meso-erythritol*
B. neotomae	Desert wood rats	Smooth	Tb,[d] Bk2	+	−	+	L-Glutamic, L-asparagine	L-Arabinose, D-galactose, D-ribose**, *meso*-erythritol
B. canis	Dogs	Rough	R/C	+	+	+	L-Glutamic, L-asparagine, L-arginine, DL-ornithine	D-Ribose*, *meso*-erythritol**
B. ovis	Sheep	Rough	R/C	−	−	−[e]	L-Glutamic, L-asparagine	

Symbols and abbreviations: +, present; –, absent; LPS, lipopolysacchande.
[a]For additional information see (Table 13).
[b]The QO_2 (N) level for the isolate of a given species or biovar is most probably 100–300 but for the cases marked with an asterisk, which produce a QO_2 (N) level above 300, or with two asterisks, which show a large variability. Substrates giving values below 100 are not indicated.
[c]A few strains are negative in this test.
[d]Not lysed at the routine test dilution but lysed at $10^4 \times$ (routine test dilution).
[e]Some strains are reported to give a positive result in the urease test.
Data from Alton et al. (1998).

Table 3. Characteristics of the biovars of the classical *Brucella* spp.[a]

Species	Biovar	Type strain[b]	CO_2 requirement on primary isolation	H_2S production	Growth on dyes at 20µg/ml		Differential LPS epitope(s)
					Thionin	Basic fuchsin	
B. melitensis	1	16M	–	–	+	+	M
	2	63/9	–	–	+	+	A
	3	Ether	–	–	+	+	AM
B. abortus	1	544	+	+	–	+	A
	2	86/8/95	+	+	–	–	A
	3	Tulya	+	+	+	+	A
	4	292	+	+	–	+[c]	M
	5	B3196	–	–	+	+	M
	6	870	–	–	+	+	A
	9	C68	+ or –	+	+	+	M
B. suis	1	1330	–	+	+	–[d]	A
	2	Thomsen	–	–	+	–	A
	3	686	–	–	+	+	A
	4	40	–	–	+	–[e]	AM
	5	513	–	–	+	–	M
B. neotomae		5K33	–	+	–[f]	–	A
B. canis		RM6/66	–	–	+	–[e]	R
B. ovis		63/290	+	–	+	–[e]	R

[a]Adapted from Alton et al. (1988).
[b]Type strains can be obtained from ATCC (http://www.atcc.org/).
[c]Some isolates do not grow on dyes.
[d]Some isolates are resistant to fuchsin.
[e]Negative for most strains.
[f]*B. neotomae* grows on thionin at 10µg/ml.

plant-associated and phototrophic relatives, but similar to their animal pathogen relatives, such as *Bartonella*, *Rickettsia* and *Anaplasma* species. Therefore, it seems that the ancestral *Brucella* genome evolved through reduction of a larger genome of a chemoautotrophic free-living ancestor and became rich in adenine and thymidine, as compared to the genomes of its precursors (Moreno, 1998).

Under suitable circumstances of temperature and the presence of nutrients, *Brucella* organisms can survive in an open environment, but there is no evidence that they replicate to a significant extent in soil, water or manure under natural conditions (Corbel and Brinley-Morgan, 1984). Thus, *Brucella* species behave essentially as strict parasites and rely for their survival, reproduction and persistence in nature on transmission between animal hosts. This picture differs from that occurring in their plasmid-containing human opportunistic and plant-associated relatives, which shift from external to host environments (Alnor et al., 1994; Bouzar et al., 1993; Brenner et al., 1991; Hulse et al., 1993; Jordan, 1984). In their hosts, *Brucella* organisms do not confront adverse local conditions found in external surroundings, such as antibiotics, heavy metals, bacterial competition and rapid environmental changes. Since *Brucella* organisms only reproduce in their hosts, the advantage of plasmids carrying genes to cope with these conditions would be trivial. It could be argued that plasmids carrying genes (e.g., for adhesion/invasion and antiphagocytic proteins) necessary to deal with variable local conditions, such as the host immune response, would be maintained by positive selection (Eberhard, 1990). However, the *Brucella* lifestyle seems to favor the presence of these genes in chromosomes rather than in plasmids. On the one hand, the intracellular milieu protects the bacteria against intermittent variable defenses, such as antibodies and complement (Survival Outside Host Cells); on the other hand, *Brucella* evades the digestive mechanisms and attains control of the cell (Controlling the Host Cell). Since the cellular environment is relatively constant concerning temperature, acidity, oxygen tension, availability of substrates and presence of bactericidal molecules, the *Brucella* physiology must match that of their hosts, in that the bacteria must be able to salvage the metabolites they need (Life Within the Replicating Niche). Housekeeping genes must secure these delicate functions. Without the positive selection imposed by external or immune local forces, the plasmid-bearing bacteria would be at a slight disadvantage (in comparison with plasmid-free bacteria that would reproduce more rapidly). It is known that unpaired segregation and loss of plasmids

are likely to occur when plasmid carriage reduces bacterial fitness in the absence of selection for specific functions (Levin and Lenski, 1983). Accordingly, the periodic invasion by parasitic bacteria of higher fitness (arising from one or very few individuals without transmissible plasmids) would clonally expand. Transmission of the bacteria to a naïve host would generate a founder effect and the diversity of the "new parasite" would be restricted to chromosomal changes (Frank, 1994). Some of the genes involved in parasitism were, however, probably acquired by horizontal transference exclusively by the *Brucella* ancestor; among these are some genes coding for enzymes for the synthesis of the *O*-polysaccharide (Table 5; Acquisition of Ancestral Genes by Horizontal Transference).

The characteristics and genome sizes in the *Brucella* extant species suggest that a second smaller chromosome evolved from megaplasmids by the acquisition of housekeeping genes (i.e., RNA genes). For instance, the *Agrobacterium* arginase and ornithine cyclodeaminase genes, present in the virulence Ti plasmid, are necessary for inducing tumors in plants and display high similarity with homologous genes located in the same operon and in the same order in the smaller *Brucella* chromosome (Kim and Mayfield, 1997). Moreover, the *vir* genes necessary to build the type IV secretion apparatus in *Agrobacterium* and *Brucella* are located in the Ti megaplasmid of *Agrobacterium* and in the smaller chromosome of *Brucella*, respectively (O'Callaghan et al., 1999; Sánchez et al., 2001). The *Brucella vir* set of genes seems to be regulated in a similar fashion as the *vir* genes located in the plasmid of the *Agrobacterium* plant pathogen (O'Callaghan et al., 1999).

Derivation of Ancestral Preexisting Structures for Virulence

The fine adjustments between *Brucella* parasites and their host cells are the result of a prolonged and intimate association. The evolutionary process that has shaped *Brucella* parasites does not operate by inventing but rather by reinventing on the grounds of pre-existing structures that are constantly confronted with specific local conditions. For instance, the periplasmic domains (involved in environmental sensing) of the sensory proteins (ChvG, ExoS and BvrS) of the two-component, sensory-regulatory systems necessary for bacterial parasitism of *Agrobacterium*, *Sinorhizobium* and *Brucella* (The BvrR-BvrS System) show less similarity than do other protein domains, implying that they were derived for sensing different stimuli (Sola-Landa et al., 1998). Although it is remarkable that the two intracellular bacteria (*Sinorhizobium meliloti* and *B. abortus*) are more similar in this region than is the pericellular one (*Agrobacterium tumefaciens*), the *Sinorhizobium* system is involved in the production of succinoglycan (Cheng and Walker, 1998), whereas the *Brucella* Bvr system seems to be involved in the structural homeostasis of the outer-membrane (C. Guzmán-Verri et al., unpublished results).

The Vir systems of *Brucella* and *Agrobacterium*, although similar in many respects, function in different cellular environments (Fig. 5). In *Agrobacterium* this secretion system delivers Ti DNA and possibly some Vir proteins to govern the plant host cell (Christie and Covacci, 2000), whereas in *Brucella*, it is likely that the system transfers molecules inside phagosomes for controlling intracellular trafficking (Escaping from the Endocytic Pathway). Type IV secretion systems are probably derived from the basal body necessary to propel the flagellum. Although *Brucella* organisms possess genes for flagellin, the bacteria are nonmotile, stressing the notion that the system is derived directly from pre-existing secretion systems (GenBank accession number AFO19251).

The absence of cyclic glucan renders *Brucella*, *Agrobacterium* and *Rhizobium* incompetent to parasitize. The putative protein coded by the β (1–2) cyclic glucan synthetase *cgs* gene of *B. abortus* is highly similar to the NvdB protein of *S. meliloti* (Ugalde, 1999) and is capable of complementing that of *Agrobacterium* and *Sinorhizobium* for parasitism (and vice versa). Moreover, *Brucella cgs* restores mobility in these plant pathogens, suggesting that this protein has different functions depending upon the bacterial background where it is expressed. In *Brucella* organisms, the cyclic glucan is not osmoregulated (Briones et al., 1997) and captures cholesterol (I. Moriyón, unpublished observations), implying that its function may differ from that in *Agrobacterium* and *Rhizobium*. Among the proposed functions for Cgs, one is to serve as transporter for several components in the bacterial inner membrane (Ugalde, 1999).

Another important example of the adaptation of pre-existing structures designed for different purposes may be found in the characteristics of the outer-membranes, which are shared by various members of this group (Moreno, 1992; Moreno, 1998; Velasco et al., 2000). For instance, in spite of having almost identical lipid As and sharing many outer-membrane physical and chemical properties, *Ochrobactrum* is sensitive to bactericidal cationic peptides, whereas *Brucella* is highly resistant (Velasco et al., 2000). As in other α-2 Proteobacteria, the core oligosaccharide of *Ochrobactrum* (but not that of

Table 4. Molecular markers and tests for the identification and typing of *Brucella*.

Gene or genetic element	Method	Strains (n)	Major result	References
Unknown	RFLP (*Xab*I digests and PFGE)	Reference strains of all species and isolates from marine mammals	Differentiation of species and isolates from marine mammals; isolates from porpoises and seals group together and those from dolphins form a distinct group	Allardet-Servent, 1988; Jensen et al., 1999
	RFLP (*Bam*HI, *Hind*III, *Sma*I, *Eco*RI and *Xho*I digests probed with random *Brucella* DNA fragments)	A total of 112 strains including the reference strains of all species and biovars	Variable results depending on probe or probes combination. Loose correlation with species and biovars	Grimont et al., 1992
	RAPD (AP-PCR with 5 primers)	A total of 25 strains including the reference strains of all species and biovars	Fingerprint differences down to strain level	Fekete et al., 1992
16S and 23S rRNA	RFLP (*Bam*HI, *Hind*III, *Sma*I, *Eco*RI and *Xho*I digests probed with *E. coli* 16S + 23S rRNA)	A total of 112 strains including reference strains of all species and biovars	Single rRNA restriction pattern	Grimont et al., 1992
16S rRNA	PCR and nested PCR	Reference strains of all species plus related and unrelated bacteria	Identification at genus level. Cross-detection of *Phyllobacterium* and *Ochrobactrum* spp. depending on the primer	Herman and De Ridder, 1992; Romero et al., 1995a; 1995b; Da Costa et al., 1996
omp2 locus (porins)	RFLP (*Bam*HI, *Kpn*I and *Pst*I digests probed with *omp2* or fragments thereof); PCR and PCR-RFLP	Strains of all species	Differentiation of tested strains at species level	Ficht et al., 1990, 1996; Sifuentes-Rincón et al., 1997
	PCR-RFLP (13 restriction enzymes)	A total of 77 strains, including reference strains of all species and biovars	High polymorphism within species and biovar; some *B. melitensis* strains are indistinguishable from *B. abortus* biovars 3, 5, 6 and 9; *B. canis* is similar to *B. suis* biovars 3 and 4	Cloeckaert et al., 1995a
*omp*31	PCR; RFLP (*Hind*III digest probed with *omp31* and adjacent DNA); PCR-RFLP	A total of 73 strains, including reference strains of all species and biovars	Large deletion encompassing *omp*31 in *B. abortus*. Species-specific markers for *B. abortus* and *B. melitensis* (RFLP); specific markers for *B. ovis*, *B. canis* and *B. suis* biovar 2 (PCR-RFLP). The combined methods discriminate all species but *B. neotomae* (grouped with some *B. suis* biovars)	Vizcaño et al., 1997
*omp*3A (*omp* 25)	PCR-RFLP (9 restriction enzymes)	A total of 77 strains, including reference strains of all species and biovars	Three restriction patterns: *B. ovis* and *B. melitensis* have species-specific patterns; a third pattern is shared by *B. abortus*, *B. suis*, *B. canis* and *B. neotomae*	Cloeckaert et al., 1995a

Table 4. *Continued*

Gene or genetic element	Method	Strains (n)	Major result	References
Envelope lipoproteins (*omp*10, *omp*16, and *omp*19)	RFLP (*Hind*III digest probed with *omp*16)	A total of 92 strains, including reference strains of all species and biovars	No significant differences with *omp*19 and *omp*10; two patterns with *omp*16 (one specific for *B. suis* biovar 2)	Verger et al., 1998
*bcsp*31K	RFLP (*Hind*III digest probed with *bcsp*31K)	*B. abortus* and *B. ovis* (20)	Specific *B. ovis* pattern due to IS711 insertion in its *bcsp*31K (see below)	Halling and Zehr, 1990; Baily et al., 1992; Da Costa et al., 1996
	PCR and nested PCR	Reference strains of all species plus related and unrelated bacteria	Identification at genus level. Cross-detection of *Phyllobacterium* and *Ochrobactrum* spp. depending on the primer	Da Costa et al., 1996
htrA	PCR and nested PCR			
groEl	PCR and nested PCR			
dnaJ	PCR and nested PCR			
dnaK	PCR and nested PCR			
	RFLP (*Eco*RV digest probed with *DnaK*) or PCR-RFLP	A total of 77 strains, including reference strains of all species and biovars	Differentiation of *B. melitensis* from the other classical species	Cloeckaert, 1996
39*ugpa*	RFLP (*Hind*III digest probed with *39ugpa*)		Two patterns, one specific for *B. ovis*	
*br*25	RFLP (*Hind*III digest probed with *br25*)		Three patterns, one specific for *B. melitensis* and one specific for *B. ovis*	
*p*39	RFLP (*Hind*III digest probed with *p39*)		No polymorphism	
*Is*711 (*Is*6501) insertion site	Multiplex-*Brucella*-PCR (5 primers)	Reference strains all species and isolates of *B. abortus* biovar 1(107), 2 (8), 4 (7), *B. ovis* (5), *B. canis* (5) and *B. suis* biovar 1 (2)	Identification of *B. abortus* (biovars 1, 2, and 4), *B. melitensis*, *B. ovis*, and *B. suis* (biovar 1)	Bricker and Halling, 1994
	RFLP (*Eco*RI digest probed with *Is*711)	Reference strains of all species and biovars; mink whale isolate	No species-specific or biovar-specific patterns but for *B. ovis* and the mink whale isolate	Clavareau et al., 1998
	IS-anchored-PCR (IS primer plus a primer chosen arbitrarily)	Reference strains of *B. melitensis* biovar 3, all *B. abortus* biovars plus strain 19, one strain each of *B. suis* and *B. canis*, and 8 *B. canis* strains	Discriminates between different species and strains within a species. Also distinguishes strain 19 from other *B. abortus* tested	Ouahrani et al., 1996
	RNA mismatch cleavage	Reference strains of all species and biovars	Differentiation of the six classical species and 11 of the 18 biovars studied	Bricker, 1999
REP and ERIC sequences	REP-PCR and ERIC-PCR	Reference strains of all species and biovars and isolates of *B. melitensis* biovar 3 (10), *B. abortus* biovar 3 (5) and 9 (2) and *B. canis* (20)	Differentiation of species and biovars tested but for some *B. abortus* biovars 3 and 9 isolates	Mercier et al., 1996; Tcherneva et al., 1996
Bru-RS1 and Bru-RS2	RFLP (*Hind*III digest probed with corresponding DNAs)	Reference strains of all species	Differentiation of reference strains	Halling and Bricker, 1994

Abbreviations used: RFLP, restriction fragment length polymorphism; PGEF, pulse field gel electrophoresis; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA; AP-PCR, arbitraily primed-PCR; REP, repetitive extragenic palindromic; and ERIC, enterobacterial intragenic consensus.

Table 5. *Brucella* lipopolysaccharide genes.

Gene (Accession no.)	*Brucella* spp.[a]	Properties of the deduced protein: Similar protein (ID code)[b] (identity)	Properties of the deduced protein: Putative function[c]	Phenotype of mutant	References
pgm (AF232056)	*B. abortus*	*A. tumefaciens* Pgm (PGMU_AGRTU) (75%)	Phosphoglucomutase (glucose-1-P↔glucose-6-P)	Rough LPS; also unpaired in synthesis of β(1,2) cyclic glucans (but not part of the glycogen operon): attenuated in mice but able to multiply in HeLa cells	Ugalde et al., 2000
pmm (manB) (AF022366)	*B. abortus*, *B. suis*	*Rhizobium sp.* Pmm (NOEK_RHISN) (50%) and *V. cholerae* Pmm (RFBB_VIBCH) (24%)	Phosphomannomutase (mannose-6-P↔mannose-1-P)	Rough LPS; may also have defects in LPS core (mannose-1-P is a perosamine precursor but core synthesis may require UDP-mannose); attenuated in mice HeLa and THP1 cells	Allen et al., 1998; Foulongne et al., 2000
wboA (AF107768)	*B. abortus*	*S. typhimurium* (RFBU_SALTY) (25%)	Glycosyltransferase (mannosyl transferase?)	Rough LPS with no quinovosamine and reduced mannose content; attenuated in mice; disrupted by IS711 in *B. abortus* RB51 vaccine	McQuinston et al., 1999; Vemulapalli et al., 1999
gmd (AF047478)	All classical *Brucella* spp.	*E. coli* K-12 Gm4D (GM4D_ECOLI) (73%)	GDP-mannose-4-6-dehydratase (perosamine biosynthetic pathway)	Rough LPS with presumably intact core	Godfroid et al., 2000; Cloeckaert et al., 2000
perA (AF047478)	All classical *Brucella* spp.	*V. cholerae* O:1 RfbE (Q06953) (53%) and *E. coli* O157:H7 RfbE (O07894) (52%)	Perosamine synthetase	Rough LPS with presumably intact core; attenuated in mice but still able to multiply normally in bovine macrophages	Godfroid et al., 1998, 2000 Cloeckaert et al., 2000
wzm (AF047478)	All classical *Brucella* spp.	*Y. enterocolitica* O:3 RfbD (RFBD_YEREN) (26%) and *V. cholerae* O:1 RfbH (Q06955) (23%)	ABC transporter involved in *O*-polysaccharide export	Rough LPS; intracellular accumulation of lipid-linked *O*-polysaccharide	Godfroid et al., 2000; Cloeckaert et al., 2000

Table 5. *Continued*

Gene (Accession no.)	*Brucella* spp.[a]	Properties of the deduced protein: Similar protein (ID code)[b] (identity)	Properties of the deduced protein: Putative function[c]	Phenotype of mutant	References
wzt (AF047478)	All classical *Brucella* spp.	*V. cholerae* RfbI (Q06956) (40%) and *Y. enterocolitica* O:3 (RFBE_YEREN) (39%)	ABC transporter involved in *O*-polysaccharide export	Rough LPS; intracellular accumulation of lipid-linked *O*-polysaccharide: *B. melitensis* 115-like mutant (*findlink Native hapten polysaccharides)	Godfroid et al., 2000; Cloeckaert et al., 2000
wbkb (AF047478)	All classical *Brucella* spp.	No significant match	Inner membrane protein of unknown function	S-LPS	Godfroidet al., 2000; Cloeckaert et al., 2000
wbkc (AF047478)	All classical *Brucella* spp.	*Thermus thermophilus* Fmt (FMT_THETH) (29%)	Methionyl-tRNA formyltransferase	Rough LPS (devoid of intra and extracellular perosamine polymers)	Godfroid et al., 2000; Cloeckaert et al., 2000
wbka (AF047478)	All classical *Brucella* spp.	*S. cholerasuis* MtfB (Q00481) (31%) and *E. coli* WbdB (Q47594) (29%)	Second mannosyl transferase	Rough LPS; affected in *O*-polysaccharide elongation	Cloeckaert et al., 2000; Godfroid et al., 2000

Abbreviations: Pgm, phosphoglucomutase; Pmm, phosphomannomutase; Gm4D, GDP-mannose-4-6-dehydratase; Fmt, formyltransferase; Mtf, mannosyl transferase; LPS, lipopolysaccharide; and S-LPS, smooth lipopolysaccharide.

[a]Where the gene has been identified.

[b]ID code in SWALL database.

[c]Step affected by mutation.

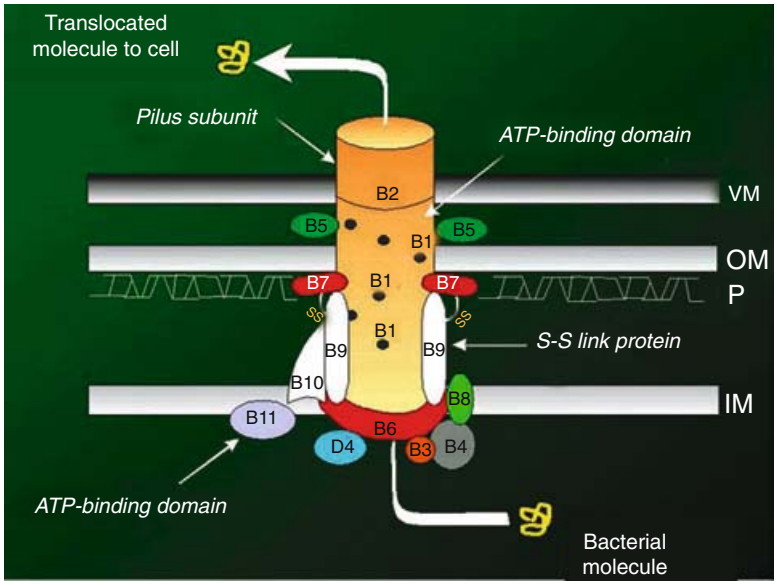


Fig. 5. Hypothetical model of a type-IV secretion system necessary for dispatching "virulent" proteins from the *Brucella* cell to the cytoplasm of the animal cell. Impairment of this system makes *Brucella* avirulent. Nonpolar *virB*$^{-10}$ mutant displays altered intracellular trafficking in epithelial cells (Fig. 36).

Brucella) possesses a negatively charged uronic acid residue that in all likelihood accounts for this different sensitivity (Response to Environmental Stress). Because the resistance to bactericidal cationic peptides is an essential property related to *Brucella* parasitism, the absence of uronic acids in *Brucella* LPS core is conspicuous and likely to be a key evolutionary variation linked to its adaptation as an intracellular pathogen (Fig. 6).

It has been determined recently that, in contrast to the LPS of free-living bacteria, the LPS of *Rhizobium* intracellular bacteroids become highly hydrophobic (Kannenberg and Carlson, 2001). It was suggested that the switch from hydrophilic to a predominantly hydrophobic LPS is the result of an adaptive response for changing environments. In the case of *Brucella*, the highly hydrophobic outer-membrane (Moriyón and Berman, 1982; Martínez de Tejada and Moriyón, 1993) seems to be well adapted to intracellular life (Moreno, 1992; Moreno, 1998; Velasco et al., 2000). This pattern offers a starting point on which the strong hydrophobicity of the lipid A of many α Proteobacteria is complemented in *Brucella* by modification of the LPS core and by the horizontal acquisition of *N*-formylperosamine genes for the synthesis of the hydrophobic *O*-polysaccharide and native hapten (NH)-polysaccharide (Godfroid et al., 1998; Velasco et al., 1996; Velasco et al., 1998a). The significance of this adaptive evolution of *Brucella* to its intracellular niche is stressed by carefully comparing the LPS of *Legionella* and *Brucella* (Fig. 7) since both intracellular pathogens have arrived from the extremes of different phylogenies to a very similar solution (Velasco et al., 1998b; Velasco et al., 2000; Zähringer et al., 1995; Acquisition of Ancestral Genes by Horizontal Transference).

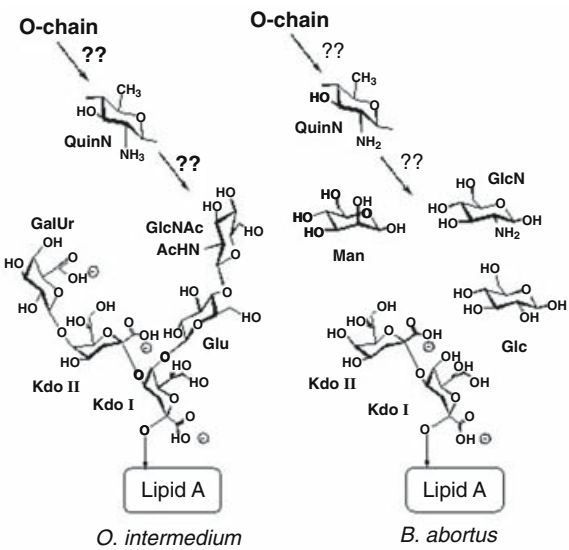


Fig. 6. Core oligosaccharides of the LPS of *O. intermedium* and *B. abortus*. The *O. intermedium* structure lacks heptose and phosphate also absent in *B. abortus* LPS. On the other hand, the *O. intermedium* carries anionic galacturonic acid that has not been detected in *B. abortus* or *B. melitensis*. The structure presented for *O. intermedium* LMG 3301 may represent only a partial structure, since sugar analysis of *Ochrobactrum* S-LPS suggests quinovosamine in the core and there is also evidence for an additional unknown sugar linked to glucuronic acid. Quinovosamine seems to be the sugar that links the core with the *O*-polysaccharide.

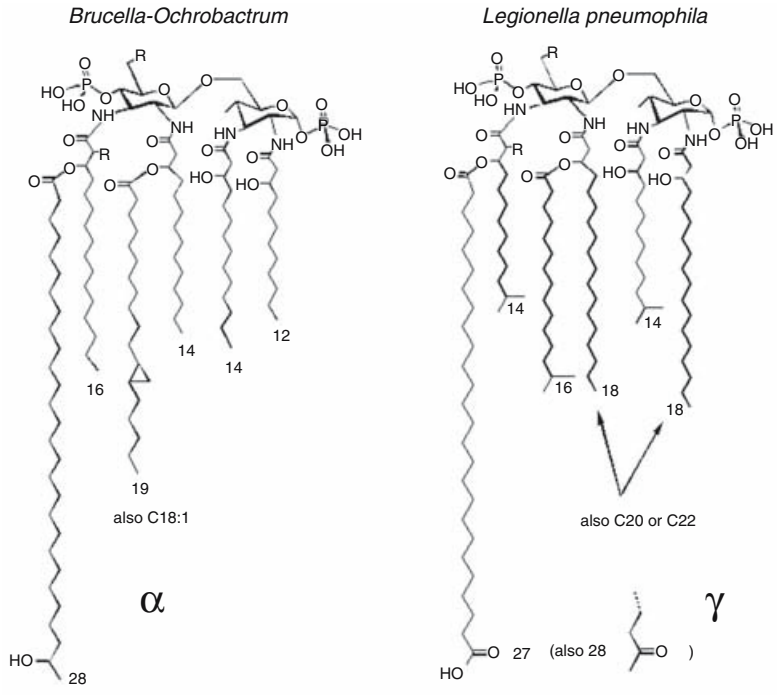


Fig. 7. Proposed structures of the lipids A of the *Brucella-Ochrobactrum* cluster and of *Legionella pneumophila*. The structures do not take into account heterogeneity due to different degrees of acylation or additional microheterogeneity due to variations in the type of acyl substitutions (based on the data of references Velasco et al., 2000; Zähringer et al., 1995). While *Brucella* and *Ochrobactrum* cluster within the α-2 subclass of Proteobacteria, *Legionella* belongs to the γ subclass. In spite of the far phylogenetic relationship between *Brucella* and *Legionella*, both intracellular parasites share a similar lipid A structure, likely to be the product of coevolution.

Acquisition of Ancestral Genes by Horizontal Transference

Local conditions may determine the plasticity and size of the genome during evolution (Population Genetic Composition of the *Brucella* Species) as well as the plasticity of the outer-membrane (Kannenberg et al., 2001; Moreno, 1997; Velasco et al., 2000). In contrast to other parasitic bacteria, most of the virulence genes identified in *Brucella* so far have been received vertically from a common ancestor, as revealed by the extensive amelioration of the sequences and by the fact that *Brucella* organisms do not possess plasmids (Genome Evolution; Genetic Exchange, Plasmids and Lysogenic Phages). One conspicuous exception may be the horizontal acquisition of the perosamine synthetase gene necessary for the synthesis of the *N*-formyl perosamine homopolymers (Godfroid et al., 1998), a gene which is also present in several pathogens, such as *Salmonella*, *Yersinia enterocolitica* and *Vibrio cholera* strains (Cherwonogrodzky et al., 1990; Table 6). This and other genes necessary to assemble the *O*-polysaccharide and NH polysaccharides were probably acquired exclusively by the *Brucella* ancestor, since none of the other members of the α-2 Proteobacteria seem to synthesize this sugar, not even the closest phylogenetic relative of *Brucella*, *Ochrobactrum* spp. (Velasco et al., 1998a; Velasco et al., 2000). The incorporation of *N*-formylperosamine polymers on the framework of a quinovosamine-containing core and lipid A basic structures (shared by many other α-2 Proteobacteria) was a crucial step for the development of the pathogenic properties of smooth *Brucella* (Inhibition of Lysosome-phagosome Function). Moreover, genes required for the synthesis of *O*-polysaccharides are present in the "natural rough" *B. ovis* and *B. canis* (Cloeckaert et al., 2000), suggesting that this was an ancestral character. Because dissociation from smooth to rough phenotype is a common event among brucellae (Colonies and Dissociation), it is likely that the loss of *O*-polysaccharide occurred separately during evolution of these two rough brucellae and not as an ancestor-descendent relationship. Changes that generated a low endotoxic core-lipid A structure, which also conferred resistance to bactericidal substances and proportioned the hydrophobic properties of the *Brucella* outer-membrane (Outer Membrane Versus Bactericidal Substances), must be added to the characteristics already described.

Taxonomy

The brucellae are included in a single genus placed in the α-2 subclass of the Proteobacteria (Population Genetic Composition of the *Brucella* Species). Through the years, classical *Brucella* taxonomists developed a classification system that established the six species (*B. melitensis*, *B. abortus*, *B. suis*, *B. neotomae*, *B.*

Table 6. Structures of the LPS *O*-polysaccharides of *Brucella abortus* and serologically crossreacting bacteria.[a]

Bacterium	Structure
Brucella abortus biovar 1	-2) - α -D- Rhap4*N*Fo-(1-
Yersinia enterocolitica O:9[b]	-2) - α -D- Rhap4*N*Fo-(1-
Vibrio cholerae O1[c]	-2) - α -D- Rhap4*N*(3-deoxy -L- tetronic acid) - (1
Escherichia coli 0157	-4) - β -D- Glcp - (1-3) - α -D- Galp*N*Ac-(1–2) - α -D- Rhap4*N*Ac-(1-3) - α -L- Fucp - (1
Escherichia hermanni [d]	-2) - α -D- Rhap4*N*Ac-(1-…-3) - α -D- Rhap4*N*Ac-(1- (1:5 and 1:6)
Stenotrophomonas maltophila[e]	-3) - α -D- Rhap4*N*Ac-(1-3) - α -D- Rhap4*N*Ac-(1-2) - α -D- Rhap-(1-3) - α -D- Rhap4*N*Ac-(1 2 | 1 α -D- Fucp3*N*Ac
Salmonella group N (O:30)	-4 - β -D- Glcp-(1-3) - α -D- Galp*N*Ac-(1-2) - α -D- Rhap4*N*Ac-(1-3) - α -L- Fucp-(1 or: -4 - β -D- Glcp-(1-3) - α -D- Galp*N*Ac-(1-2) - α -D- Rhap4*N*Ac-(1-3) - α -L- Fucp-(1 4 | 1 β -D- Glcp

Abbreviations: Rhap4*N*Fo, *N*-formyl-perosamine (pyranose); Rhap4*N*(3-deoxy-L-tetronic acid), *N*-3-deoxy-tetronyl-perosamine (pyranose); Glcp, glucopyranose; Galp*N*Ac, *N*-acetyl-galactosamine (pyranose); Rhap4*N*Ac, *N*-acetyl-perosamine (pyranose); and Fucp, fucopyranose.

[a]Structures are from Perry and Bundle (1990b). *Francisella tularensis* crossreacts with smooth *Brucella*, but its *O*-polysaccharide structure is not known.

[b]Although indistinguishable from the *O*-polysaccharide of *B. abortus* biotype 1, the reactivity with monoclonal antibodies suggests subtle chemical differences perhaps related to the degree of *N*-formylation.

[c]Other *V. cholerae* containing perosamine derivatives are Hakata (Rhap4*N*Ac), O76 and O144 (Rhap4*N*[2-hydroxypropionate]) and 1875 (Rhap4*N*[3-hydroxypropionate]). See Isshiki et al. (1995); Kondo et al. (1993, 1996); Sano et al. (1996).

[d]Only some serovars (such as *E. hermanni* NRCC 4262, 4279, 4298, 4299 and 4300; Perry and Bundle, 1990a).

[e]Only some serovars (such as *S. maltophila* 555 and 556 [CECT 112 and 113]).

ovis and *B. canis*) that are presently recognized by the Subcommittee on Taxonomy of *Brucella*. Main characters used in this classification are the host range and the cell surface features (Table 2). These six species are further subdivided into biovars (or biotypes) that have been useful in epidemiological studies. Although the system has proven to be useful in the past, revision is required in the light of recent discoveries. Several *Brucella* strains that do not fit into the classical species have been isolated from marine mammals and show internal diversity (Jahans et al., 1997). Depending upon whether marine strains are included as one species (*B. maris*) containing three biovars or as three different species, the genus may increase from 6 up to 9 species in the coming years (Jahans et al., 1997). This traditional view on the subdivision of the genus into species was challenged over a decade ago on the grounds that the high degree of DNA homology demonstrated in the DNA hybridization experiments conformed better with the definition of a single species of which the six classical species would not be but biovars (Verger et al., 1985). The view that the genus is divided into at least the six classical species is adopted in this chapter because the controversy arises from the use of different concepts of species, not all of which are valid or have a relevant biological value when applied to *Brucella*. This issue has been, and still is, a matter of confusion that has affected some important culture collections. This controversy is discussed in depth in the next sections (Population Genetic Composition of the *Brucella* Species; Defining the *Brucella* Species).

The Genus *Brucella*

The brucellae can be readily distinguished from other bacteria on the basis of the phenotypic and biochemical characteristics, host preferences, phylogenetic studies and serological analysis (Ficht et al., 1996; Moreno, 1998; Velasco et al., 1998b; Tables 1, 2, and 4). Comprehensive revisions of the genus based on traditional characteristics have been examined in several works (Alton et al., 1988; Corbel and Brinley-Morgan, 1984; Moyer and Hausler, 1992). The cellular and colonial characteristics (Identification), the nutritional and physicochemical requirements (Isolation), and the structural (Metabolism) and overall genetic (Genetics) properties are presented elsewhere in the chapter.

Although *Brucella* shares several features with other α-2 Proteobacteria, various idiosyncratic characters are remarkable (Table 1). All mem-

bers of the genus are primary parasites that do not require predisposing host factors. Host preference, predilection for reproductive tissues, affinity for fetal tissues and the induction of abortion and male epididymitis are some of the most marked biological features of the *Brucella* members (Pathology). Other distinctive biological characteristics of members of the genus are their ability to grow within the endoplasmic reticulum of infected cells (Replication Within the Endoplasmic Reticulum), the low endotoxicity of their LPS (Maintaining the Host Cell Alive), their strong resistance to the action of bactericidal cationic peptides (Outer Membrane Versus Bactericidal Substances), and the permeability of the outer-membrane to hydrophobic substances (Role of the Outer Membrane in Nutrient Uptake) (Freer et al., 1999; Freer et al., 1996; Martínez de Tejada and Moriyón, 1993; Martínez de Tejada et al., 1995; Velasco et al., 2000). In addition, many differential properties of *Brucella* are negative characters. For instance, all *Brucella* members are nonmotile, do not possess flagella, fimbriae, pili or capsules, are unable to ferment sugars or produce gas, and lack a functional glycolysis or an Entner-Doudoroff pathway. Exotoxins, hemolysins or proteolytic enzymes or resting forms have never been reported. Some of the negative distinctive features of *Brucella* concern the LPS: absence of heptose and uronic acids among the core sugars. Although shared with other intracellular parasites of the α Proteobacteria (Fig. 1), the absence of plasmids or lysogenic bacteriophages is one of the most distinctive characteristics of the genus.

Population Genetic Composition of the *Brucella* Species

A major problem in the definition of the *Brucella* species is to know whether these bacteria are clonal or reticulate evolutionary units (Moreno, 1997). In the first case, the inheritance is vertically transmitted as a result of binary fission and clonal expansion. In the second case, adaptive changes occurring within an individual can be horizontally transferred to many or all members of the group. A third alternative lies between these two possibilities (Fig. 8). Genetic drift and speciation in clonal bacteria will depend almost exclusively on mutation and internal genetic rearrangement processes, whereas speciation in reticulate bacteria will depend also on genetic exchanges.

In nonclonal reticulate bacteria, recombination may be a dominant force driving the divergence among the different members (Guttman and Dykhuizen, 1994). In general, recombination seems to take place more often between closely related bacteria than across species. The genetic relationships between closely related individuals could be regarded as tokogenetic (Moreno, 1997), in contrast to the phylogenetic links that interconnect the different species (Fig.

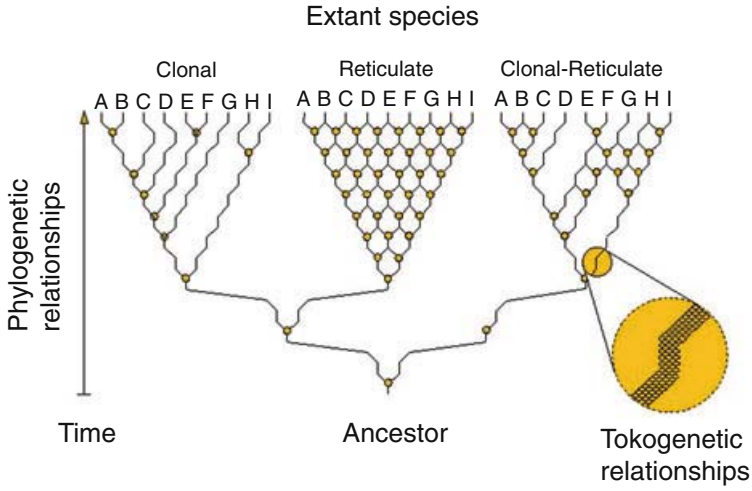


Fig. 8. Hypothetical phylogenetic trees and tokogenetic relationships among bacteria. Three different alternatives for generating bacterial species or strains (A-I) during evolution are presented. Speciation will depend on the probabilistic transmission and expansion of exogenous and endogenous genetic events as well as natural selection. Endogenous events such as mutation, internal recombination of genes and duplication or deletion of sequences are vertically transmitted and clonally expanded by the ancestor (O). Exogenous events include the horizontal acquisition or recombination of foreign bacteria sequences, plasmids and lysogenic phages (/\). Examples of clonal species are the *Brucella* spp., *Bartonella* spp., *Anaplasma* spp. and *Rickettsiae* spp.; examples of reticulate or clonal-reticulate bacteria are members of the Rhizobiaceae and Enterobacteriaceae families. Tokogenetic relationships have been documented in enterobacterial species (Guttman and Dykhuizen, 1994). Modified from Moreno (1997), with permission.

8). Tokogenetic recombinations are expected to homogenize the gene pool among the interacting organisms, thereby restricting the network structure of the species to a limited level. In comparison, recombination between different species (Amábile-Cuevas and Chicurel, 1992) results in genetic diversification promoting not only the expansion of the network structure of the species, but also favoring a fast and severe genetic drift, which may eventually cause speciation (Syvanen, 1994). A recombinational phenomenon between chromosomes and plasmids and between homologous sequences within chromosomes (Kim and Mayfield, 1997; Küdig et al., 1994; Lambert et al., 1990; Nikolskaya et al., 1995; Prakosh and Schilperoort, 1982; Rivilla and Downie, 1994; Schwedock and Long, 1994) may explain the difficulties in recognizing distinct genus and species among certain groups of α Proteobacteria, such as *Agrobacterium*, *Phyllobacterium*, *Rhizobium*, *Sinorhizobium*, *Bradhyrizobium* and *Rhodobacter* species (Laguerre et al., 1994; So et al., 1994).

In *Brucella*, like in other intracellular bacteria, recombination appears to be precluded or exceptional. The local conditions of intracellular parasites alternating from one host to another generate a founder effect in the population. Because the bacteria do not recombine, the expansion in the host must be considered of clonal origin. *Brucella*, therefore, must be contemplated within the context of clonality rather than a reticulate structure. Since clonality is maintained by the vertical transmission of genetic material, all evolutionary changes must be a result of mutational processes or autogenous rearrangement of genes mediated by insertion sequences, retrons, transposons or homologous recombinations (Ficht et al., 1996; Andersson and Andersson, 1997; Jumas-Bilak et al., 1998a; Jumas-Bilak et al., 1998b; Michaux-Charachon et al., 1997; Ouahrani et al., 1993; Sniegowski, 1997). These paths are comparatively limited in generating diversity, and accordingly, have generated a *Brucella* genus possessing a tight monophyletic clonal structure different from other members of the α-2 Proteobacteria. These concepts are relevant to understand not only because the problems posed by the species definition within the *Brucella* genus, but also because the strong tendency of the genus to maintain constant antibiotic susceptibility, antigenicity, cultural and serological properties, and genetic organization.

Defining the *Brucella* Species

Three different species concepts and one perception dominate in prokaryote classification: the phylogenetic species concept (Cracraft, 1983), the taxonomic species concept (Staley and Krieg, 1984), the biological species concept (Dobzhansky, 1937), and the microbiologists' perception (Pitt, 1994; Sneath, 1984a). The three concepts have strengths and weaknesses; each emphasizes different aspects of the evolutionary process and are within the boundaries of science. Because the taxonomic and phylogenetic concepts are based on numerical analysis (Sneath, 1984b; Williams et al., 1995; Woese, 1994), they are the most distinctly understood. The biological species concept may be the ultimate definition, but owing to the complexity involved in understanding the biology of a bacterial species, it has been relegated to last among the three scientific views (Krieg and Holt, 1984). The microbiologists' species perception is anthropocentric and nonscientific in nature. Nevertheless, it must be taken into account since sooner or later "a classification that is of little use to the microbiologist, no matter how fine a scheme or who devised it, will be ignored or significantly modified" (Staley and Krieg, 1984).

Speciation Concept Based on Phylogenetic Analysis A phylogenetic species is "the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descendence" (Cracraft, 1983). This concept has the advantage that species are defined as the terminal organism in a lineage that has an ancestor common to other terminal organisms, represented by the node joining their two branches, thus avoiding the meaningless classical ranked classification into kingdoms, phyla, classes, order and families. In the light of this definition, debates concerning bacterial ranks above the genus level are trivial and unimportant (O'Hara, 1994). A problem with this concept in defining *Brucella* species involves determining how monophily is to be recognized and how to distinguish gene phylogenies from pedigrees (Avise, 1990). The main question is which *Brucella* genes can better represent this genus and which of these genes best represent the dispersion into different species. *Brucella* phylogenies based on the sequences of conserved molecules, such as ribosomal RNAs and chaperone proteins, have greatly contributed to discerning the evolutionary relationships of *Brucella* with other organisms (Figs. 1 and 2). However, at the species level, a classification system based on these molecules does not have enough resolution for defining the different *Brucella* species as separate entities (Stackebrandt and Goebel, 1994). In this respect, it seems that phylogenies based in outer-membrane protein sequences and restriction maps seem to have better resolution at the *Brucella* species level (Allardet-Servent et al., 1988; Bricker et al., 2000; Ficht et al., 1996;

Higgins, 2000; Michaux-Charachon et al., 1997). The main problem is the absence of limits in the prediction of a terminal taxa: if one tries hard enough, sequence differences can be found to diagnose virtually any bacterial population. In spite of this, the phylogenetic approach based on outer-membrane protein sequences, restriction enzyme analysis or classical numerical analysis based on biochemical characteristics has clearly contributed to the discernment of what may be a natural system of classification, in agreement with the biological attributes displayed by the different *Brucella* species (Fig. 4). This approach has made us look more carefully at the common factors of *Brucella* belonging to the same branch, sometimes in spite of the diverse host preference or chromosomal structure displayed by sister brucellae, as is the case of *B. suis* biovars (Fig. 1).

Species Definition by the Polyphasic Approach The taxonomic species concept is a definition clearly linked to its method (see also Defining Taxonomic Ranks in Volume 1). The classical common procedures of *Brucella* classification are based on the analysis of phenotypic features, biochemical characteristics, genotypic similarities and immunochemical reactions. Taxonomical schemes have been then illustrated in the form of clades or dendrograms (Gargani, 1977; Moreno, 1992). The same procedures used to determine a species have been employed to further characterize the various *Brucella* species into biovars. The virtue of the polyphasic approach is that it allows high resolution at the terminal branches of dendrograms; its drawback is the inaccuracy in the earlier nodes of the branches (Moreno, 1997). This is due mainly to the fact that many characteristics displayed by the different brucellae (i.e., *B. canis* and *B. ovis* rough strains) may be the product of coevolution or parallel evolution. The combination of phylogenetic grouping based on sequence comparisons with taxonomic classification has been a very powerful method for approaching a practical definition of the *Brucella* species.

In spite of the advantages, the polyphasic approach possesses some drawbacks when coming to the interpretation of the *Brucella* DNA-DNA reassociation values. In other phylogenetically related genera, this interpretation is straightforward. For example, *Bartonella* members can be differentiated into at least 16 individual species according not only to different phenotypic, chemotypic and immunochemical criteria, but also by their DNA-DNA reassociation values (Birtles et al., 1995). The latter procedure has been pragmatically established as a "superior method" for the classification of species (Stackebrandt and Goebel, 1994). Owing that high DNA-DNA reassociation values among the different *Brucella* strains approach 100%, this has led to the proposal that the genus is constituted by one single species (*B. melitensis*) with several biovars (Verger et al., 1985). In spite of this, the different *Brucella* species may be resolved on the basis of DNA restriction patterns (Allardet-Servent et al., 1988; Bricker et al., 2000; Fekete et al., 1992a; Fekete et al., 1992b; Jensen et al., 1999; Tcherneva et al., 2000), phylogenetic analysis of idiosyncratic sequences (Ficht et al., 1996), as well as phenotypic, chemotypic and antigenic characteristics (Cloeckaert et al., 1999; Moreno, 1992; Velasco et al., 1998b). More important, *Brucella* species can be distinguished one from the other on the basis of their distinct biological behavior (Corbel, 1989; Meyer, 1990). Therefore, in the case of *Brucella* organisms, the DNA-DNA reassociation technique does not resolve as well as the observed phenotypic and biological characteristics between the different recognized classical species do (Meyer, 1990).

Biological Attributes in the Context of *Brucella* Species Definition In contrast to the numerical analyses used in the phylogenetic and taxonomic studies for the definition of bacterial species, the biological concept is based upon the identification of fundamental qualitative characteristics. In modern biological usage, species are defined as a particular group of organisms that retain their distinctness from other kinds in nature over a period of successive generations. *Brucella* "distinctness" refers to its biological characteristics, such as the basic comprehension of the life cycle (preferred habitat, potential habitat, preferred host, secondary hosts, vectors, reservoirs, interaction with other bacteria, commensalism, virulence and transmission), physiology (metabolism, generation time, optimal growth temperature, defense mechanisms, offensive mechanisms, repair systems and internal rearrangement of genes), and reproductive behavior (vertical versus horizontal inheritance). The bacterial species defined on the basis of qualitative biological characteristics is sometimes referred to as genospecies (Sneath, 1984b). Since in *Brucella* similar biological traits may occur owing to convergent or parallel evolution (i.e., roughness), the traditional biological species concept must be modified in favor of a more versatile notion.

Experience has demonstrated that biological characteristics by themselves are of little use in defining bacterial species (Weisburg et al., 1989). However, when phylogeny and taxonomy are considered, the biological properties shown by a bacterium contribute to resolving important uncertainties. For instance, brucellosis is a highly contagious bacterial disease of animals and humans. The six recognized species and the

marine strains display different affinities for mammals (Tables 2 and 7). *Brucella abortus* is a parasite of bovines, *Brucella melitensis* of goats and ovines, *B. suis* of swines, hares (biovar 2) and reindeer (biovar 4), *Brucella canis* of canines, *Brucella ovis* of male ovines, *Brucella neotomae* of wood rats, and the marine isolates parasites of dolphins, porpoises, whales and seals (Bricker et al., 2000; Corbel and Brinley-Morgan, 1984; Higgins, 2000; Jahans et al., 1997). Sporadically, *Brucella* organisms have been seen in the reproductive organs of ticks, fleas, lice and worms (Burgdorfer, 1967; Crespo-Leń, 1994; Meyer, 1977). It may be that some of the *Brucella* species use these animal parasites as sporadic vectors or reservoirs.

The known molecular and biochemical characteristics that distinguish the various *Brucella* species and strains, although important, do not explain the subtle and different biological behaviors among the species. Most *Brucella* are very aggressive pathogens that may infect other hosts; however, when this occurs, infection is often terminal and of minor epidemiological relevance. What makes the biological attributes of these microorganisms important in the context of the species definition is the fact that (under natural conditions) the different *Brucella* strains have the tendency to remain in their host animals, and cross infections only occur when the natural hosts are in close contact with a second mammal species. The most susceptible *Brucella* victim is the fetus of the preferred host (The Fetus, the Most Susceptible Host). However, the course of infection in the natural host (when nonpregnant) usually progresses without being noticed. After abortion, infection may recede and self-cure may proceed, and self-cure is the most frequent outcome in the natural host (Enright, 1990a) (Outcome of the Disease and Self Cure). On the one hand, abortion is an efficient condition that ensures dispersion of the bacteria (Infecting the Host); on the other hand, the immune response is a competent mechanism necessary to ensure the survival of the infected host (Control of the Infection). In general, *Brucella* species are well adapted to their natural hosts, reflecting the good equilibrium between both parties. It is important to point out that occurrence of live offspring from *Brucella*-infected mothers is relatively frequent (from 10–20% of cases) and that these animals could later in life abort or give live births (Fig. 9).

One of the important biological distinctions among *Brucella* is their virulence. For many years it has been known that *B. melitensis* is a "vicious bug" considered as one of the candidates for bacteriological warfare (*Brucella* as a Biological Weapon), whereas *B. neotomae* is a "nice bug" confined to desert wood rats (Corbel and Brinley-Morgan, 1984). This is not a matter

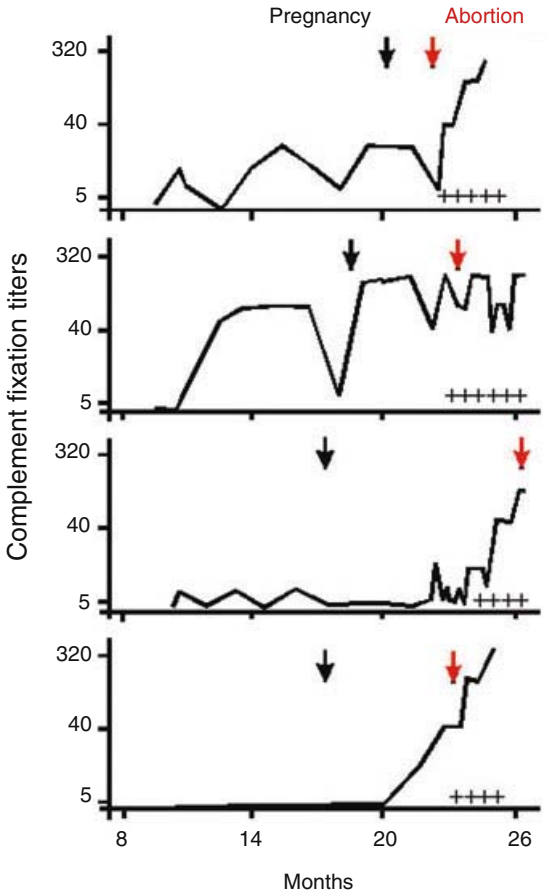


Fig. 9. Antibody responses of bovines born from *B. abortus* strain 554-infected mothers. The serological tests were performed in time before pregnancy, after pregnancy, during abortion and after abortion. After birth, calves were isolated with no possibility for secondary *Brucella* contamination. Then, insemination proceeded and abortion was recorded several months later. Bacteriological isolation was carried out after abortion. The *B. abortus* isolates corresponded to the same biovars isolated from the mothers. Notice that all calves at one time or during extended periods showed negative serological responses. One bovine demonstrated antibodies only after pregnancy. From Plommet (1977), with permission.

of semantics; in every single country, people responsible for control and eradication programs know the different pathological behavior and epidemiological circumstances that distinguish the various *Brucella* species. *Brucella neotomae* under certain conditions, however, displays virulence for mice (Gibby and Gibby, 1965). It is known that humans are more susceptible to *B. melitensis* than to *B. abortus* (Flores-Castro, 1979), whereas mice are more susceptible to *B. abortus* than to *B. melitensis* (Young et al., 1979). It has been determined that *B. melitensis* is more resistant than *B. abortus* to intracellular killing mediated by human neutrophils (Young et al., 1985), a fact that may explain the greater suscep-

tibility of humans to *B. melitensis* infections. Similarly, while rough *B. abortus* 2308 *per*A mutants incapable of generating *O*-chain or NH are readily killed by macrophages, rough *B. melitensis* 16M *per*A mutants are capable of replicating within these cells (Godfroid et al., 1998; E. Moreno et al., unpublished observations). The fact that the same gene mutation in each of these two species causes different biological behavior with respect to intracellular parasitism illustrates the importance in understanding the small but consistent genetic differences that distinguish the various *Brucella* species.

Although from the phylogenetic and taxonomical point of view *B. suis* strains form a closely related cluster, from the biological point of view the first three biovars are separated from biovars 4 and 5. While biovars 1, 2 and 3 have affinity for porcines, biovar 4 preferentially infects reindeer and biovar 5 infects wild rodents (Alton, 1990b; Table 2). In some cases, one biovar could have preference for two different hosts, as in *B. suis* biovar 2 that parasitizes swines and hares (Table 2). It is important to notice that the host preference is not related to chromosome number or size (Fig. 1). Therefore, the subtle genetic and biochemical differences that distinguish these *B. suis* biovars seem to be more relevant for host specificity than are the dramatic differences in the number of chromosomes. This further supports the hypothesis that the chromosome of *B. suis* biotype 3 is the result of the fusion of the two chromosomes with no major consequences for gene expression and competence of infection.

Regarding the *Brucella* marine strains, referred to as *Brucella* "maris" by some authors (Jahans et al., 1997), the cluster is composed of at least three subclusters, according to their biochemical, genetic and animal host preference (Bricker et al., 2000; Jensen et al., 1999; Tcherneva et al., 2000). Their exclusive isolation from marine mammals from diverse habitats suggests that, in spite of being widespread, marine strains do not normally infect terrestrial animals, including humans (Higgins, 2000). Because the transmission of terrestrial *Brucella* organisms mainly occurs by close contact between the infected hosts or tissues (Infecting the Host), the question arises as to how *Brucella* is transmitted under marine conditions. It has been found that filaria lungworms isolated from seals are infected with *Brucella*, suggesting a means of transmission among marine animals (Garner et al., 1997). Since some of these filaria worms cycle in fish, it is reasonable to propose that marine mammals eat fish containing lungworms infected with *Brucella*, and thereby fish become the source of *Brucella* infection for these mammals. If true, then this biological attribute would be characteristic of at least some marine *Brucella*, distinguishing them clearly from the remaining species.

Brucella ovis and (to a lesser extent) *B. canis* are not very aggressive pathogens for secondary hosts when compared to the smooth *Brucella* species, with the exception of *B. neotomae*, which seems to be the least virulent. At the surface level, *B. ovis* and *B. canis* are described as "natural rough" strains (Response to Environmental Stress; Outer Membrane Topology; Moreno et al., 1984a). In contrast to the rough mutants of *Brucella* derived from smooth strains, *B. ovis* and *B. canis* attach in lower numbers to cell surfaces and are more prone to intracellular destruction within lysosomes in epithelial and phagocytic cells (Freer et al., 1999; Pizarro-Cerdá, 1998a). In this respect, it seems that *B. ovis* and *B. canis*, although capable of invading and replicating within cells of their host's reproductive system, are not very efficient in invading cells in vitro (Fig. 21). The singular behavior of these "natural rough" *Brucella* may not only reflect an important biological difference with respect to smooth *Brucella*, but also reflect the restricted preferences of these bacteria for ovines and canines, along with their reduced virulence for other animal hosts, including humans (Corbel and Brinley-Morgan, 1984).

What makes the biological attributes of these microorganisms important in the context of the species definition is that under natural conditions the distinct biochemical, phenotypic, immunochemical and genomic characteristics that identify each of the different *Brucella* species have remained constant in space and time (Corbel, 1989; Meyer, 1990). Since the biological behavior of *Brucella* species does not depend on the presence of accessory genetic elements, it is feasible to consider the qualitative biological characteristics as a valid alternative for use in defining a scheme of *Brucella* species relationships.

At the molecular level, several differences among the various *Brucella* species and strains have been described (Bricker et al., 2000; Ficht et al., 1996; Jensen et al., 1999; Jumas-Bilak et al., 1995; Jumas-Bilak et al., 1998a; Michaux-Charachon et al., 1997; Ouahrani et al., 1993; Tcherneva et al., 2000). For instance, the absence of operons coding for putative proteins involved in the synthesis of polysaccharides and inversions in the genes coding for outer-membrane proteins in *B. abortus* strains are striking features that may be related to the different biological behavior of these brucellae with respect to other species (Vizcaíno et al., 1997; Vizcaino et al., 1999). The different distribution and number of insertion sequences that distinguish the various *Brucella* species may be an indicator not only of distinct gene locations around the two chromo-

somes, but also of the level of expression of these genes (Ouahrani et al., 1993). The absence of *O*-polysaccharide in *B. canis* and *B. ovis* species may be related to the reduced virulence of these species in relation to *B. melitensis*, *B. suis*, and *B. abortus*. The identification and characterization of these genetic and structural variations in relation to host preference and parasitism will help to define host preference and parasitism of the various *Brucella* species within a broader biological context.

Microbiologists' Perception of *Brucella* Species *Brucella* species are a closely related phylogenetic and taxonomic cluster with DNA-DNA reassociation values close to 100%, and on this basis, it has been proposed that *Brucella* is a monospecific genus (Verger et al., 1985). Even though this proposition may suggest a "superior rank" of classification (Stackebrandt and Goebel, 1994), this molecular technique fails to reveal the differences demonstrable in the biological behaviors of the different *Brucella* organisms. Although this is one of the reasons why this proposition has had little acceptance within the scientific community devoted to *Brucella* research (Meyer, 1990; Moreno, 1997), the ultimate judgment has come from veterinarians, microbiologists, physicians, epidemiologists and many other professionals who do not consider the new recommendation relevant for their purposes (Nielsen and Duncan, 1990b; Wong et al., 1992). Therefore, within this context, the name of the species and strains is not a matter of semantics. In every country, people responsible for *Brucella* control and eradication programs know the different pathological behavior and epidemiological circumstances that distinguish the various *Brucella* species. Talk of *B. abortus* is one thing, but the mention of *B. melitensis* is a very different matter. The name of the latter species would dispatch a general alarm among all public health units (Flores-Castro and Baer, 1979).

In modern times, very few scientists attempt to define species on the basis of a single or a few bacterial isolates. In general, the characterization of many distinct isolates is required before a serious committee in bacterial nomenclature would consider it appropriate to define a species as "new" (Graham et al., 1984). Nevertheless, this is not a straightforward subject. No one really knows how many *Brucella* isolates are necessary, and whether these isolates should be obtained during certain time periods from different niches, habitats, hosts, localities and so on. In this sense, the marine *Brucella* strains seem to fulfill the requirements for classification as a distinct cluster of separate species. The issue at this point is not whether the marine strains are different from the terrestrial brucellae, but rather what the differences are between them; that is, do the proposed *Brucella* "maris" comprise several species or only one species with several biovars? Similar questions may be raised with *B. suis* biovar 4 and 5 to which some investigators have delegated as different species owing to their preference for reindeer and wild rodents, respectively (Table 2). In this respect, it is likely that our human self-centered view of the world frequently makes us forget that the brucellae themselves are not as susceptible to systematics as we are.

Habitat

Traditionally, *Brucella* organisms have been defined as facultative intracellular pathogens, since they multiply in bacteriological media and also replicate within cells (Corbel and Brinley-Morgan, 1984). Their biology, however, seems to point in the opposite direction: *Brucella* organisms are pathogens whose ultimate goal is to propagate in their preferred niche, the cell (*Brucella* Traffics and Replicates Within Host Cells). In this sense the brucellae are better described as facultatively extracellular intracellular parasites (Moreno and Moriyón, 2002). With this perspective, it may well be that the most risky and uncomfortable environment for *Brucella* is the extracellular milieu from which these bacteria must escape to survive and persist as intracellular pathogens from one generation to another (Survival Outside Host Cells).

The *Brucella* species display host preference, although they may infect secondary hosts (Geographical Distribution). Under certain circumstances, *Brucella* can reproduce within fleas, lice, ticks and worms, which occasionally may serve as vectors for transmission to mammalian hosts. In temperate zones, *Brucella* could survive in soil, manure or water, depending upon the temperature. Normally in low temperatures (between 2–8°C), the bacteria live up to several weeks or even months, as long as enough organic material is available and the bacterium is protected from the sun's rays. When exposed to sun's rays in the open, *Brucella* organisms steadily die, a phenomenon that takes place more quickly when the bacteria are exposed to sun's rays above 18°C. At temperatures of 56°C or higher, *Brucella* organisms are quickly destroyed (Ray, 1977). Pasteurization (performed at 62°C for 30 min) of dairy products eliminates *Brucella* organisms and the risk of human contamination.

Isolation

All *Brucella* spp. are pathogens that are seldom obtained from sources other than human

patients and sick animals or their products, and their isolation is an essential part of the diagnostic procedures. Thus, the practical aspects of isolation are better understood in the context of the laboratory diagnosis of brucellosis, which includes the use of molecular methods in both humans (Direct Diagnosis of Human Brucellosis) and animals (Direct Diagnosis in Animal Brucellosis). Since they most often differ in feasibility, sample type, media, and criteria used in the evaluation of the results, the bacteriological diagnosis of human and animal brucellosis is described and discussed in two separate sections (Direct Diagnosis of Human Brucellosis; Direct Diagnosis in Animal Brucellosis). The present section deals only with the culture of *Brucella* in the microbiology laboratory.

Biosafety

Brucella has been classified as a group 3 microorganism and should be handled accordingly (*Brucella* as a Biological Weapon). Brucellosis is the most commonly reported laboratory infection, and although the infectious dose for humans is not known, it is likely that only very low numbers of bacteria (estimated at less than 10 cells) are enough to cause infection through the mucosae. Aerosols generated during homogenization of tissues, centrifugation, pouring or transferring of broths, shaking or stirring of tubes and flasks, inoculation of Petri dishes and other laboratory manipulations represent the highest risk. Biosafety level 2 practices can be used for activities involving clinical materials of human or animal origin, but all procedures likely to generate aerosols should be carried out in an appropriate biosafety cabinet. Biosafety level 3 containment practices and facilities are required for all manipulations involving large-scale culture and for experimental animal studies (http://www.hc-sc.gc.ca/hpb/lcdc/biosafty/msds/msds23e.html). Material safety data sheets can be obtained at (http://www.hc-sc.gc.ca/pphb-dgspsp/ols-bsl/index.html) and general laboratory precautions are described by Alton et al. (1988).

Culture

The brucellae are chemoheterotrophic bacteria that grow well in a variety of lifeless media and under relatively simple laboratory conditions, but for those biotypes and species that require CO_2 (Table 3).

Physicochemical Requirements and Atmosphere The temperature growth range of brucellae is 18–42°C, with variations that depend on the strain and medium. The optimal temperature is, however, between 34–37°C. The brucellae grow best at pH 6.6–7.4, and because they do not acidify the growth medium (a slight increase in pH is sometimes observed), buffering is not strictly necessary. The optimal osmotic pressure is reported to be somewhat lower (2–6 atmospheres) than that found in the usual culture media (about 7–8 atm; Gerhardt, 1958).

All *Brucella* spp. are strictly aerobic, but some *B. abortus* biovars, some *Brucella* isolates from marine mammals, and *B. ovis* only grow in atmospheres containing 5–10% CO_2 (Tables 3 and 7). This does not relate to a decreased pO_2 but to a specific requirement of CO_2 per se, and at least for the CO_2-dependent *B. abortus* strains, subculture frequently leads to selection of spontaneously occurring CO_2-independent mutants. Although the exact redox potential (E_h) and oxygen tension required for optimal growth are not known, the brucellae need vigorous aeration when grown in broth. For small-to-medium volumes, shaking is adequate, but for large volumes sparging and antifoaming agents are necessary. In this case, attention has to be paid to the antifoaming agents, since all brucellae are sensitive to many of these compounds. For laboratory fermentors with dissolved oxygen control, stirring and sparging conditions maintaining a 20–50% oxygen saturation at 37°C give good results (Rousseau et al.,1987). These and other conditions used in less sophisticated fermentors are summarized in Table 8.

Nutritional Requirements On primary isolation, most *Brucella* strains grow slowly or poorly in media devoid of blood, serum or tissue extracts. Accordingly, the brucellae are often described as fastidious, and in fact, reported doubling times in vitro (2.5–3.5 h) are longer than those of many pathogenic bacteria. However, careful analyses (Gerhardt, 1958; Plommet, 1991) show that at least the growth requirements of *B. suis*, *B. abortus* and *B. melitensis* are not excessive. Thus, the slow growth of brucellae upon primary isolation may reflect, in part, a lag phase required for adapting to an environment very different from the host, low efficiency in nutrient uptake, basic genetic features or other factors. Moreover, these bacteria are sensitive to low concentrations of inhibitors (such as fatty acids) present in some media (Huddleson, 1955) or resulting from heat sterilization (Gerhardt, 1958; Rousseau et al., 1987; Yantorno et al., 1978), and this explains the improvement of growth caused by agents with detoxifying action, such as charcoal, serum or agar itself (Robertson et al., 1977). Amino acid imbalance has also been suggested as a cause of reduced growth (Gerhardt, 1958). The variable growth rates observed

Table 7. Characteristics of the *Brucella* isolates from marine mammals.[a]

Host	No. of isolates	LPS differential epitope	Sensitivity to phage(s)[b] Tb	Wb	Bk2	Iz	Fi	CO_2 requirements	Reference
Seals									
Phoca vitulina (common seal)	8	A	–[c]	–[d]	++	+	v[e]	+	Jahans et al., 1997
P. vitulina richardsi (Pacific common seal)	1	A	–	n.t.[f]	n.t.[f]	n.t.[f]	n.t.[f]	+	Garnert et al., 1997
Cystophora cristata (hooded seal)	1	A	+	+	++	++	+	+	Jahans et al., 1997
Halichoerus grypus (grey seal)	1	A	+	+	++	++	+	+	Jahans et al., 1997
Porpoises									
Phocoena phocoena (harbor porpoise)	5	A	–	+	++/+	v[e]	+	–	Jahans et al., 1997
Dolphins									
Delphinus delphis (common dolphin)	2	A	+[g]	+	++	++	+	–	Jahans et al., 1997
Stenella coeruleoalba (striped dolphin)	2	A	v[e,g]	+	++	++	+	–	Jahans et al., 1997
Lagenorhynchus actus (white-sided dolphin)	1	A	–	+	+	–	–		Jahans et al., 1997
Tursiops truncatus (bottlenose dolphin)	1	M	–	++	–	n.t.[f]	–	–	Ewalt et al., 1994
Whales									
Balaenoptera acutorostrata (mink whale)	1	A	–[g]	++	n.t.[f]	++	n.t.[f]	–	Claraveau et al., 1998

[a]All isolates are smooth, grow on fuchsin and thionin (at 20µg/ml), are urease positive and do not produce H_2S.
[b]Lysis (++) or partial lysis (+) at routine test dilution; all isolates are resistant to phage R/C.
[c]Some isolates are lysed (+ to ++) at $10^4 \times$ (routine test dilution).
[d]One isolate was lysed (+) by phage Wb.
[e]v, variable (from ++ to –) responses depending on the isolate.
[f]n.t., not tested.
[g]Lysed at at $10^4 \times$ (routine test dilution).

with different batches of commercial media and among manufacturers can be attributed to some of the above-summarized circumstances. An additional factor retarding growth detection on primary isolation is that brucellae may be present in only low numbers in some samples (Diagnosis).

In chemically defined media containing appropriate mineral salts (Gerhardt, 1958; Plommet, 1991; Table 9), *B. suis*, *B. abortus* and *B. melitensis* require nicotinic acid and thiamine for growth. Pantothenic acid is required by some strains and is only stimulatory for others, and biotin is stimulatory. But for some strains that require sulfur-containing amino acids, these three species can use ammonium sulfate and thiosulfate as nitrogen and sulfur sources and glucose as the sole carbon and energy source. Many *B. suis* strains can also use glutamate as the sole nitrogen and carbon source, and this ability is also displayed by some *B. melitensis* strains when grown in the presence of CO_2 but not by *B. abortus*, even though this species can actively oxidize this amino acid (Table 2). A combination of glutamate, lactate and glycerol seems also satisfactory as a carbon source. All these data show that most strains of these three species are endowed with prototrophic capacities for all organic constituents, with the exception of thiamine and nicotinic acid, and that *B. suis* is the closest to prototrophy. This last observation probably accounts for the fact that *B. suis* usually shows higher growth rates than those of *B. melitensis* or *B. abortus* and also for its comparatively greater variability in oxidative metabolic patterns (Table 2). On these bases, it has been suggested that *B. suis* may represent the metabolic type closest to that of the soil- or plant-associated α-2 Proteobacteria from which the brucellae probably evolved (Plommet, 1991). There are incomplete data or no nutritional studies performed with *B. canis*, *B. neotomae*, *B. ovis*, or the isolates from marine mammals.

The nutritional requirements of the brucellae are met by a variety of standard laboratory media (Tables 8 and 10). Most often these media contain a combination of peptones and glucose. Serum or blood supplements are not strictly required, although they would enhance the growth of some strains. *Brucella ovis* is an exception, since almost all strains will grow poorly or not at all in standard media that lack serum or blood. *Brucella abortus* biovar 2 also requires serum but only on primary isolation. As discussed above, this requirement may represent increased sensitivity to hydrophobic inhibitors rather than true

Table 8. Growth of *Brucella* in laboratory fermentors.

Medium	Sterilization	Growth conditions	Anti-foaming mixture[a]	Yield	Comments	References
3% Glucose, 1.5% enzymatic digest of casein,[b] 1.5% enzymatic digest of animal tissue,[c] 1% yeast extract, 0.9% NaH_2PO_4, 0.33% Na_2HPO_4	Filtration	Aeration increased progressively from 10 liters of air ·min $^{-1}$ at 400rpm to 14 liters of air ·min $^{-1}$ at 600rpm	0.0002% AF 60 silicone emulsion[d]	—	Mix the ingredients while boiling the water. The vessels and the antifoaming agent are sterilized before adding the medium (35min at 121°C). It is the standard USDA procedure for growing *B. abortus* 19 (vaccine strain).	Alton et al., 1988
2% Glucose, 2% fructose, 3.1% enzymatic digest of animal tissue,[c] 1% yeast extract, and 0.16% Na_2HPO_4	Heat 5min at 121°C followed by rapid cooling.[e]	Oxygen dissolved control set at 20–50% O_2 saturation	0.001–0.005% Silicone A-N-butyl acetate (1:3)[f]	2×10^{11} cfu ·ml $^{-1}$	All materials but the antifoaming mixture are sterilized together in the fermentor vessel. Tested with *B. abortus* 19 (vaccine strain). Twelve liters of medium in a 20-liter vessel.	Rousseau et al., 1987
2% Glucose, 1.7% enzymatic digest of casein,[b] 0.3% enzymatic digest of soya,[g] 0.5% yeast extract, 0.25% K_2HPO_4, and 0.5% NaCl	Heat 10min at 121°C followed by rapid cooling.[e]	Oxygen dissolved control set at 35% O_2 saturation	0.003% Silicone A-N-butyl acetate (1:3)[f]	2×10^{11} cfu ·ml $^{-1}$	Glucose sterilized (121°C, 20min) separately. Tested with *B. abortus* 19 (vaccine strain) and 2308 and with *B. melitensis* 16M. Eight liters of medium in a 15-liter vessel.	Aragón et al., 1996

[a]The sensitivity to antifoaming agents may vary among strains, and it increases for rough mutants and at least for the rough species *B. ovis*.
[b]Bacto-casitone (Difco) or similar.
[c]Bacto-peptone (Difco) or similar.
[d]General Electric, Silicone Products Division, Waterford, New York. This product may be identical to Sigma antifoaming silicone A.
[e]Owing to the large volume of fermentor vessels, sterilization times have to be corrected for thermal inertia. The actual sterilization time at 121°C, plus the time the vessel is above 110° plus the cooling time must be equivalent to the standard sterilization conditions in autoclaves. Prolonged heat sterilization produces degradation compounds toxic for brucellae.
[f]Sigma Chemical Co., St. Louis, MO.
[g]Soytone (Difco) or similar.

Table 9. Chemically defined media for *B. abortus*, *B. melitensis* and *B. suis*.

Characteristics	Medium:[a] A	B	C
Organic macronutrients			
Glucose	1.0g	—	1.0g
Glycerol	—	30.0g	—
Lactic acid	—	5.0g	—
Glutamic acid (Na salt)	—	1500mg	—
Vitamins[b]			
Thiamine·HCl	0.2mg	0.2mg	0.2mg
Nicotinic acid	0.2mg	0.2mg	0.2mg
Pantothenic acid (Ca salt)	0.04mg	0.04mg	0.04mg
Biotin	0.0001mg	0.0001mg	0.0001mg
Minerals			
$(NH_4)_2SO_4$	0.5g	—	0.5g
K_2HPO_4	1.0g	10.0g	7.0g
KH_2PO_4	—	—	3.0g
$Na_2S_2O_3 \cdot 5H_2O$	0.1g	0.1g	0.1g
Mg^{2+} (as SO_4^{2-})	10.0mg	10.0mg	10.0mg
Mn^{2+} (as SO_4^{2-})	—	0.1mg	0.1mg
Fe^{2+} (as SO_4^{2-})	—	0.1mg	0.1mg
NaCl	7.5g	7.5g	5.0g
pH	6.8–7.2	6.8–7.0	7.0

[a]Amounts per liter of broth; medium A is from McCullough and Dick (1943), medium B from Gerhardt (1958), and medium C from Plommet (1991).
[b]Vitamins can be replaced by 1.0–0.5g of yeast extract (López-Goñet al., 1992; Plommet, 1991).

nutritional requirements. Synthetic or semisynthetic media have been reviewed by Gerhardt (1958) and Plommet (1991). This last author recommends a simple mineral salt-glucose-vitamin media for physiological studies, which can be prepared synthetically or semisynthetically with yeast extract (Table 9).

Selective Agents and Media The brucellae are inhibited by the hydrophobic dyes and surfactants used in media selective for classical Gram-negative bacteria, such as most enteric bacteria, pseudomonads and others (Permeability). Early formulations of *Brucella*-selective media that include crystal violet, ethyl violet or gentian violet are not satisfactory because some biovars and species are sensitive to these agents. On the other hand, the brucellae grow well in the presence of polymyxins (polymyxin B and colistin [polymyxin E]) at concentrations that are inhibitory for many Gram-negative bacteria (Lipopolysaccharide and Resistance to Outer-membrane Destabilizing Agents). Thus, selective and enrichment media have been designed that include these antibiotics plus others that, at the concentrations used, act preferentially on Gram-positive bacteria and fungi (Table 11). It has to be noted that present formulations of *Brucella*-selective media have only been tested extensively on *B. abortus*, *B. melitensis* and *B. ovis* and that, although unquestionably useful, they are inhibitory for some strains. Moreover, present formulations of selective media are not equivalent because of differences in antibiotic sensitivity (in particular, bacitracin and nalidixic acid) between *B. abortus* and *B. melitensis* (Marín et al., 1996). Although it is not known to what extent, these media are probably satisfactory for other *Brucella* species.

Identification

Identification of *Brucella* can be performed after isolation in pure culture by conventional bacteriological methods, including serological and phage typing, and also using Molecular Biology tools. The use of molecular probes to identify the presence of *Brucella* cells or their debris in blood, milk, tissues and other materials without prior isolation is discussed in the context of the diagnosis of human and animal brucellosis. Typing at biovar and strain level is necessary only for epidemiological studies and for the identification of the vaccine strains.

Cellular and Colonial Characteristics

These are important characteristics because the brucellae have a cell morphology, staining properties and colonial appearance on isolation media such that, along with the epidemiological evidence, enable experienced bacteriologists

Table 10. Standard media most commonly used to grow *Brucella*.

Common name	Basal medium	Commercial source[a]	Supplements	Comments
Tryptic soy broth (or agar)	1.5% enzymatic digest of casein, 0.5–0.3% enzymatic digest of soybean meal, 0.25% glucose, 0.25% K_2HPO_4, and 0.5% NaCl; (1.5–2.0% agar)	Trypticase soy broth (BBL); Trycase soy broth (bioMérieux); Bacto-tryptic soy broth (Difco); Tryptone soy (GIBCO); and Tryptone soy broth (Oxoid)	None or, depending on the purpose, one or several of the following: 2–5% filter-sterilized serum;[b] 0.2–0.5% yeast extract; 5–10% defibrinated blood.	General purpose media. Yeast extract is beneficial, in particular for *B. ovis*.
Blood broth (or agar) base	1.0% enzymatic digest of casein, 1.0% enzymatic digest of animal tissue, 0.1% glucose, 0.2% yeast extract, 0.5% NaCl, and 0.01% $NaHSO_4$; (1.5–2.0% agar)	*Brucella* broth (BBL); Bacto-*Brucella* broth (Difco); and *Brucella* medium base (Oxoid)[c]	None or, depending on the purpose, one or several of the following: 2–5% filter-sterilized serum;[b] 0.2% yeast extract; 5–10% defibrinated blood	General purpose media; it is equivalent to Albimi broth. Can be used to prepare selective media (**Table 11**)
Serum-dextrose agar	0.3–0.5% beef or meat extract, 1.0–0.5% enzymatic meat digest, 0.8–0.5% NaCl, and 1.5–2.0% agar	Nutrient agar (bioMérieux); and Bacto-nutrient agar (Difco)	One: 5% of a filter-sterilized mixture of 20% dextrose in serum	General purpose media; not identical but equivalent to BBL's and Oxoid's Nutrient agar. Can be used to prepare selective media (**Table 11**)
Glycerol-dextrose agar	0.3–0.5% beef or meat extract, 1.0–0.5% enzymatic meat digest, 5% serum, 1% glucose, 0.8–0.5% NaCl, and 1.5–2.0% agar	Bacto-nutrient agar (Difco)	One: 2% glycerol and 1% glucose (added independently from filter-sterilized stocks)	Recommended for dissociation testing
Glycerol-potato agar	20% potato infusion, 0.5% beef or meat extract, enzymatic meat digest, 1% glucose, 0.5% NaCl, and 1.5–2.0% agar	Bacto-Potato infusion agar (Difco)[d]	One: a 2% glycerol (added before autoclaving)	Propagation of smooth strains in Roux bottles for antigen or vaccine production
Modified Thayer-Martin's	1.5% enzymatic digest of protein, 0.1% corn starch, 0.4% K_2HPO_4, 0.1% KH_2PO_4, 0.5% NaCl, and 1% agar	Bacto-GC medium base (Difco); GC medium base (GIBCO); GC agar base (Oxoid); and GC agar base (BBL)	One of two: 0.7% blood (added to the molten medium equilibrated at 55–60°C) or 1% hemoglobin	Recommended for *B. ovis*. Can be used to prepare selective media (**Table 11**)

[a]BBL (Baltimore Biological Laboratories), Baltimore, Md, USA; bioMérieux, Marcy l'Etoile, France; Difco Laboratories, Detroit, Mich., USA; GIBCO (Grand Island Biological Company) Laboratories, Grand Island, N.Y., USA; Oxoid Ltd., London, England.

[b]Equine or bovine serum; serum is required at least for *B. abortus* biovar 2 primary isolation and for *B. ovis* growth.

[c]Oxoid suggests several alternative basal media for using with antibiotic supplements.

[d]Several manufacturers market potato agars than contain no peptone or meat extracts and that can be supplemented with these materials.

Table 11. Selective and enrichment media used to isolate *B. abortus*, *B. melitensis* and *B. ovis*.

	Medium:[a]				
Characteristics	USDA[b]	A	B[c]	C	D[d]
Composition					
Basal medium	Any of the peptone media in Table 10	Tryptic soy broth 5% serum[e]	Blood agar base 5% serum[e]	Tryptic soy agar 5% serum[e]	Modified Thayer-Martin's[e]
Selective agents					
Polymyxin B sulfate	1,800 units	5,000 units	5,000 units	1,800 units	
Colistin methane-sulfonate	—	—	—	—	86,000 units
Bacitracin	7,500 units	20,000 units	25,000 units	25,000 units	
Vancomycin		20mg	20mg	—	3mg
Nalidixic acid		5mg	5mg	—	
Cycloheximide	30mg	100mg	100mg	100mg	
Lincomycin				6,000 units	
Nitrofurantoin	—	—	—	—	10mg
Cycloserine		312mg		—	
Nystatin		100,000 units	100,000 units	100,000 units	12,500 units
Amphotericin B		4mg		—	
Agar	20g	—	20g	20g	10g
Recommended use	Isolation of *B. abortus* from samples not heavily contaminated with other bacteria	*B. abortus* enrichment from milk	Isolation of *B. abortus* from milk or other heavily contaminated samples	Isolation of *B. abortus* from milk or other heavily contaminated samples	Isolation of *B. melitensis*, *B. ovis* and *B. canis*

Abbreviation: USDA, United States Department of Agriculture.

[a]Figures are the amount per liter of basal medium. Medium A is from Brodie and Sinton (1975), medium B from Farrell (1974), medium C from Ewalt et al. (1983). and medium D from Brown et al. (1971).

[b]Modified Kuzdas and Morse medium described by Alton et al. 1988.

[c]The antibiotic mixture can be obtained from Oxoid as a freeze-dried additive.

[d]The antibiotic mixture can be obtained from BBL, Difco and GIBCO as a freeze-dried additive (VCN).

[e]See (Table 10).

to perform an immediate presumptive identification.

Bacterial Cells The brucellae are coccobacilli or short rods of 0.5–0.7 by 0.6–1.5 μm, but at least some *B. suis* strains develop larger cells in rich media. They are arranged individually, less often in pairs or small groups. At least under standard growth conditions, all *Brucella* spp. are devoid of flagella or capsules. They are Gram-negative, and although not truly acid-fast, they are stained by the modified Ziehl-Neelsen method of Stamp (basic fuchsin staining followed by decolorization with diluted acetic acid; Fig. 10). All *Brucella* spp. but *B. ovis* are reported to be positive by the modified Köster's method (safranin-NaOH staining followed by decolorization with diluted sulfuric acid). For detailed staining protocols, consult Alton et al. (1988).

Colonies and Dissociation Colonies on translucid media are transparent, convex and have an entire edge. They are usually small (0.5–1.0 mm after 2–3 days of incubation of a fresh inoculum), but there are variations that depend on the medium and strain. When examined with obliquely reflected light, the colonies of the smooth species (Table 2) appear moist and glistening and somewhat bluish, whereas those of the rough species have a dry, granular aspect.

The smooth *Brucella* species often dissociate during growth to yield mixtures of typically smooth and rough colonies along with others of intermediate characteristics. The cell surface is responsible for the appearance of the colonies and dissociation is caused by spontaneous mutations leading to the synthesis of LPS lacking the *O*-polysaccharide (rough-LPS) (Response to Environmental Stress). Moreover, mucoid colonies derived from the smooth strains have been repeatedly described and can be distinguished by their slimy consistency, but the structural and genetic defects behind this phenotype are not known with certainty. The rough *B. canis* often produces mucoid colonies. The early literature contains descriptions of many environmental factors favoring dissociation, but the only ones clearly demonstrating effect are low oxygen and age of the culture (reviewed in Gerhardt, 1958). Dissociation is favored by anoxia and hence by growth in nonaerated broth or on humid solid media. Accumulation of rough mutants occurs late in the stationary phase of growth.

Since the smooth-LPS is a major surface antigen and a virulence factor, control of dissociation is essential in production of vaccines (Vaccines and Vaccination) and diagnostic antigens (Immunological Tests for Brucellosis). Specific and detailed control protocols are given in the manual *Techniques for the Brucellosis Laboratory* by Alton et al. (1988). Controls can be carried out by examination with obliquely reflected light but other tests requiring less experience may be more practical. Agglutination tests are particularly useful to test individual colonies. Smooth brucellae agglutinate only with sera of *O*-polysaccharide specificity, and semi-rough mutants can be detected by their simultaneous agglutination with sera of *O*-polysaccharide and rough-LPS specificities. A failure to agglutinate with sera of *O*-polysaccharide (LPS) specificity and a positive agglutination with anti-rough-LPS sera are characteristics of the rough mutants (and species). Moreover, the rough-type cells also agglutinate with neutral acriflavine at 1 : 1000. To examine cultures for dissociation, they can be plated out and plates briefly flooded with a 1 : 4 mixture of 10% crystal violet in ethanol and 1% ammonium oxalate in distilled water. By this method, and in opposition to the smooth-type colonies, the rough type colonies become stained (Fig. 11). Finally, smooth cultures sediment slowly in broth, whereas those of the rough mutants and rough species autoagglutinate rapidly. All these differential tests and properties reflect the profound alteration in outer-membrane topology (Outer Membrane Topology) caused by the defects in LPS.

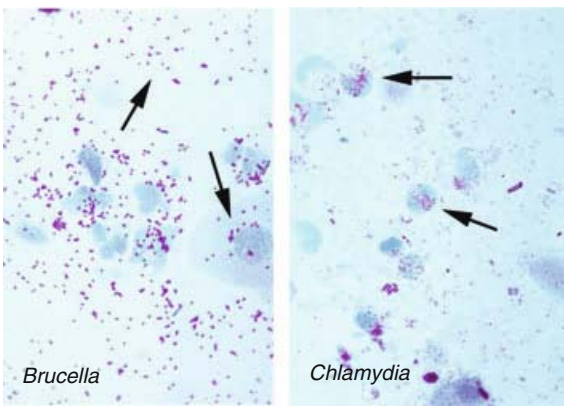


Fig. 10. *Brucella* staining by Stamp's method. The images show smears made with material taken from sheep abortions caused by *B. melitensis* or *Chlamydia* spp. Both parasites stain positive by the Stamp's method, but while *Chlamydia* bodies are mostly associated with cells and are of a very small size and of irregular appearance (due to the presence of both reticulate and elementary bodies), *Brucella* cells are larger and found everywhere. Courtesy of J. M. Blasco, SIA, DGA, Zaragoza, Spain.

Identification and Typing

Identification of brucellae at genus level is simple and can be achieved in most laboratories,

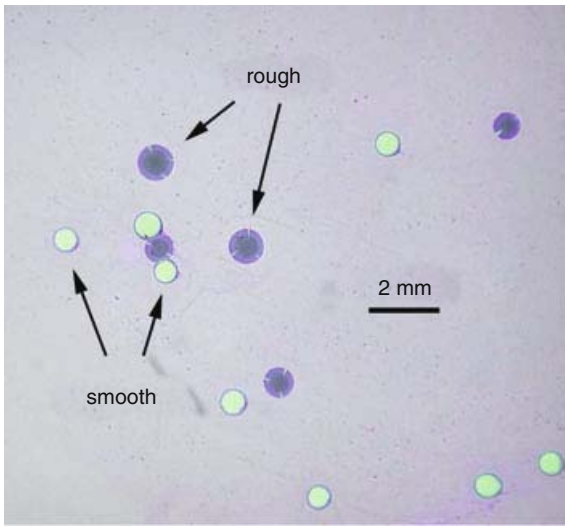


Fig. 11. Smooth and rough *Brucella* colonies demonstrated by the crystal violet method.

most conveniently by serological methods. Precise identification to species and biovar level is only possible in reference laboratories.

STANDARD TESTS Standard identification is based first on the morphological and staining characteristics of cells and colonies and on the growth requirements, including CO_2, and conditions of primary isolation (Isolation). Pure *Brucella* cultures are nonhemolytic. Catalase is positive and their metabolism is that of a strictly respiratory microorganism, although *B. neotomae* is reported to be able to show some fermentation from glucose. Since they are oxidative rather than fermentative microorganisms, they do not acidify the medium and are negative in tests that, like the methyl red and Voges-Proskauer tests, detect acidic metabolites or other fermentation products. With the exception of *B. ovis* and *B. neotomae*, all species are positive in the cytochrome *c*-oxidase test, and all but *B. ovis* are able to reduce nitrates to nitrites and have urease activity (Table 2). They do not produce indole from tryptophan and, depending on the strain and species, they have limited or no ability to use citrate as the sole carbon source.

Only few bacteria show serological crossreactivity with the *O*-polysaccharide of the *Brucella* smooth-LPS (Table 6), and they are easily differentiated on the basis of the standard bacteriological tests. Thus, slide agglutination tests with antisera of the appropriate specificity are very useful in the identification of *Brucella* and easily separate the rough from the smooth *Brucella* species. Those carrying a smooth-type LPS (Table 2) agglutinate with sera-containing antibodies to the *O*-polysaccharide but not with sera containing antibodies to the core-lipid A antibodies, owing to the topology of the outer-membrane of smooth cells (Outer Membrane Topology). The rough species and rough mutant strains agglutinate with sera of the latter but not of the former specificity. Sensitivity to the smooth- and rough-specific phages carries the same information (Table 13). Semi-rough strains or dissociated cultures agglutinate with both sorts of antibody and show intermediate sensitivities to the smooth- and rough-specific phages.

For species differentiation, cytochrome *c*-oxidase, urease and nitrate reduction tests are partially useful since, used along with the agglutination tests, they discriminate *B. ovis*, *B. canis* and *B. neotomae* but not *B. melitensis*, *B. abortus* and *B. suis*. Typing with the Tb phage discriminates these three species (Table 2). Further assessments to assign the isolates to biovars also confirm the species identification but require specific means and protocols (Alton et al., 1988). For identification to biovar level, the distribution of the A and M epitopes (Response to Environmental Stress), CO_2 requirements on primary isolation, H_2S production and growth on serum dextrose agar supplemented with thionin and fuchsin (and sometimes safranin) are used (Table 3), even though there are atypical strains in some of those tests. Metabolic oxidative profiles have an intermediate value: they discriminate species and, for *B. suis*, some groups of biovars (Table 2).

The *Brucella* isolates from marine mammals have not been assigned to species yet, and the number of isolates is too reduced to assess a possible distribution into biovars. So far, all isolates have smooth colonial morphology and are resistant to the rough specific RC phage. With one exception, they carry the A epitope and no intermediate A+M strains have been described. All grow on thionin and fuchsin at the standard (20 μg/ml) dye concentrations, show urease activity, and do not produce H_2S but vary in the CO_2 requirement for growth and sensitivity to the standard phages (Table 7).

MOLECULAR METHODS A number of probes have been used for the detection by polymerase chain reaction (PCR) of *Brucella* DNA in samples of human or animal origin (Bacteriological Culture for Humans; Molecular Tests for Humans). Identification at genus level is easily achieved under laboratory conditions by PCR with primers taken from a variety of available sequences, such as those of the 16S rRNA, heat shock proteins, outer-membrane proteins, insertion sequence IS*711* (also known as IS*6501*), and others (Table 4). Cross-identification or detec-

tion of phylogenetically related bacteria with some of the primers is a demonstrated possibility. In addition, gene polymorphism has been examined for the differentiation at species and biovar levels with partial success (Vizcaíno et al., 2000). To date, there is no established single molecular method that can fully replace the classical identification tests and biotyping scheme, even though species can be differentiated by some protocols, and markers specific for *B. melitensis*, *B. abortus*, *B. ovis* and *B. canis* and for some biotypes or groups of biotypes have been found (Table 4). This promising line of research will require further assessments of numbers of strains large enough to draw definite conclusions on the usefulness of particular protocols or methods.

Identification of Vaccine Strains *B. abortus* 19 (*B. abortus* US-19) can be differentiated from typical field isolates of *B. abortus* biovar 1 by its inhibition on media containing appropriate concentrations of thionin (2 µg/ml) and penicillin (5 IU/ml). Moreover, in opposition to typical *B. abortus* biovar 1 primary isolates, this vaccine strain does not require CO_2 for growth. Typical strain 19 is also inhibited by *i*-erythritol (1 mg/ml) because it carries a 702-bp deletion in the *ery* region (Sangari et al., 1994) causing a metabolic deficiency that probably leads to the toxic build-up of intermediate catabolites (Intermediary Metabolism). However, a few strain 19 isolates grow on *i*-erythritol (Alton et al., 1988) possibly because they carry compensatory mutations, and in this case, the phenotypic test fails. The deletion in the *ery* region can be directly examined by hybridization of *Eco*RI-digested DNA with appropriate *ery* probes (Sangari and Agüero, 1994a) or by PCR with primers taken either from the sequences at both sides of the deletion (Sangari et al., 1994) or from the 702-bp deletion plus the *ery* region conserved in strain 19 (Bricker and Halling, 1995).

B. melitensis strain Rev. 1 can be differentiated from typical field isolates of *B. melitensis* biovar 1 by its inability to grow on thionin and fuchsin (both at 20 µg/ml) or penicillin (5 IU/ml). Moreover, Rev. 1 is resistant to streptomycin at 2.5 µg/ml. (Rev. 1 is a revertant of a streptomycin-dependent mutant.) Molecular markers for this vaccine will certainly be developed, taking advantage of the expected mutation(s) in some of the ribosomal proteins involved in streptomycin resistance.

B. abortus RB51 is a rough strain that has been proposed as a live vaccine for cattle brucellosis. Because field strains infecting cattle are smooth, this strain can be easily differentiated from most field strains by the characteristics associated with the rough phenotype (Colonies and Dissociation). Resistance to rifampicin is also a characteristic of this strain. Moreover, strain RB51 carries two IS*711* elements in a specific locus, and this allows its differentiation from field isolates of *B. abortus* biovar 1, 2 and 4 (but not from the parental virulent strain *B. abortus* 2308; Ewalt and Bricker, 2000).

The Brucellaphages

Over twenty brucellaphages have been isolated from several natural sources, including the bacteria themselves, manure, fetuses, blood, milk and other materials from *Brucella*-infected animals. Although there is evidence compatible with the existence of a lysogenic state in some cases (Corbel and Thomas, 1980a), this has never been proven and all brucellaphages in use are lytic. The origin, species host range, and other basic features of the most significant brucellaphages are presented in Table 13, and data

Table 12. The major free lipids of *Brucella abortus* and *Brucella melitensis*.

	Phospholipids				Neutral lipids		
Fatty acid[a]	Phosphatidyl-choline (37.6%)[b]	Phosphatidyl-ethanolamine (32.9%)[b]	Phosphatidyl-glycerol (9.4%)[b]	Diphosphatidyl-glycerol (20.1%)[b]	Ornithine lipids (32%)[c]	Di-, monolgycerides and other acyl esters (28%)[c]	Wax-like esters (6%)[c]
16:0	30.1	32.3	56.5	32.5	30.4	27.3	34.5
18:0	3.8	5.6	5.3	5.8	12.2	9.0	8.9
16:1	16.0	6.4	7.0	20.4	1.9	4.1	8.8
18:1	6.0	12.4	4.3	8.7	18.4	17.8	9.8
19:0	43.3	38.5	23.3	28.6	29.3	31.3	—
other	6.9	4.8	3.6	4.0	7.8	10.5	38.0

[a]Expressed as % of the total fatty acid in the particular lipid.
[b]Expressed as % of the total phospholipid.
[c]Expressed as % of the total neutral lipid.
From Cherwonogrodzky et al. (1990).

Table 13. Characteristics of the main *Brucella* phages.[a]

Phage (group)[a]	Origin	Propagating strain	Host range lysis[b]		Other properties
			at RTD and 10^4 RTD	only at 10^4 RTD	
Tb (1)	Isolated from liquid manure of a cowshed in Tbilisi (Russia)	*B. abortus* 544[c] and S19[d]	*B. abortus*	*B. suis* and *B. neotomae*	Also known as TB; does not lyse rough mutants
Fi (2)	Isolated from a *B. abortus* culture collected in Firenze (Italy). Fi/13 is the type strain	*B. abortus* S19[d]	*B. abortus* and *B. neotomae*	*B. suis*	Also knonwn as Fz75/13; does not lyse rough mutants
Wb (3)	Isolated from a *B. suis* culture in Weybridge (England)	*B. abortus* S19[d], *B. suis* 1330[c]	*B. abortus*, *B. suis* and *B. neotomae*	Reported for some *B. melitensis* strains	Also knonwn as W; does not lyse rough mutants
Bk2 (4)	Obtained in Berkley (USA) by plating Bk0[e] on *B. melitensis* 16M[c]	*B. melitensis* Isfahan[f] and 16M[c]	*B. abortus*, *B. melitensis*, *B. suis* and *B. neotomae*	None reported	Does not lyse rough mutants; relative of Bk1
R/C (5)	Obtained from R/O[g] by serial propagation on *B. canis* Mex.51[h]	*B. canis* Mex.51[h] and RM 6/66[c]	*B. canis*, *B. ovis* and rough *B. abortus*	Reported for some rough *B. melitensis* mutant strains	Not active on smooth *brucellae*
R/M (5)	Obtained from R/C by serial propagation on *B. melitensis* 115	*B. melitensis* 115[i]	n.d.[j]	n.d.[j]	Reported to be more consistently lytic for rough *B. melitensis*
Iz (6)	Isolated from sheep and goat feces in Izatnagar (India)	*B. abortus* S19,[d] *B. melitensis* 16M[c], *B. melitensis* 115[i]	*B. abortus*, *B. melitensis*, *B. suis* and *B. neotomae*	Some *B. suis* strains	Lyses *B. melitensis*, *B. abortus* and *B. suis* rough mutants
Np (7)	Isolated from atypical *B. abortus* strains in Nepean (Canada)	*B. abortus* S19[d]	*B. abortus*	*B. neotomae*	Does not lyse rough mutants

[a]Phage stocks are maintained at the FAO/WHO Center for Brucellosis Reference and Research, OIE-Brucellosis Reference Laboratory Central Veterinary Laboratory, New Haw, Weybridge, Surrey KT15 3NB, England; some brucellaphages can be obtained from ATCC (http://www.atcc.org/). An exhaustive list of the brucellaphages is given in Ackerman et al. (1981).
[b]RTD is defined as the highest phage dilution producing confluent lysis on a lawn of the propagating strain. At 10^4 RTD, there is a "lysis from without" and no phage multiplication occurs.
[c]See Table 3.
[d]See 9.3.
[e]Bk0 was obtained by passage of Wb on *B. melitensis* Isfahan.
[f]A biotype 1 strain isolated in Isfahan (Iran).
[g]R/O was obtained from phage R by serial propagation on *B. ovis* 63/290; although it multiplies in some rough brucellas, some phages active on some smooth forms are produced. Phage R was obtained by simultaneous incubation of Wb, MC/75 and D with *B. abortus* 544 in the presence of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; it is also unstable, and although it multiplies on some rough mutants, some phages are active on smooth *B. abortus*. Phages MC/75 and D are Tb derivatives obtained after storage at 4°C in toluene for 5 years and by propagation on *B. abortus* 544 in the presence of mitomycin, respectively.
[h]A strain isolated in México.
[i]*B. melitensis* 115 (also known as B115) is a goat isolate that showed the rough phenotype on the isolation plates and not as a result of subsequent dissociation.
[j]n.d., no data available.
From Corbel and Thomas (1980); Rigby (1990).

available on other brucellaphages can be found in the review of Ackermann et al. (1981).

The first brucellaphages isolated were fully active only on *B. abortus* or on *B. abortus*, *B. suis* and *B. neotomae*, and since they proved useful for *Brucella* identification, derivatives active on *B. melitensis* and rough *Brucella* species were obtained from them under laboratory conditions. A phage (Iz) active on both S and R brucellae was isolated in the eighties. The brucellaphages active on smooth brucellae are often named after the place where they were isolated or obtained, whereas those active on rough brucellae are named using the letter R followed by the initial letter of the species used to adapt the parental phage to multiply in rough brucellae. They are usually grouped by their *Brucella* host range, with numbers that reflect the order in which they were isolated or developed (Corbel and Thomas, 1980a; Rigby, 1990).

Structural Characteristics The brucellaphages are footed viruses with a 50–80 nm diameter isometric icosahedral head carrying double-stranded DNA and a 10–30 × 7–9 nm long tail (morphological group C1 of *Podoviridae*). Tail fibers are apparent in some preparations. All brucellaphages tested are serologically related, and where determined (Tb and a few others not listed in Table 13), the DNA has a molecular weight of about 25×10^6, with a G+C content of 45.3–46.7% (Ackermann et al., 1981). Restriction endonuclease (*Eco*RI, *Hind*III, *Pvu*II, *Ava*I and *Bg*II) analyses have not shown major differences among DNAs from phages Tb, Fz, Wb, R/C and Iz, but the DNA of phage Np shows some differences when digested with *Eco*RI and *Hind*III (Rigby, 1990).

Multiplication *Host Range* The brucellaphages fail to replicate in a large variety of Gram-negative bacteria tested, including *Yersinia enterocolitica* O:9, *Salmonella* group N, *Vibrio cholerae*, *Stenotrophomonas maltophilia* 555 and *Francisella tularensis*, all of which produce LPS *O*-polysaccharides with structural similarities to that of smooth brucellae (Table 6). To date, there is no report indicating that the brucellaphages are not specific for brucellae. Thus, variations in host range are limited to those existing within the genus (Table 13).

Receptors Adsorption kinetics are similarly slow in all brucellaphages tested, with K values ranging from 0.3×10^{-10} to 4.9×10^{-11} ml/min depending on phage and host. The molecular nature of the receptors is not known. Although the rough-specific brucellaphages are derived from smooth-specific ones, the Tb, Fi, Wb and Bk2 phages (active on smooth brucellae) are not absorbed efficiently by rough strains or species. As far as it is known, the major difference between the surface of rough and smooth *Brucella* lays on the structure of the LPS and the exposure of the outer-membrane proteins (Omps) (Outer Membrane Topologyz), and this suggests that the LPS *O*-polysaccharide should be part of the receptor of Tb, Fi, Wb and Bk2. However, *Y. enterocolitica* O:9, which has an LPS *O*-polysaccharide structurally very similar to that of *B. abortus*, does not absorb any of those phages (Corbel and Thomas, 1980a), and this is also true for other bacteria with structurally related *O*-polysaccharides (Table 6). Absorption of phages Tb, Fi, Wb and Bk2 by smooth *B. melitensis*, *B. abortus*, *B. suis* and *B. neotomae* roughly corresponds to their species host range (Table 13) and occurs regardless of the intraspecies variations in *O*-polysaccharide structure associated with the distributions of the A and M epitopes among the biotypes (Table 3). Taken together, all these observations show that the fine structure of the smooth LPS is not relevant and that, in addition to LPS, some other and hitherto undetermined surface feature in which *B. melitensis* differs from other smooth brucellae plays a role in the receptor activity. In *B. neotomae*, the receptor has been associated with a thermoresistant periodate- and protease sensitive protein-phospholipid-LPS complex (Corbel and Thomas, 1980a).

Latent Period and Burst Size These parameters have been estimated to be 30–140 min and 22–121, respectively, depending on phage and host (Ackermann et al., 1981). In comparative experiments carried out under the same conditions, the values were 60–150 min and 8–26 (Corbel and Thomas, 1980a).

Use in Typing Owing to their narrow host-range, the brucellaphages are useful in the identification of *Brucella* at the genus and, along with other tests, species (Table 2) levels. For this purpose, phages Tb and R/C are routinely used. The strains to be tested are inoculated onto plates of an appropriate medium, such as serum-dextrose agar (Table 10), so as to produce a lawn, spotted with phage suspensions at the appropriate concentrations, and incubated. Detailed protocols for the maintenance and titration of brucellaphages can be found in the following references: Alton et al. (1988); Corbel and Thomas (1980a).

Preservation

For short-term preservation, *Brucella* cultures can be kept at 4°C and transferred periodically (in sealed tubes, they usually last longer than 6 months) on slopes of an appropriate medium,

provided precautions to minimize the establishment of laboratory-favored mutants are taken. For the smooth-to-rough dissociation, these precautions include avoiding the use of media with excessive moisture, incubation times as short as possible, and unnecessary holding of cultures at room temperature. Even so, many strains tend to dissociate, and in this case, the rough mutants can be eliminated by inoculation into suitable laboratory animals and recovery of the strain from spleens a few weeks later.

For long-term preservation, freeze-drying is the method of choice, keeping in mind the hazards posed by the aerosols that can be created during this kind of manipulation. Stabilizers described for *Brucella* include water solutions of 5% bovine serum albumin, 7.5% saccharose, 1% monosodium glutamate, or 2.5% enzymatic digest of casein, 5.0% sucrose, 1% monosodium glutamate (Alton et al., 1988). It is advisable to keep the freeze-dried bacteria at 4°C. At least *B. abortus* and *B. melitensis* can be maintained in skim milk suspensions at –80°C for more than 5 years, but the method is not suitable for *B. ovis*. *Brucella* stored on potato infusion agar slopes directly at –80°C survives for more than 5 years without significant loss of viability.

Physiology

The brucellae are chemoorganotrophic microorganisms that are able to grow both on lifeless media and within host cells (Habitat). As discussed elsewhere in this chapter, this behavior is based on a complex set of properties, and among them, some have a structural basis (Metabolism). Presumably, these bacteria are able to adapt their metabolism to the changing conditions they encounter in the external and intracellular environments. However, little is known on this important aspect of the biology of *Brucella*, and this is explained by both the experimental limits inherent to the work with highly virulent microorganisms and, until recently, the paucity of suitable genetic tools.

Structure

The brucellae are Gram-negative bacteria (Extracellular Enzymes and Uptake) and, therefore, their cell envelopes have inner- and outer-membranes enclosing a periplasm (Intermediary Metabolism) that contains the peptidoglycan mesh and some periplasmic soluble components. Free lipids (phospholipids and neutral lipids) are structural elements of the outer-membrane and inner membranes. In addition, the outer-membrane (Fig. 20) contains LPS (Response to Environmental Stress) and several Omps, and the inner-membrane contains proteins involved in substrate transport and other metabolic processes. Our knowledge of the topology of this cell envelope is hampered by the lack of adequate methods to physically separate the outer-and inner-membranes. These methods often include a step in which the outer-membrane is destabilized to be rendered either susceptible to detergent action or permeable to lysozyme. However, *Brucella* shows a marked outer-membrane stability that prevents the application of such methods and is also the probable reason for the failure of classical protocols to preferentially extract soluble periplasmic components. Despite these problems, a combination of chemical, genetic and morphological approaches has yielded a substantial amount of information on the *Brucella* cell envelope.

Ultrastructure of the Cell Envelope

Electron microscopy of *Brucella* shows the classical structure of the Gram-negative cell envelope with an outer-membrane of 6.5–8.0 nm and a cytoplasmic membrane of similar thickness, both separated by a periplasmic space of variable dimensions, possibly depending on the section examined and the fixation procedure (De Petris, 1964; Dubray, 1972; Dubray, 1975). Differences between smooth and rough *Brucella* cells can be observed by using phosphotungstic acid (Dubray, 1976). Positively charged stains and polycationic ferritin derivatives densely stain the surface of rough *Brucella* (Weber et al., 1977), possibly revealing that the negatively charged groups of the inner core of the LPS become exposed in rough cells (Fig. 12). Intracytoplasmic membranous structures have been reported (De Petris, 1964; Dubray, 1972). With differences among strains and species, exponentially growing *Brucella* cells release significant amounts of outer-membrane fragments enriched in several Omps (Gamazo and Moriyón, 1987; Gamazo et al., 1989). Although a capsule-like material associated with smooth cells (Vysotskii et al., 1967; Vysotskii, 1968) or reacting with anti-smooth LPS antibodies (Oberti et al., 1982) has been reported, the nature of the material has not been clarified.

Comparative studies show that the layer corresponding to the peptidoglycan is both thicker (5 versus 2–3 nm) and in an interaction with the outer-membrane much tighter in *Brucella* than in *Escherichia coli* (Dubray, 1976). In addition, it has been noted that whereas heat inactivation of cells causes the *B. abortus* cytoplasmic membrane to collapse, the outer-membrane keeps its morphological appearance despite release of important amounts of LPS and other outer-membrane materials (Rubbi, 1991). These observations suggest that the outer-membrane-

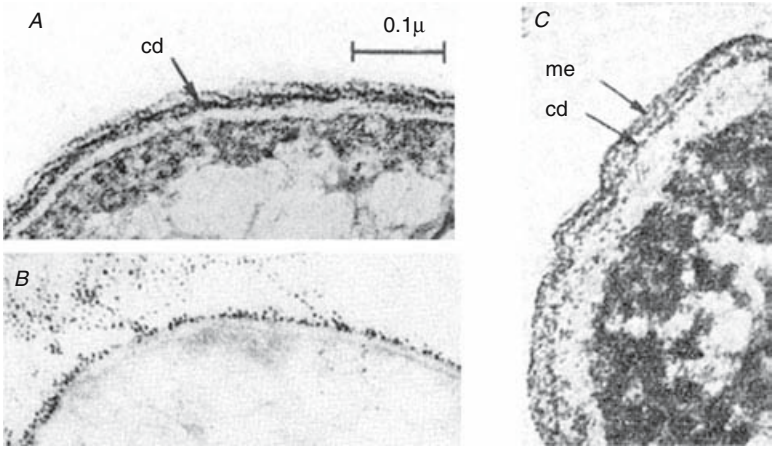


Fig. 12. Ultrastructure of *Brucella*. Thin sections of *B. abortus* 99 (A), *B. canis* (B), and *Escherichia coli* B (C) stained by the glutaraldehyde-uranyl acetate-lead citrate method (A and C) or with polycationic ferritin (B). Arrows mark the outer membrane (me) and the peptidoglycan layer (cd). Panel A adapted from Dubray (1975), panel B from Weber et al. (1977), and panel C from Dubray (1976), with permission.

peptidoglycan interaction could result in outer-membrane stiffness greater in *Brucella* than in other Gram-negative organisms. This could be related to the tight association of some Omps with the peptidoglycan (Sugar Catabolism and Tricarboxylic Acid Cycle).

Periplasmic Components

Peptidoglycan There is no detailed analysis of the structure of *Brucella* peptidoglycan or specific studies on its mechanism of synthesis. Based on qualitative analysis (Mardarowicz, 1966), the taxonomical position of the group, its sensitivity to muramidases and the action of antibiotics on *Brucella*, this peptidoglycan seems to be similar to that of most Gram-negative organisms. Although early studies reported quantitative and qualitative interspecies differences in peptidoglycan amino acid composition (Lacave and Roux, 1965), these data should be confirmed because *Brucella* peptidoglycan interacts with a variety of Omps difficult to remove (Omp-peptidoglycan Association). L-Forms devoid of peptidoglycan have been repeatedly observed and can be obtained with penicillin in vitro (Hatten et al., 1969) and from tissue cultures infected with *Brucella* (Elberg and Ralston, 1980; Hatten and Sulkin, 1966a; Hatten and Sulkin, 1966b) or isolated from infected animals treated (Schmitt-Slomska et al., 1981) or not treated (Corbel et al., 1980b) with antibiotics.

Soluble Components The *Brucella* periplasm contains comparatively large amounts of cyclic β-glucans (β-(1→2)-linked D-glucopyranose) made of 16 to 25 sugar units identical to some found in other members of the α-2 Proteobacteria (Moreno, 1992), such as *Agrobacterium*, *Rhizobium* and *Sinorhizobium* (Bohin, 2000). At least in these three bacteria, cyclic β-glucans synthesis is stimulated at low osmotic pressure, and it has been postulated that they take part in the regulation of the periplasmic colloid osmotic pressure. In *Brucella*, however, cyclic β-glucans synthesis is not osmoregulated (Iñ de Iannino et al., 2000), and they must accomplish a different function. Considering *Brucella* mutants defective in cyclic β-glucans are attenuated and show an altered intracellular trafficking, such function seems essential for full virulence (Escaping from the Endocytic Pathway) and may relate to the activity that closely related compounds, such as the methyl-cyclodextrins, has on animal cell membranes. Some authors have mistakenly equated these periplasmic glucans with polysaccharide B (see below) (Native Hapten Polysaccharides).

Two important detoxifying enzymes, a Cu^{++}/Zn^{++} superoxide dismutase (different from the cytosolic Mn^{++}-superoxide dismutase) and a catalase, have been identified in the periplasm of *B. abortus* (Kim et al., 2000; Stabel et al., 1994). Both enzymes protect against oxidative stress (Response to Environmental Stress), but their role in virulence is controversial (Neutralization of Oxygen and Nitrogen Intermediates). Other proteins identified in the periplasm include proteins BP26 (Immunological Diagnosis of Human Brucellosis Caused by Smooth *Brucella*; Rossetti et al., 1996) and P39, which are likely to be substrate-binding proteins involved in transport (De Fays et al., 1999).

The Free-Lipids The free lipids (i.e., the lipids that can be extracted with organic solvents from whole cells) include the overall cell envelope lipid composition and represent about 4.5% of the total cell dry weight. They have been the topic of an extensive review (Cherwonogrodzky et al., 1990) and their composition (Table 12) reflects the taxonomical position of *Brucella* (Fig. 4). There are interspecies differences in the

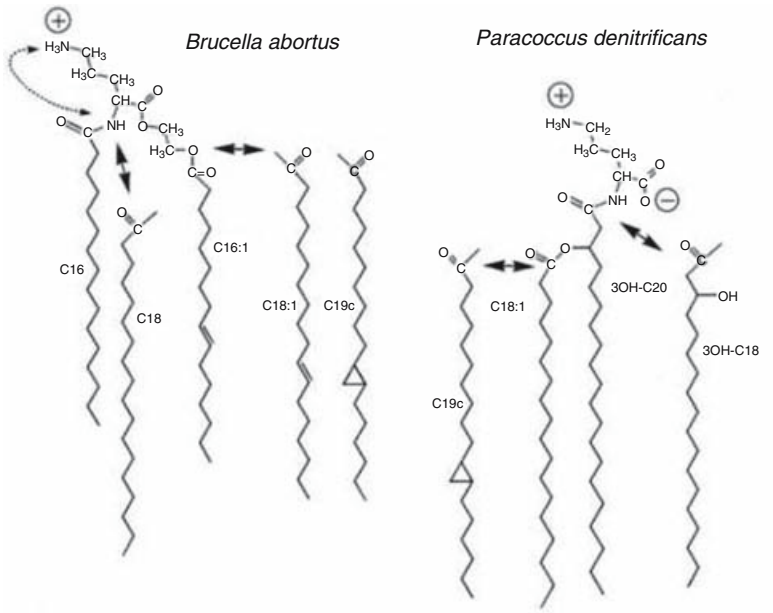


Fig. 13. Structures proposed for the ornithine-lipids of several Proteobacteria. Whereas *B. abortus* ornithine lipid carries an ester-linked acyl-ethyleneglycol (and is thus diacylated) and a positive net charge, other ornithine-lipids carry acyl-oxyacyl substitutions and are zwitterionic. Double arrows mark alternative acyl groups, and the dashed double arrow in the *B. abortus* ornithine-lipid marks an alternative acylation in the δ-amino group.

fatty acid profiles obtained by methanolysis of whole cells (Tanaka et al., 1977), and it is also known that the exact lipid composition depends on the medium and growth phase.

Phospholipids Although the precise lipid composition depends on growth medium and age of the culture, the major *Brucella* phospholipid is always phosphatidylcholine (Outer Membrane Topology) or its immediate biosynthetic precursors. Phosphatidylethanolamine is in close proportion to phosphatidylcholine, and cardiolipin and phosphatidylglycerol are also present in significant amounts. The dominant fatty acids are C16, usually at position C1 of glycerol, and C18:1 and C19 cyclopropane (lactobacillic acid), mostly at C2.

Neutral Lipids *Brucella* cells contain a substantial amount (over half of the total free lipids) of neutral lipids of which the ornithine lipids are particularly remarkable. These ornithine lipids differ from those described in other bacteria, are ester-linked to an acylated ethylene glycol residue (Figs. 13 and 14) and should carry a positive charge under most conditions (Outer Membrane Topology). Other neutral lipids described in *Brucella* include mono-, di- and triglycerides and other acyl-esters.

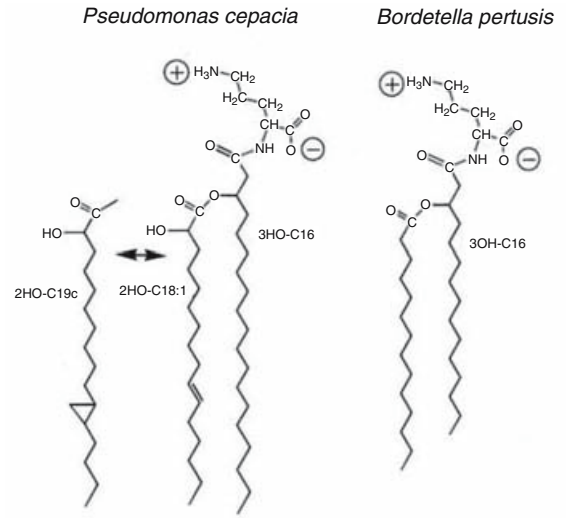


Fig. 14. Structures proposed for the ornithine-lipids of several Proteobacteria (second part). See first part in Fig. 13. Whereas *B. abortus* ornithine lipid carries an ester-linked acyl-ethyleneglycol (and is thus diacylated) and a positive net charge, other ornithine-lipids carry acyl-oxyacyl substitutions and are zwitterionic. Double arrows mark alternative acyl groups, and the dashed double arrow in the *B. abortus* ornithine-lipid marks an alternative acylation in the δ-amino group.

The Lipopolysaccharide *Brucella* is unique among Gram-negative bacteria in that some species have smooth-type LPS and others have naturally rough-type LPS (i.e., lipo-oligosaccharides; Table 2) different from the LPS of the rough mutants of the smooth types. The *Brucella* LPS have not been completely characterized. Presumably, the smooth- and rough-type LPS of *Brucella* have a closely similar lipid A structure and differ mostly in the saccharide moiety. *Brucella* LPS extracts are constituted by a

heterogeneous group of LPS molecules (Freer et al., 1995). This heterogeneity arises from the different length and different degree of acylation of the O polysaccharide, from the different acylation of the lipid A moiety and possibly from various substitutions of the core oligosaccharide.

Lipid A The structures proposed for the lipid A of *B. abortus* and *O. intermedium* (Velasco et al., 2000; Zähringer et al., 1995) are shown in Fig. 7. The more remarkable features are i) the 2,3-dideoxy, 2,3-diaminoglucose (diaminoglucose) rather than 2-deoxy, 2-aminoglucose (glucosamine) backbone found in classical lipid A, ii) the fact that all acyl substitutions on the disaccharide are in amide linkages (rather than in both amide and ester linkages), iii) and the long chain acyl substituents, which include not only C18:1 and C19 cyclopropane but also 27-OH-C28:0 and minor amounts of 29-OH-C30:0, 27-Keto-C 28:0, and 29-Keto-C30:0. It is remarkable that this structure shows a basic similarity with that of *Legionella pneumophila* (Fig. 7), a taxonomically distant bacterium that shares with *Brucella* the acid-fastness and the ability to multiply within host cells.

Core Oligosaccharide The qualitative composition of the *B. abortus* LPS core can be inferred from partial chemical analyses and some genetic evidence (Table 5) but the detailed structure is not known. Sugars present in the core oligosaccharide of the *Brucella* smooth-LPS are 3-deoxy-D-manno-octulosonic acid (Kdo), glucosamine, 2-amino, 2,6-dideoxy-D-glucose (quinovosamine), glucose and mannose (Velasco et al., 2000). Like the LPS of other members of the α-2 Proteobacteria, the LPS core oligosaccharide of *Brucella* lacks heptose and phosphates. It is interesting to compare this core oligosaccharide with that of *O. intermedium* (Fig. 6). The comparison shows that in *O. intermedium*, but not in *B. abortus*, LPS contains galacturonic acid, which would carry a negative charge under physiological conditions. Galacturonic or glucuronic acids are present in the LPS core of other α-2 Proteobacteria examined, and therefore its absence in *Brucella* is remarkable (Lipopolysaccharide and Resistance to Outer-membrane Destabilizing Agents; Outer Membrane Versus Bactericidal Substances). Two epitopes have been described in the core oligosaccharide of *B. abortus* R mutants: R1, which includes the outermost sugars, and R2, which includes Kdo and sugars immediate to it (Rojas et al., 1994; Fig. 15).

O-polysaccharide The *O*-polysaccharides are synthesized by the smooth but not by the rough *Brucella* species (Table 2). This structure is also missing in the surface of the rough mutants of the smooth species that appear spontaneously during culture dissociation (Colonies and Dissociation). They are homopolymers of 4,6 dideoxy-4-formamido-D-mannose (*N*-formylperosamine; Perry and Bundle, 1990b). Their basic structure is represented by the *O*-polysaccharides of *B. abortus* biovar 1 and *B. melitensis* biovar 1 in which *N*-formylperosamine is in exclusively α-(1→2) linkages or in repeating blocks of five sugar residues, four α-(1→2)-linked and one α-(1→3)-linked, respectively (Fig. 16). The bacteria that significantly crossreact with smooth brucellae at LPS level carry *O*-polysaccharides that contain perosamine (Table 6).

Like in other smooth-type LPS, the *O*-polysaccharides show molecular weight heterogeneity within a given strain, and there is heterogeneity in average length among strains. The average number of 96 sugar units has been estimated for the *O*-polysaccharide of *B. abortus* 1119-3. From the molecular weight estimated by gel permeation chromatography, a range of 25–92 and of 35–130 sugar units can be calculated for the *O*-polysaccharides of *B. abortus* 2308 and *B. melitensis* 16M, respectively. *N*-formylperosamine has just one free hydroxyl group, and this peculiarity makes these smooth-LPSs phenol-soluble in opposition to most smooth-LPSs that partition into water when extracted by the hot water-phenol protocol. However, *N*-formylperosamine polysaccharides have a dual character, since they are also readily soluble in water where they show a marked tendency to self-aggregate at low ionic strength (Aragón et al., 1996b).

The smooth-LPS is the major antigen of the cell surface of all smooth *Brucella* species, and its *O*-polysaccharide is the serologically immunodominant section. Smooth brucellae show strong interspecies serological crossreactivity. Studies with monoclonal antibodies show that biovar 1 of *B. abortus* and *B. melitensis* carry the A (abortus) and M (melitensis) epitopes, respectively, and that there is a common *Brucella* (C/B) epitope (Douglas and Palmer, 1988; Fig. 15). There are three combinations of these epitopes (A-C/B, M-C/B and A-M-C/B) distributed among the different biovars of smooth brucellae in a nonspecies-specific manner (Table 3). Similar studies show a second common epitope (C/Y) shared by all the smooth brucellae and by *Yersinia enterocolitica* O:9 whose *O*-polysaccharide is also a homopolymer of *N*-formylperosamine in a-(1→2) linkages (Table 6). Although conceptually useful, the A-M-C epitopic scheme is a simplification because there are antibodies that recognize more than one of those epitopes, although with different intensity (Weynants et al., 1997). Thus, in polyclonal antibody responses, such as those occurring

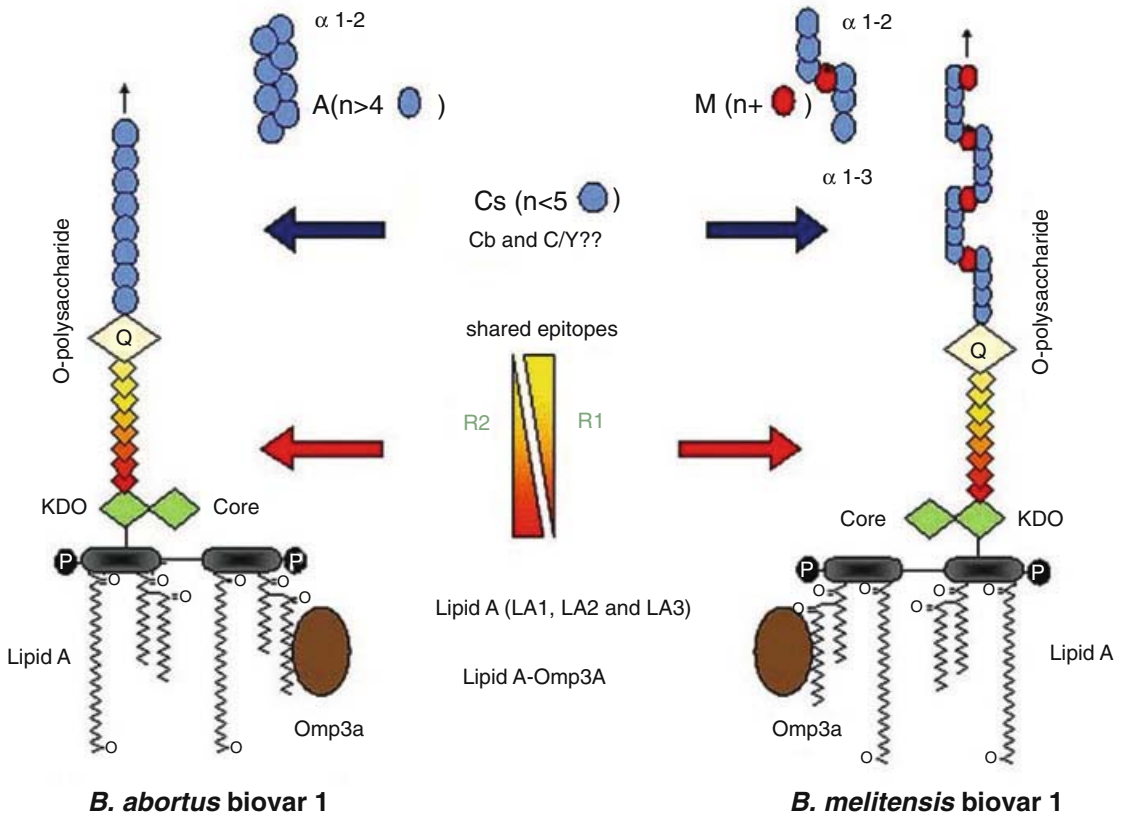


Fig. 15. Simplified epitopic structure of the smooth LPS of *B. abortus* and *B. melitensis*. Sugars in α (1-2) and α (1-3) linkages are represented in light blue or red, respectively. The *O*-polysaccharide A and M epitopes are specific of *B. abortus* (biovar 1) and *B. melitensis* (biovar 1), respectively. Shared epitopes are the *O*-polysaccharides C epitopes, the outer (R1) and inner (R2) core epitopes, the lipid A epitopes (LA1, LA2 and LA3) and some epitopes created by the interaction of the rough LPS with some Omps (possibly Omp3a [Omp25]). The beginning of the core (K do linked to the lipid A) is marked as a green diamond, while the end of the core (quinovosamine linked to the *O*-polysaccharide) is represented by a light yellow diamond (Q) structure.

during infection, the *Brucella O*-polysaccharides behave as structures made of overlapping epitopes, and this contributes to the intensity of the crossreactivity among smooth brucellae. These considerations are relevant to understand the serological diagnosis of brucellosis (Immunological Tests for Brucellosis).

The epitopic structure of the *O*-polysaccharide is explained by the relative frequency of α-(1→2) and α-(1→3) linkages. The α-(1→2) linkages relate to both the A and C epitope(s) and the α-(1→3) linkages to the M epitope, and intermediate biovars expressing both the A and M-type epitopes (such as *B. melitensis* biovar 3) and M-type *B. abortus* biovars have the expected proportions of α-(1→2)- and α-(1→3)-linked sugars. Indeed, the structural similarities between the A- and the M-type homopolymers also explain the concept of overlapping epitopes. On the other hand, the structural details accounting for the differences between the C/Y and C/B epitopes are not known. According to the ^{13}C-nuclear magnetic resonance analyses performed so far, the *O*-polysaccharides of *B. abortus* and *Y. enterocolitica* O:9 are indistinguishable, and this would leave no room for a C/B epitope carried by the *O*-polysaccharides of smooth-brucellae but not of *Y. enterocolitica* O:9. Since there is evidence that the smooth brucellae carry perosamine polysaccharides (Physiology of CO_2 Requirements) in which not all amino groups are formylated (M. Staaf et al., unpublished observations) a possibility is that differences in the degree of *N*-formylation not detectable by conventional ^{13}C-nuclear magnetic resonance methods account for the C/B and C/Y epitopes.

The Lipooligosaccharide of Rough Brucella Species *Brucella ovis* and *B. canis* carry lipooligosaccharides (naturally rough-LPS) in their outer-membranes rather than smooth-type LPS (Table 2). Our present knowledge of these rough LPSs is rather incomplete. *Brucella ovis* rough-LPS contains Kdo and up to 7 additional sugars, including glucose, mannose, glucosamine and

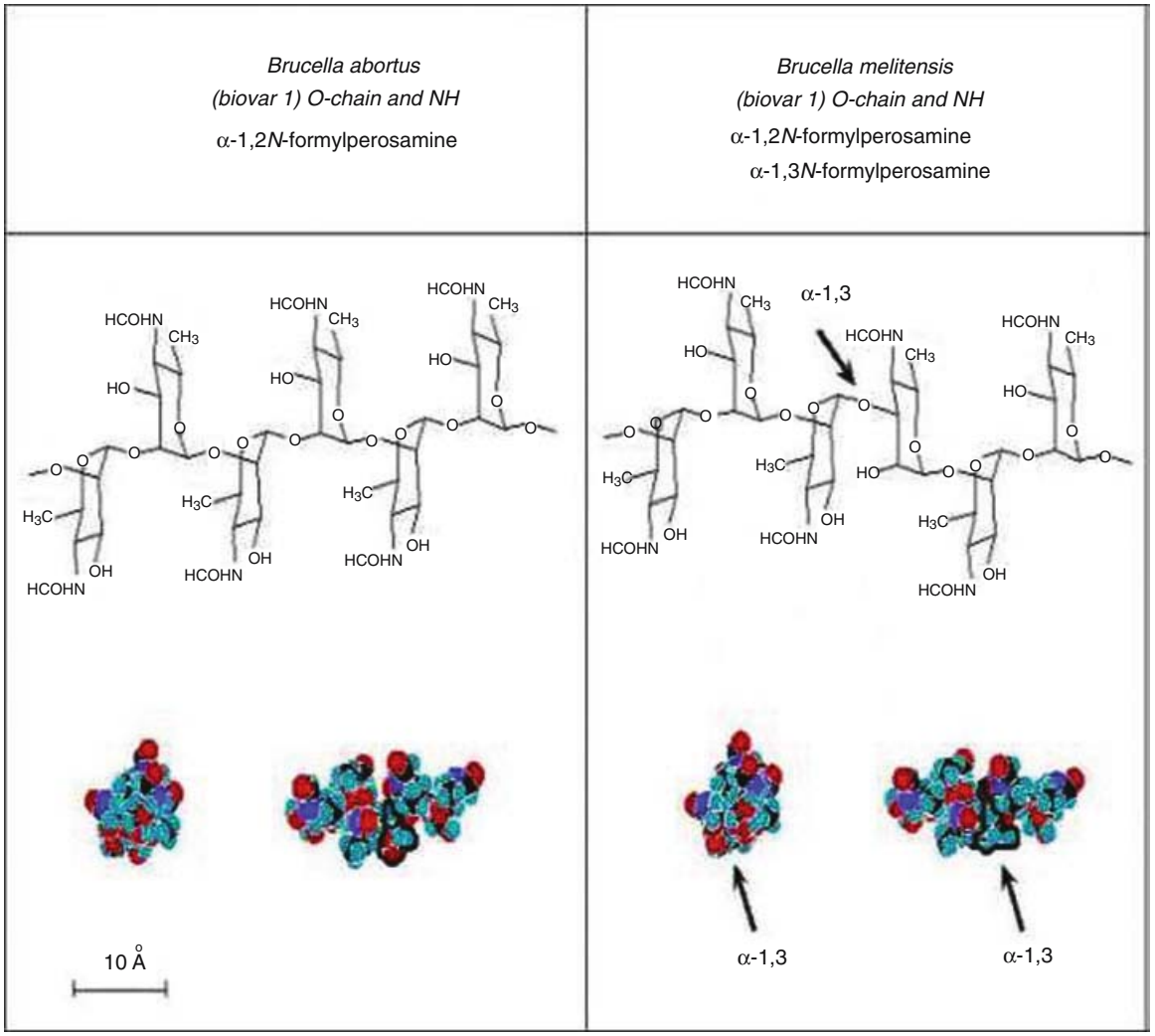


Fig. 16. Tridimensional and structural model of the *O*- and NH-polysaccharide molecules of *B. abortus* (biovar 1) and *B. melitensis* (biovar 1). Notice how the α 1,3 linkages interrupt each string of four α 1,2 linked *N*-formylperosamine residues in the *B. melitensis* homopolymer, unlike the continuity of the α 1,2 linkages observed in the *B. abortus* polysaccharides. Although formyl groups are substituents on most perosamine residues, the degree of formylation varies among the different homopolymer polysaccharides (*O*-chain > NH > Poly B).

unidentified sugars (Moreno et al., 1984a; Suárez et al., 1990). This short oligosaccharide stub bears the antigenic determinants of the *B. ovis* rough-LPS, which include epitopes shared with the LPS-core of smooth-*Brucella* species and *B. canis*, as well as *B. ovis*-specific epitopes (Díaz and Bosseray, 1973a; Moreno et al., 1984a). Likewise, *B. canis* rough-LPS seems to carry their own antigenic determinants (Carmichael, 1990). Although *B. ovis* and *B. canis* produce only rough-LPS, it is remarkable that they contain genes required for the synthesis of the *O*-polysaccharide (Table 5).

Genetics and Biosynthesis of Lipopolysaccharide Little is known on the biosynthesis of *Brucella* LPS. Indirect evidence shows that the three main pathways (lipid A, core and *O*-polysaccharide) used by the best studied Proteobacteria are as expected present in *Brucella*. Several genes involved in LPS biosynthesis have been identified, including those involved in the synthesis of perosamine and its formylation, polymerization and transport of the *O*-polysaccharide (Table 5). Six of these genes are most likely in a single operon. Interestingly, the G+C content of the open reading frames likely to be involved in *O*-polysaccharide synthesis is lower (44–49%) than that of the whole genome of *Brucella* (56–58% for *B. melitensis*; Godfroid et al., 2000). This suggests that these genes could have been acquired by horizontal transfer, and indeed, *O*-polysaccharides close to or identical to those of *Brucella* are found in some phyloge-

Table 14. Some biological activities of the lipopolysaccharides of *Brucella* and *E. coli*.

Effect	μg[a]		Reference
	Brucella LPS	*E. coli*/ *S. enterica* LPS	
LD_{50} in C3H/HeJ mice[b]	70	>500	Cherwonogrodzky et al., 1990
Chick embryo lethality	10	0.003	Cherwonogrodzky et al., 1990
Local Schwartzman reaction	>1000	20	Cherwonogrodzky et al., 1990
Pyrogenicity in rabbits	>300	0.15	Cherwonogrodzky et al., 1990
Lymulus lysate gelation	0.001	0.002	Cherwonogrodzky et al., 1990
Inactivation by polymyxin B	no	yes	Cherwonogrodzky et al., 1990
Anti-C′	50 (weak)	20 (strong)	Cherwonogrodzky et al., 1990
Oxydative burst in polymorphonuclear neutrophiles	200	0.2	Rasool et al., 1992
Macrophage toxicity	>200	2	Rasool et al., 1992
Maximal induction of:			
IFN-γ	200	50	Goldstein et al., 1992
IL-1β	≥10	0.007	Goldstein et al., 1992
TNF-α	≥10	0.023	Goldstein et al., 1992

Abbreviations: LPS, lipopolysaccharide(s); C′, complement; IFN, interferon; IL, interleukin; and TNF, tumor necrosis factor.
[a]μg causing the indicated effect.
[b]Carrageenan-treated endotoxin-resistant mice.

netically distant bacteria (Table 6). Since the genetic and ecological characteristics of the brucellae suggest that they presently have little opportunities for such exchanges (Genetic Exchange, Plasmids and Lysogenic Phages), acquisition of *O*-polysaccharide genes could have been an early event in the evolution of the genus (Acquisition of Ancestral Genes by Horizontal Transference). This is in keeping with the structural homogeneity of the *N*-formylperosamine homopolymers and with the presence in *B. ovis* and *B. canis* of genes involved in its synthesis (Cloeckaert et al., 2000).

LPS Biological Activities Lipid A is the structure directly involved in the endotoxin-related properties of LPS. Since the lipid A of *Brucella* has a structure that clearly departs from that of the classical enterobacterial-type lipid A, it is not surprising that its LPS shows lower biological activities (Table 14). In general, the lower endotoxicity of *Brucella* LPS is manifested as a marked reduction in toxicity linked to a reduced ability to stimulate susceptible host cells to release immune mediators. Indeed, *Brucella* LPS is from 200 to 2,000 times less active than *Salmonella* LPS (Fig. 17). *Brucella abortus* LPS activates the complement system poorly by the alternative pathway, and the *O*-polysaccharide has been shown to be involved in this phenomenon (Fig. 18). Both reduced endotoxicity and poor complement activation contribute to virulence (Opsonization and Complement Susceptibility). There is no evidence of interaction between the *O*-polysaccharide of *B. abortus* and collectins (mannose-binding protein and surfac-

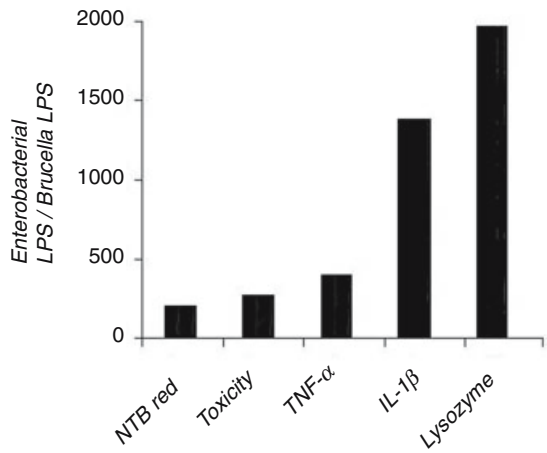


Fig. 17. Comparison of the biological activities of *Brucella* LPS with *S. typhimurium* and *E. coli* LPSs in phagocytic cells. The concentration of *Brucella* LPS at which the maximum biological effect is generated was recorded and compared with the concentration of *S. typhimurium* or *E. coli* for the same effect. Respiratory shunt in human polymorphonuclear leukocytes was measured as reduction of nitro-blue tetrazolium (*NTB-red*); maximum level of toxicity (*Toxicity*) reached with *B. abortus* LPS was less that 10% at 500 mg/ml $\times 10^6$ macrophages; TNFα production (*TNF-α*) in murine macrophages; IL-1β production (*IL-1β*) by murine macrophages; and lysozyme release (*Lysozyme*) by human polymorphonuclear leukocytes. Notice that *Brucella* LPS is from 200 to 2,000 times less potent than *Salmonella* or *Escherichia* LPSs. Adapted from Rasool et al. (1992); Goldstein et al. (1992).

tant proteins), although *B. abortus* has been shown to bind to human B cells and macrophages by a lectin-type mechanism (*Brucella* Bacterins and Lipopolysaccharide as Immunological Tools).

NATIVE HAPTEN POLYSACCHARIDES These are haptenic polysaccharides that can be demonstrated in extracts of smooth *Brucella* by immunoprecipitation with sera from *B. abortus*- or *B. melitensis*-infected animals in the presence of hypertonic buffers (Fig. 19). The *B. melitensis* 115 rough mutant is unable to produce smooth-LPS but also accumulates a similar compound (named "polysaccharide B") in the cell envelope and its phenotype is close to that of LPS *wzm* mutants (Table 5). Both NH and polysaccharide B are *N*-formylperosamine homopolymers with a frequency of α-(1→2) and α-(1→3) linkages similar to that of the *O*-polysaccharide (Fig. 16) of the strain from which they are isolated (Aragón et al., 1996b). However, in contrast to the *O*-polysaccharide of LPS, neither polysaccharide B nor NH is linked to core oligosaccharide sugars and both are partially (up to 50%) deformylated (M. Staaf et al., unpublished observations). This last feature is intriguing because polymerization of at least *B. melitensis* LPS *O*-polysaccharides is blocked by disruption of the gene coding for the putative formyltransferase (Godfroid et al., 2000; Table 5). NH and polysaccharide B may represent biosynthetic precursors of the *O*-polysaccharide of smooth-LPS, but NH also has been demonstrated in the outer-membrane fragments that are released spontaneously by growing *B. melitensis*. Thus, NH is in the outer-membrane as a structure that, although similar to the *O*-polysaccharides, is not linked to core-lipid A (Outer Membrane Topology). This type of polysaccharide was described long ago in several Gram-negative bacteria (Anacker et al., 1964) and it is known as their presence in the outer-membrane results from variations in the standard polymer-lipid A-core ligase reaction (Whitfield et al., 1997).

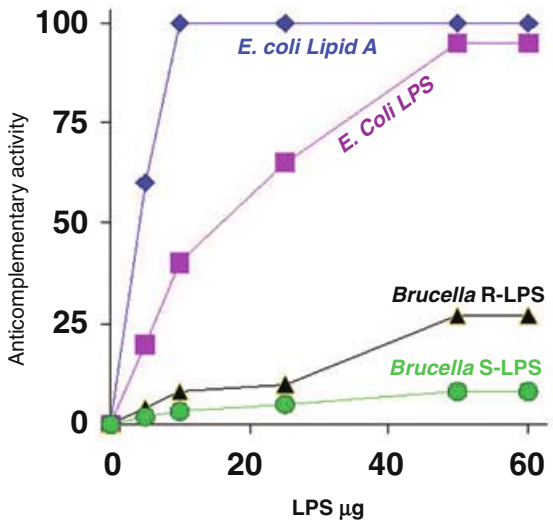


Fig. 18. Anticomplementary activity of LPS and lipid A. Increasing amounts of each preparation were incubated with guinea pig complement and percentage loss of hemolytic activity was measured. Notice that *B. abortus* smooth and rough LPSs exert low anticomplementary activity only at high concentrations of material, whereas *E. coli* lipid A and *Salmonella* LPS display a powerful anticomplementary activity. Adapted from Moreno et al. (1981), with permission.

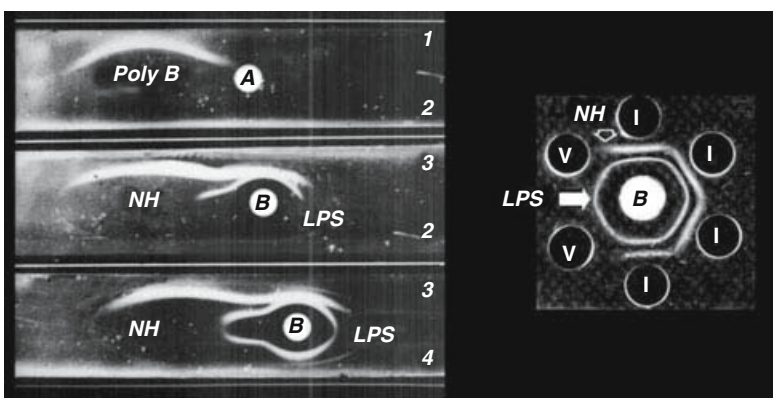


Fig. 19. Immunoprecipitation analysis of *Brucella* polysaccharides and lipopolysaccharides. Left panel: immunoelectrophoresis of rough *B. melitensis* 115 polysaccharide B (A); and *B. melitensis* extracts containing native hapten (NH) and smooth LPS (B). The troughs contained sera from infected cattle either plain (1 and 3) or absorbed with smooth *Brucella* cells (2) or polysaccharide B (4). Right panel: Double gel immunodiffusion of *B. melitensis* extracts containing NH and smooth LPS and sera from infected (I) or *B. abortus* strain 19 vaccinated (V) cattle. Notice that while infected bovines react with both LPS and NH, vaccinated animals only react against the LPS, with no traces of reaction against the NH.

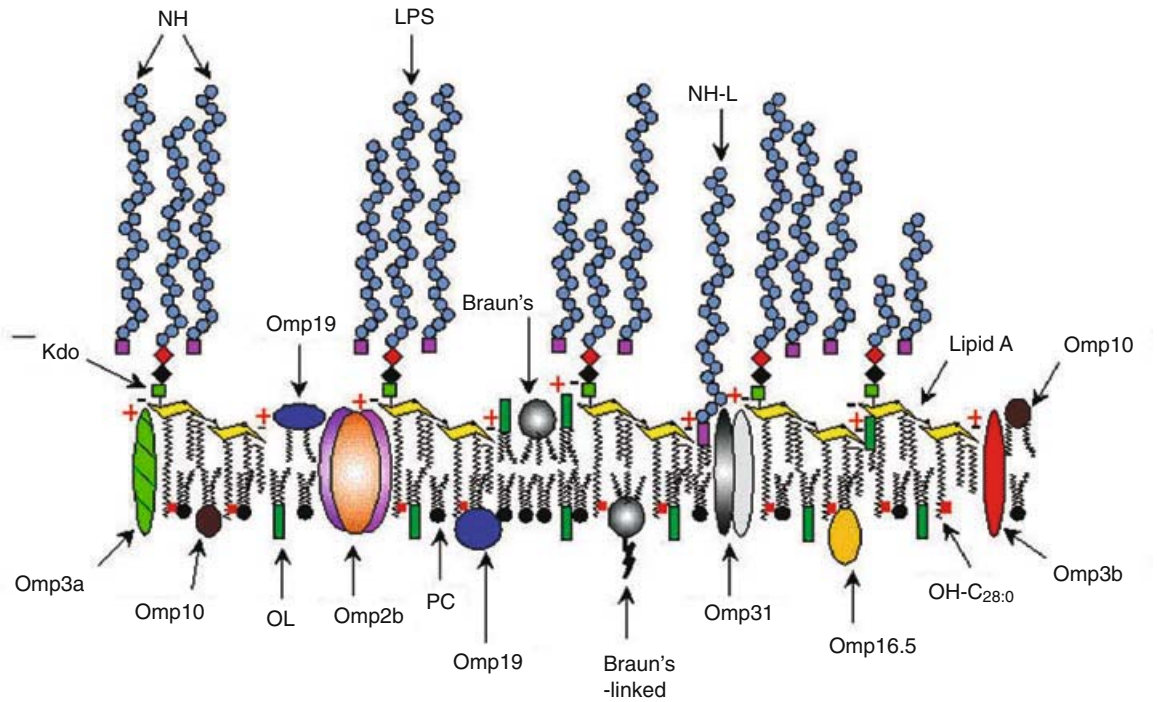


Fig. 20. A hypothetical model of the *Brucella* outer membrane. Omps are indicated by their mass in kDa, except for two group 3 proteins (Omp3a and Omp3b) with molecular weights of 26 kDa and 23 kDa, respectively. Lipoproteins have their acyl groups inserted in the bilayer. The lipid A diaminoglucose disaccharide backbone (represented by yellow trapezoids) is linked to fatty acids (black zigzag structures). The red squares in the bottom of the structure mark the hydroxyl groups of the LPS acyl chains that span the outer membrane. Purple squares mark the reducing ends of the NHs, an unknown sugar that in some cases may be linked to a lipid (NH-L). Braun's lipoprotein may be linked to peptidoglycan or free in the outer membrane. The polar heads of ornithine lipids (OL) are marked as green rectangles. Dark circles represent phospholipids, mainly phosphatidylcholine (PC). Notice that the NH of various sizes are intertwined with the *O*-polysaccharides of the LPS in the outer membrane, forming a dense layer. The negative charges of the LPS are neutralized by the positive charges of the ornithine lipids (OL) and cationic Omp such as Omp3b. Group 3 proteins (Omp3a [Omp25], Omp3b and Omp31) are strongly bound to LPS. Omp2b is a porin.

Outer Membrane Proteins The major Omps of *Brucella* were first identified by detergent extraction of cell envelopes and classified according to their apparent molecular weights as group 1 (94 or 88 kDa), group 2 (36–38 kDa), and group 3 (31–34 kDa and 25–27 kDa). Moreover, a lipoprotein linked to the peptidoglycan and three other lipoproteins have been identified in the cell envelope. Group 2 are porins and group 3 contains at least three unrelated proteins (Omp31, Omp3a or Omp25, and Omp3b), but the nature and role of group 1 remains to be elucidated (Fig. 20). Presently, little is known about the Omps of the *Brucella* isolated from marine mammals, and the data summarized in the following sections concern only the six classical species. Most information concerning the *Brucella* Omps has been reviewed by Cloeckaert et al. (1996b).

Porin Proteins The brucellae carry two related porin genes (*omp2a* and *omp2b*) with a high degree (>85%) of internal homology but with little homology to the genes of other porins described so far. This *omp2* locus is present in all *Brucella* species and is highly polymorphic at both *omp2a* and *omp2b*. This locus contains some species-specific sequences, specific markers for several of the classical biovars, and other sorts of intraspecific diversity (Fig. 4), including natural Omp2a-Omp2b chimeric proteins (Paquet et al., 2001). Thus, sequence analysis of the *omp2* genes has also taxonomical and phylogenetic implications (Table 4). Most of these variations are created by simple exchanges of conserved motifs between *omp2a* and *omp2b* and, in general, seem to have little impact on the antigenic variability of the proteins. The cognate proteins are predicted to be folded as a 16 stranded β-barrel, and most differences seem accumulated at the external loops (Paquet et al., 2001). At least Omp2b is in a trimeric state in the outer- membrane and behaves as a peptidoglycan-associated protein (Fig. 20). Obvi-

ously, *Brucella* porins span the outer-membrane and contribute to its permeability. In vitro, *B. abortus* only expresses Omp2a, and Omp2b may be expressed under other conditions. The pattern of expression in other *Brucella* species is not known.

Omp31 The *omp31* gene is present and expressed in all *Brucella* classical species but *B. abortus*, which carries a long 8-kb deletion encompassing this gene and others of unknown function (Vizcaíno et al., 1997). Gene *omp31* shows only little homology with *omp3a* (*omp25*) or *omp3b*, the other two groups of genes coding for a group 3 protein. Omp31 shows a tight interaction with LPS and is associated with peptidoglycan (Outer Membrane Topology). These features suggest that this protein plays a structural role, but if so, its absence from *B. abortus* suggests that it is not essential. Omp31 is able to form oligomers resistant to denaturation, and since this is a property characteristic of some porins, it has been suggested that it may play this role (Fig. 20).

Omp3a and Omp3b Family Two different groups of Omps, Omp3a (formerly Omp25) and Omp3b, are composed of 8 beta sheets and are related to RopA and RopB proteins of *Rhizobium* (Fig. 20). These two protein families have a molecular weight ranging from 26–23 kDa and each group displays from 5 to 8 spots in two dimensional gels (C. Guzmán-Verri et al., unpublished results). Several putative genes coding for these proteins have been identified in the *Brucella* genome, supporting the two-dimensional gel findings and supporting that they constitute a family of Omps with unknown functions. Similarly to Omp31, they show characteristically tight interaction with the LPS and are also associated with the peptidoglycan (Outer Membrane Topology). Omp3a is highly conserved in *Brucella* species and the only remarkable variation is a 36-bp deletion at the 3' end of the *B. ovis omp3a* gene that causes an antigenic shift in the Omp3a of this species. Although both families may play a structural role, this does not exclude additional functions in virulence, since at least in *B. abortus*, its expression in the outer-membrane seems to be regulated by the BvrR-BvrS system (Mechanisms of Entry to Host Cells).

Lipoproteins The *Brucella* cell envelope contains three proteins (Omp19, Omp16.5 and Omp10) with genetic features characteristic of bacterial lipoproteins and which have been shown to incorporate palmitic acid (Tibor et al., 1999). These lipoproteins are similar to the peptidoglycan-associated lipoproteins of other Gram-negative bacteria (Outer Membrane Topology) and crossreact with homologous proteins of other members of the α-2 Proteobacteria. An additional lipoprotein of overall characteristics similar to those of Braun's lipoprotein has been shown in *B. abortus*, *B. melitensis* and *B. ovis* (Gómez-Miguel and Moriyón, 1986), but the corresponding gene has not been identified (Fig. 20).

Outer Membrane Topology Like in other Gram-negative bacteria, the LPS is located in the outer leaflet of the outer-membrane anchored by its lipid moiety (Fig. 20). The very long fatty acids of the lipid A are likely to span the outer-membrane and to be anchored on the inner surface by the hydroxyl group close to the end of the acyl chain. The phosphate groups in lipid A and the Kdo carboxyl groups of the core may be associated with ornithine lipids, because the latter lipids appear as contaminants in LPS extracts and have a free amino group with a positive charge (Freer et al., 1996). With regard to the phospholipids, there is evidence that phosphatidylcholine is preferentially distributed in the outer-membrane (Gamazo and Moriyón, 1987), perhaps in its inner leaflet. In the smooth strains, the *O*-polysaccharide extends outwards, in association with the NH. This last molecule is maintained in the outer-membrane intertwined with the LPS *O*-polysaccharide or linked to a lipid other than lipid A (Rojas et al., 1994).

The presence or the absence of the *O*-polysaccharide profoundly modifies the topology of the surface of *Brucella* cells. First, it masks the ionic groups of the proteins and the core-lipid A LPS sections. Since the latter sections are dominant, mutants lacking the *O*-polysaccharide have a negatively charged surface at physiological pH, which contrasts with the very weak charge carried by smooth cells (Schurig et al., 1981; Weber et al., 1978). This difference is the basis of some tests used to distinguish smooth strains from their rough mutants (Cellular and Colonial Characteristics). Second, access to the anionic groups of the lipid A-core is sterically hindered in the smooth strains and this contributes to resistance to polycations (Properties of the Outer Membrane). Finally, there are differences in the surface exposure of Omps between the smooth *Brucella* species on the one hand and the rough mutants and *B. ovis* and *B. canis* on the other. In general, these differences are manifested as a comparatively reduced Omp accessibility to antibodies in the smooth strains. However, not all smooth strains are identical in this property, and the variations reflect at least in part different degrees of *O*-polysaccharide polymerization and also the total amount of smooth LPS produced. In some smooth strains studied in detail, Omp accessibility to antibodies is par-

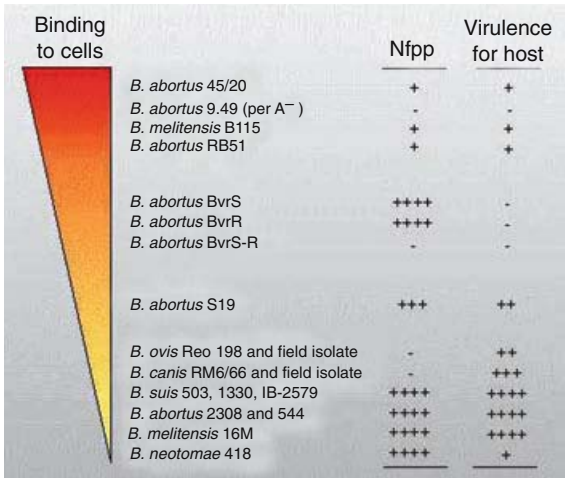


Fig. 21. Correlation among *Brucella* strains binding to epithelial cells, presence of *N*-formylperosamine polysaccharides (Nfpp) and virulence. Values are ranked from negative or absent (–), weak positive or present in low quantities r (+) to strong positive or present in large quantities (++++). The base of the triangle is compatible with the high number of bacteria binding to cells, whereas the apex of the triangle is compatible with the low number of brucellae binding to cells. Notice the absence of correlation between presence of Detilleux et al. (1990a, b); Freer et al. (1999); and Pizarro-Cerdá (1998).

alleled by an increase in both sensitivity to the R/C (rough-specific) *Brucella* phages (The Brucellaphages) and exposure of LPS core-lipid A epitopes. However, not all rough mutant strains are identical in Omp antibody accessibility, and differences between *B. ovis* and *B. canis* also have been observed (Bowden et al., 1995). Interestingly, these differences correlate well with the differences observed between the "natural rough" and rough mutants in the attachment and invasion of cells (Fig. 21).

Several Omps behave as peptidoglycan-associated proteins. If molecular weight, surface exposure and association with the peptidoglycan are used as criteria, porins, Omp31 and Omp3a are membrane-spanning proteins. The topology of Omp19, Omp16.5 and Omp10 proteins is probably more complex. They are accessible to antibodies on the surface of rough strains, and although according to the predicted amino acid sequence are hydrophilic, they also carry a lipid moiety and are extracted with detergents. Their similarities with the peptidoglycan-associated lipoproteins of other Proteobacteria suggest that they could be in the periplasmic space and play a role in the interaction of the outer-membrane with the peptidoglycan but they should also be anchored on the cell surface. The Braun's lipoprotein of *Brucella* seems to be covalently attached to the peptidoglycan but it also has been reported to be surface exposed, perhaps as a free form (Fig. 20).

Properties of the Outer Membrane Some simple observations, such as the inability to grow in media containing hydrophobic dyes, the ability to grow on selective media containing polymyxin B (Table 11), and the acid-fastness when stained by Stamp's method (Fig. 10) (Cellular and Colonial Characteristics), show that the brucellae have cell envelopes with a characteristic set of properties, some of which reflect, in all likelihood, an adaptation to intracellular parasitism (Outer Membrane Versus Bactericidal Substances).

Permeability Nutrients or precursors must passively cross the outer-membrane before they are taken up by the periplasmic and membrane proteins engaged in transport and translocation through the cytoplasmic membrane. Some early observations on the oxidation of glutamate by resting cells suggest that there is a link between outer-membrane permeability and virulence (Oxidation of Amino Acids). This could occur as a result of subtle differences in outer-membrane topology indirectly causing alterations in the pathways described (The Hydrophilic Pathway). There are two possible pathways for substrates to penetrate through Gram-negative outer-membranes and both are accessible in *Brucella* cells.

The Hydrophilic Pathway Some small hydrophilic molecules penetrate the outer-membrane of *Brucella* through Omp2 porins (Douglas et al., 1984). In classical functional assays, porins of *B. abortus*, *B. melitensis* and *B. canis* show an apparent internal diameter of about 1.2 nm, comparable to that of the OmpF porin of *E. coli*. At least for *B. abortus*, this result should correspond to Omp2b, since Omp2a is not expressed in vitro. However, cloned *B. abortus* Omp2a increases the outer-membrane permeability of *E. coli* to maltodextrins, and in planar bilayers, Omp2a forms monomeric pores smaller than those of Omp2b (the latter shows channel activity in trimeric state) (Marquis and Ficht, 1993). Although only three species and a few representative strains have been examined, there are small differences in the selectivity of porins purified from cell envelopes in functional assays according to which *B. melitensis* porin could have the smallest internal diameter and *B. canis* the largest, with *B. abortus* showing intermediate diameters (Douglas et al., 1984). Since thionin (a dye used in *Brucella* biotyping) has an exceptionally low hydrophobicity among dyes and its size is compatible with that of the *Brucella* porin channel, it has been suggested that differences in thionin

sensitivity of *B. melitensis* and *B. abortus* (Table 3) relate to different functional properties of the porins (Douglas et al., 1984). Recent comparative analyses suggest functional differences between Omp2a and Omp2b, the former being more efficient in allowing sugar diffusion (Paquet et al., 2001).

The Hydrophobic Pathway The antibiotic and dye sensitivity patterns and the direct assessment of permeability with hydrophobic probes show that the outer-membrane of *Brucella* is readily permeable to hydrophobic compounds (Martínez de Tejada and Moriyón, 1993; Fig. 22). The same evidence suggests that this permeability is more marked in *B. ovis* than in *B. abortus* and *B. melitensis* (Freer et al., 1999), but comparative studies with a representative number of strains have not been performed. The well-known variations in sensitivity to the large hydrophobic dye basic fuchsin (Table 3) also suggest species and biovar differences in this pathway, which could reflect subtle outer-membrane structural or topological differences. This pathway also accounts for the effect of sexual hormones on *Brucella* cell envelopes and metabolic rates (Meyer, 1976) and for the inability of *Brucella* to grow on media designed for bacteria that, like *Escherichia*, *Salmonella*, *Pseudomonas* and many others, have efficient outer-membrane barriers to hydrophobic permeants. In *Brucella*, permeability to hydrophobic compounds is linked to the LPS structure (Freer et al., 1996), and comparative studies with *Ochrobactrum* suggest that the absence of negatively charged groups and counterions at core level (Fig. 6) plays an important role in the inability to establish an effective barrier (Velasco et al., 2000).

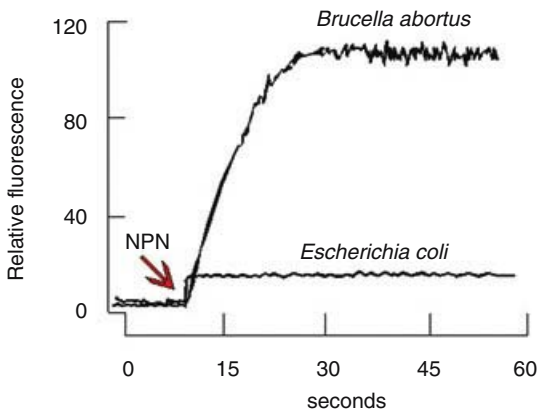


Fig. 22. Outer membrane permeability to the hydrophobic probe *N*-phenyl-1-naphthylamine (NPN). The arrow marks the time at which the bacteria were exposed to NPN and partition of the probe into the cell envelope (detected as a fluorescence increase) begins. Notice the increased uptake and permeability for the hydrophobic NPN probe by *Brucella* in comparison with the strong resistance to NPN penetration by *E. coli* cells. For details see Martinez-de-Tejada and Moriyón (1993).

Outer Membrane Stability It is a common observation that, as compared to *E. coli*, the brucellae are resistant to physical disintegration. Likewise, detergent extraction of *Brucella* cell envelope components needs conditions harsher than those used in many Gram-negative bacteria (reviewed in Moriyón and López-Goñi, 1998). Finally, *Brucella* cell envelopes are not destabilized by EDTA or polycationic microbicidal peptides and proteins that have a strong action on the majority of Gram-negative bacteria examined (Freer et al., 1996). At least in part, these observations are accounted for by the structure of the *Brucella* cell envelope.

Omp-peptidoglycan Association Electron microscopy shows that the peptidoglycan of *Brucella* is in an interaction with the outer-membrane much tighter than that observed in *Escherichia coli* (Fig. 12). Moreover, in some extraction procedures, Omps appear associated with the peptidoglycan. Although there are conflicting views on the precise structural meaning of this association (Moriyón and López-Goñi 1998), it could provide the *Brucella* cell envelope with additional stability.

Lipid Composition As compared to *Escherichia*, *Salmonella* and *Pseudomonas*, *Brucella* contains free lipids that carry longer acyl chains suggestive of a stronger hydrophobicity (Table 12). Moreover, phosphatidylcholine, which is the major phospholipid, has structural properties different from those of phosphatidylethanolamine (the major phospholipid in many other Gram-negative organisms), and at least in vitro, it forms more stable bilayers. Long acyl chains are also a characteristic of the *Brucella* lipid A (Fig. 7), and the overall hydrophobic effect could be reinforced by the topology of the very long fatty acids (Outer Membrane Topology). These features are present in other members of the α-2 Proteobacteria with which the brucellae share the property of the acid-fastness when stained by the modified Stamp's method (Fig. 10).

Lipopolysaccharide and Resistance to Outer-membrane Destabilizing Agents The reduced presence of anionic groups in the LPS core oligosaccharide and its strong hydrophobic anchorage (Fig. 20) are the main reasons for the marked resistance of *Brucella* to polymyxins (Fig. 23) and other polycationic peptides (Freer et al., 1996; Velasco et al., 2000; Fig. 24; Outer Membrane Versus Bactericidal Substances). Moreover,

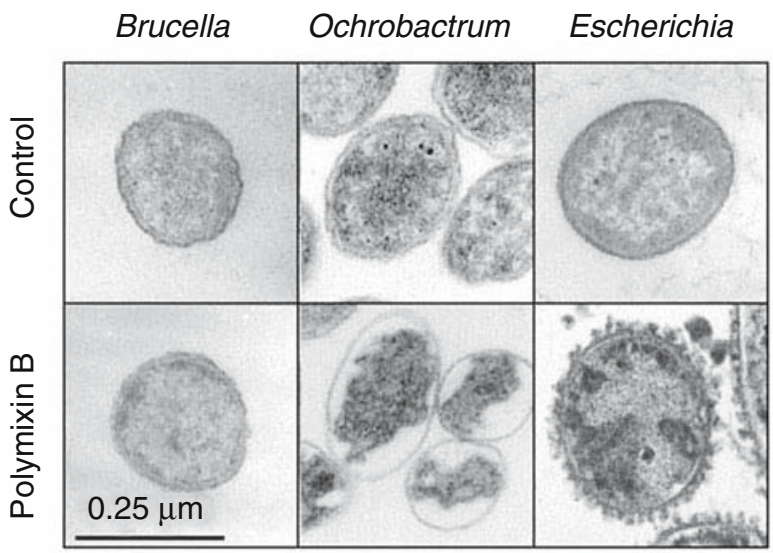


Fig. 23. Bactericidal activity of polymyxin B against *B. abortus*, *O. intermedium* and *E. coli*. Notice the characteristic outer membrane blebbing caused by polymyxin B in *E. coli* but not in *B. abortus* or *O. intermedium*. *Brucella abortus* treated with polymyxin B looks intact and not significantly different from the control. On the contrary, *E. coli* and *O. intermedium* are killed by polymyxin B. Although the outer membrane is morphologically unharmed, self-promoted uptake of polymyxin B in *O. intermedium* is inferred from the extensive damage to the cytoplasmic membrane and from the subsequent cytoplasm coagulation. From Velasco et al. (2000), with permission.

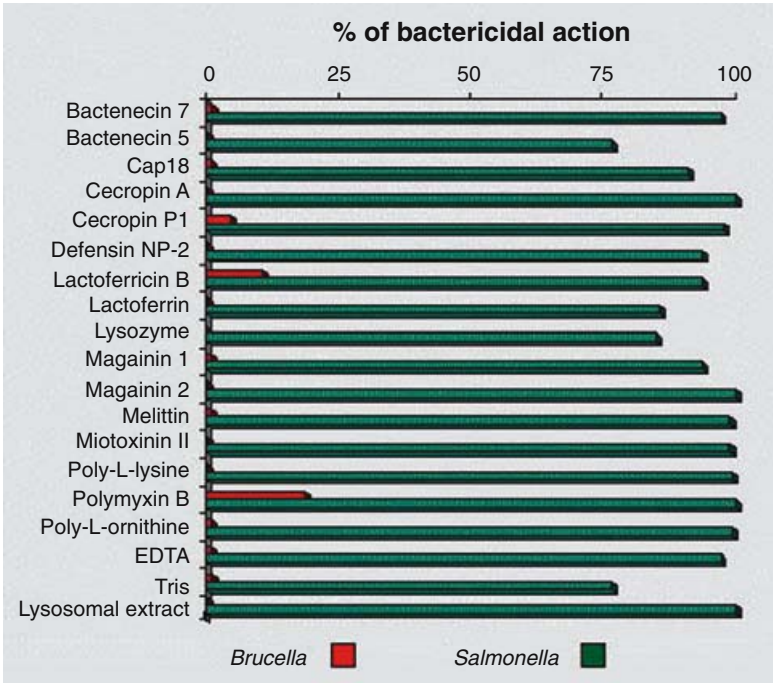


Fig. 24. Bactericidal activity of various cationic peptides and microbicidal substances against *B. abortus* and *S. typhimurium*. Concentrations used were the minimal quantity of bactericidal substance required to reduce the *Salmonella* colony-forming units (CFUs) not less than 75% in more than 99% of the controls. Incubation and number of organisms tested were the same for both bacteria: Notice the high resistance of *Brucella* to bactericidal substances in comparison to *Salmonella*. Adapted from Martínez de Tejada et al., 1995; Freer et al., 1996 and Páramo et al., 1998.

ornithine lipids could also shield the negatively charged groups of Kdo and lipid A (Outer Membrane Topology), which are the initial targets of these agents (Fig. 20). In many Gram-negative organisms, such negatively charged groups are bridged by divalent cations that play an essential role in outer-membrane stability. However, the resistance of *Brucella* to divalent cation chelators shows no significant structural role for this effect, and it can be postulated that their absence is compensated by the stronger hydrophobic anchorage of the LPS (Velasco et al., 2000).

Metabolism

This section summarizes what is known about the uptake of nutrients (Extracellular Enzymes and Uptake), intermediary metabolism (Intermediary Metabolism), and energy-yielding processes (ATP Synthesis and Respiratory Chain) of *Brucella* and its response to environmental stress (Response to Environmental Stress). Recently, the results of a large genomic sequence survey carried out in *B. abortus* (http://www.iib.unsam.edu.ar/genomelab/brucella/

index.html) (Sánchez et al., 2001) and the complete sequences of the genomes in *B. melitensis* strain 16M (http://www.genome.scranton.edu/Brucella/) (DelVecchio et al., 2002) and *B. suis* (www.tigr.org/) (Paulsen et al., 2002) have been made available to researchers. As our knowledge of the *Brucella* genome expands, it is expected that many details of its physiology will be revealed and become accessible to experimental testing. Although reference to some of the sequence data will also be made, the reader has to keep in mind the problems associated with the interpretation of this still incomplete information.

Extracellular Enzymes and Uptake The brucellae are devoid of extracellular hydrolytic enzymes acting on proteins, nucleic acids or polysaccharides. Accordingly, these bacteria depend exclusively on low molecular weight nutrients, such as sugars, amino acids or oligopeptides, etc., that are already present in the growth medium and that, depending on their hydrophobicity, cross the outer-membrane either through the porins or directly through the bilayer; both possibilities exist in *Brucella* (Permeability). The periplasmic space of many Gram-negative bacteria contains binding proteins that act coordinately with some cytoplasmic membrane transport systems. Attempts to isolate binding proteins from *Brucella* have been unsuccessful, which has been attributed to a tighter interaction of these proteins with the cell than those reported for other bacteria (Rest and Robertson, 1974), probably reflecting its outer-membrane properties (Lipopolysaccharide and Resistance to Outer-membrane Destabilizing Agents). However, the *Brucella* genome contains genes whose products have characteristics suggestive of the periplasmic binding proteins involved in transport of oligopeptides, dipeptides and amino acids (Sánchez et al., 2001). In one case (protein P39), the genetic evidence has been complemented by modeling studies that also suggest a periplasmic binding role (De Fays et al., 1999) (Auxotrophic and Cell Cycle Genes During Intracellular Life).

Sequences corresponding to aquaporins, mechanosensitive channels, transporters for the uptake of osmoprotectants, and probable ion-specific channels are also present. *B. melitensis* contains an ORF that is similar to the aquaporin of *B. abortus* (Rodriguez et al., 2000). There are genes coding for three putative ABC transporters for osmoprotectants, but not for members of the betaine/carnitine/choline transporter (DelVecchio et al., 2002).

Uptake of Organic Nutrients The transport of glucose has been examined in *B. abortus* (Rest and Robertson, 1974) and the functional studies show the absence of a phosphotransferase (phosphoenolpyruvate-dependent) system and the presence of a proton-coupled uptake. The system is equally active in cells grown in the presence of fructose, galactose, erythritol and glucose, and seems therefore constitutive. Galactose acts as a competitive inhibitor for the uptake of glucose (Rest and Robertson, 1974) and complementation studies performed in *E. coli* with the *B. abortus* gene show that both sugars are transported by the same transmembrane protein (Essenberg et al., 1997). The putative glucose-galactose transport protein belongs to the proton-coupled transporters of the major facilitator superfamily, and a genomic sequence survey suggests ABC-type transporters active on sugars (D-fructose, D-mannose, melibiose, sucrose, trehalose, maltose, xylose, and sorbitol in *B. melitensis*), amino acids and oligopeptides (Sánchez et al., 2001; DelVecchio et al., 2002). The genomic analysis (*B. melitensis*) shows no erythritol-specific transporters (see below) such as those found in *Rhizobium*, but both glycerol and erythritol could be taken up by several putative polyol ABC transporters. A homologue to the gene coding for the multiple sugar-binding proteins of *Agrobacterium* and *Azospirillum* is present in at least *B. melitensis*. Also worth mentioning is that the *bacA* gene, which encodes a putative cytoplasmic membrane transport protein, is critical for virulence (LeVier et al., 2000).

Uptake of Inorganic Nutrients Some aspects of the iron uptake systems of *Brucella* have been investigated. In early studies on the effect of iron availability, it was observed that *Brucella* releases large amounts of the monocatechol 2,3-dihydroxybenzoate but not hydroxamate-type siderophores, and 2,3-dihydroxybenzoate and other simple monocatechols were observed to promote Fe uptake by an energy-dependent mechanism (López-Goñi et al., 1992). Since monocatechols have much less affinity for iron than complex catechols (such as enterobactin or agrobactin) have, their role as siderophores is not obvious (for a possible different role of 2,3, dihydroxybenzoate, see Iron Chelation and Bactericidal Action). More recently, a *B. abortus* transposon mutant has been described that is unable to grow on iron restricted medium albeit it releases 2,3-dihydroxybenzoate (González-Carreró et al., 2002). This mutant fails to release a minor catechol which, in cross-feeding experiments, has the ability to promote growth of the mutant under Fe limiting conditions. The mutated gene shows homology with genes (*entC*) involved in the synthesis of complex catechols by other bacteria, including vibriobactin and enter-

obactin (González-Carreróet al., 2002). However, enterobactin fails to promote growth of *B. abortus* and, interestingly, neither agrobactin nor rhizobactin mediate Fe uptake by *B. abortus* (López-Goñet al., 1992). These data show that *B. abortus* produces a complex catechol siderophore (brucebactin) of unknown structure which is different from some of the catechols produced by related bacteria. A puzzling observation is that the release of catechols is not accompanied by the expression of new outer-membrane proteins that could act as siderophore receptors (López-Goñet al., 1992). However, because 2,3, dihydroxybenzoate and related compounds are moderately hydrophobic, direct penetration of siderophore chelates by the hydrophobic pathway could explain this observation. Once in the cytoplasmic compartment, iron has to be stored in such a way that the generation of free oxygen radicals, including the very toxic hydroxyl radical, is prevented, and a bacterioferritin gene with a high similarity to the corresponding *E. coli* gene has been characterized in *B. melitensis* (Denoel et al., 1995) (Iron Chelation and Bactericidal Action).

Recently, some aspects of the high affinity system of nickel (Ni) uptake have been elucidated in *B. suis* (Jubier-Maurin et al., 2001). The *nik* gene cluster of *B. suis* closely resembles that of the corresponding *E. coli* operon, and although the regulatory gene (*nikR*) is found in a different position and orientation, both systems can complement the heterologous deficiency. This system is activated by low oxygen tension and metal ion deficiency and repressed when Ni is in excess. As expected (the enzyme contains Ni), its impairment leads to a severe reduction in urease activity (The Urea Cycle and Urease Activity). Finally, sequences compatible with systems involved in Mg^{++}, Zn^{++}, NO_3^- and SO_4^{2-} transport have been identified in genomic surveys.

Efflux Systems Genomic surveys (DelVecchio et al., 2002) show the presence of genes putatively coding for members of the cation diffusion facilitator (CDF) family and P-type ATPases involved in heavy metal efflux. Moreover, there are homologues of ABC transporters involved in drug efflux and of multidrug-resistance proteins and acriflavine-resistance and quaternary ammonium resistance systems. A TolC homologue (a common outer membrane component of several of these efflux pumps) is also present.

Intermediary Metabolism

Sugar Catabolism and Tricarboxylic Acid Cycle At least *B. melitensis* (strain 16M) carries genes predicted to code for the enzymes of all pathways of central carbohydrate metabolism but those of the glycogen cycle. This includes genes for the pentose phosphate enzymes as well as for the complete Entner-Doudoroff pathway but this is in apparent conflict with the results of functional studies (DelVecchio et al., 2002). The pathways of glucose catabolism have been studied in *B. abortus* (strains 19 and 2308), *B. melitensis* (strain 16M) and *B. suis* (strain 1330) using glucose radiolabeled at positions C1, C2, C3, C4 and C6 in a complex medium (Robertson and McCullough, 1968a). The kinetics of $^{14}CO_2$ release in dependence of the labeled position is C1 > C2 > C3 > C4 > C6, and this is compatible with either the operation of the hexose monophosphate pathway by itself or the hexose monophosphate plus the Entner-Doudoroff pathways working simultaneously, in both cases in conjunction with pyruvate oxidation through the tricarboxylic acid cycle. However, analysis of pyruvate from arsenite-poisoned cells or of alanine as the major product derived from pyruvate shows that glucose C1, C2 or C3 does not contribute significantly to the labeling of these two molecules. This implies that a C3–C4 cleavage does not occur significantly in the degradation of glucose and therefore that neither the Entner-Doudoroff pathway nor the hexose diphosphate pathway is active in *Brucella*. Differences between the $^{14}CO_2$ release pattern of *B. suis* and the other species tested have been noted (Robertson and McCullough, 1968a) and attributed to a higher activity of the reactions leading from glyceraldehyde-3-phosphate to pyruvate. In cell-free studies with *B. abortus* 19, it has been observed that fructose 1,6-diphosphate is oxidized very slowly, that fructose diphosphate aldolase activity is very low and that, although phospho-2-keto-3-deoxygluconate aldolase activity is detected, there is no phosphogluconate dehydratase activity (Robertson and McCullough, 1968b). Consistent with the genomic studies, the cell-free studies also show the major enzymatic activities of the pentose cycle when they are tested during anaerobic dissimilation of ribose-5-phosphate. All these findings support the existence of an operative pathway in which glucose is phosphorylated (the glucokinase gene has been characterized; Essenberg, 1995) and oxidized first to gluconate-6-phosphate. Gluconate is then oxidized in the pentose cycle to yield 3 moles of CO_2 plus 1 mole of glyceraldehyde-3-phosphate, and the latter is channeled into the tricarboxylic acid cycle via pyruvate (Fig. 25). So far, the oxidative pentose phosphate cycle as the major glucose catabolic pathway has been described in only a few bacteria.

Glycerol is efficiently used by brucellae, and the genes that encode glycerol kinase and glycerol-3 phosphate dehydrogenase have been

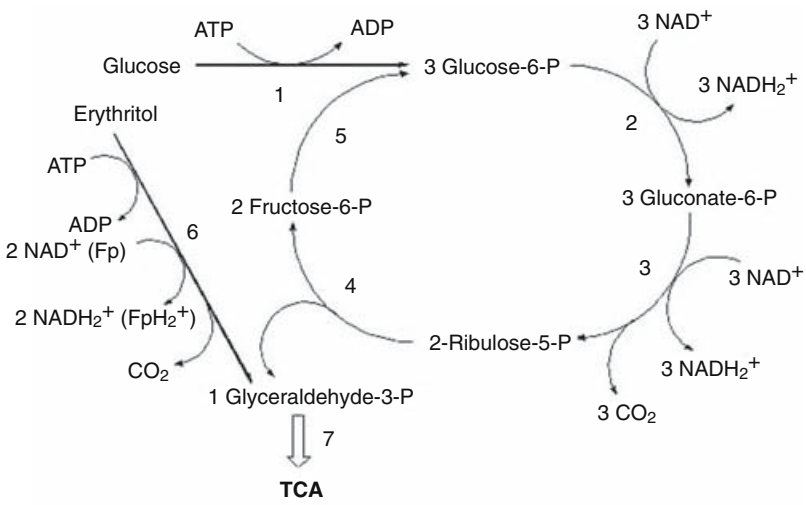


Fig. 25. Glucose and erythritol catabolism in *B. abortus*. 1) glucokinase; 2) glucose-6-phosphate dehydrogenase; 3) gluconate-6-phosphate dehydrogenase; 4) transaldolase and transketolase interconversions; 5) glucose-phosphate isomerase; 6) erythritol pathway (see Fig. 26 and 27); and 7) terminal reactions of the glycolytic pathway and pyruvate dehydrogenase leading to the tricarboxylic acid (TCA) cycle.

identified in, at least, B. melitensis (DelVecchio et al., 2002). Also, early studies on the ability to grow on McCullough and Dick minimal medium (Table 9) with sucrose, glucose, galactose, mannose, fructose, threalose, xylose, arabinose and erythritol as the sole energy and carbon sources showed that all these compounds supported the growth of the four *B. suis* strains tested, that the growth of four *B. melitensis* strains showed variable results in arabinose, mannose, sucrose and threalose, and that none of the four *B. abortus* strains grew on arabinose, mannose, sucrose and threalose (McCullough and Beal, 1951). Because McCullough and Dick medium does not furnish the minimal requirements of the less prototrophic *Brucella* species (Nutritional Requirements), absence of growth in these experiments has to be interpreted with care. Nevertheless, the results suggest that at least *B. suis* has both transport and enzymatic abilities to channel all those compounds into the tricarboxylic acid cycle, and several putative sugar kinases can be identified in genomic surveys (Sánchez et al., 2001; DelVecchio et al., 2002). Similar speculations can be made on the meaning of the oxidative metabolic patterns established for the classical species and biotypes of *Brucella* (Table 2), taking always into account that the differences may also reflect differences in uptake and not necessarily in catabolic abilities.

The tricarboxylic acid cycle has been demonstrated in *B. abortus* 19 by using pyruvate- and acetate-^{14}C (Robertson and McCullough, 1968a) and also in other *B. abortus* strains in enzymatic studies (Altenbern and Housewright, 1953; Marr et al., 1953). Evidence for the functioning of several steps of the cycle has also been provided by studies on the oxidative dissimilation of amino acids by *B. abortus*. These data are consistent with those of the genomic analyses which also show genes putatively coding for the enzymes required for anaplerotic reactions (NAD-dependent malate dehydrogenase [malic enzyme], phosphoenolpyruvate carboxykinase and pyruvate carboxylase). In addition, there is a pyruvate phosphate dikinase, which converts pyruvate to phosphoenolpyruvate (Oxidation of Amino Acids).

Erythritol The early studies described in the section Sugar Catabolism and Tricarboxylic Acid Cycle showed that erythritol is able to support the growth of all *B. abortus*, *B. suis* and *B. melitensis* strains (McCullough and Beal, 1951) and this has been confirmed repeatedly. Other characteristics also make the metabolism of this compound by *Brucella* particularly interesting: growth with erythritol occurs earlier than with other substrates, and at least in *B. abortus*, erythritol is used preferentially over glucose in complex media (Anderson and Smith, 1965). These observations, and the presence of erythritol in the placenta of ungulates, led to the controversy on the possible relationship(s) between erythritol metabolism and virulence and viscerotropism in *Brucella*.

The metabolic pathway of erythritol was elucidated in *B. abortus* B19 (British strain 19) (Sperry and Robertson, 1975). *Meso*-erythritol is oxidized through five steps that involve a kinase, three dehydrogenases and a decarboxylase to CO_2 and dihydroxyacetone-phosphate, which is then converted to pyruvic acid through the final steps of glycolysis (Figs. 25 and 26). Two dehydrogenases are membrane bound and require NAD, but the third (3-keto-L-erythrose-4-phosphate dehydrogenase) is coupled directly to the electron transport chain. The pathway is different from that described in other bacteria that either transform erythritol in L-erythrulose

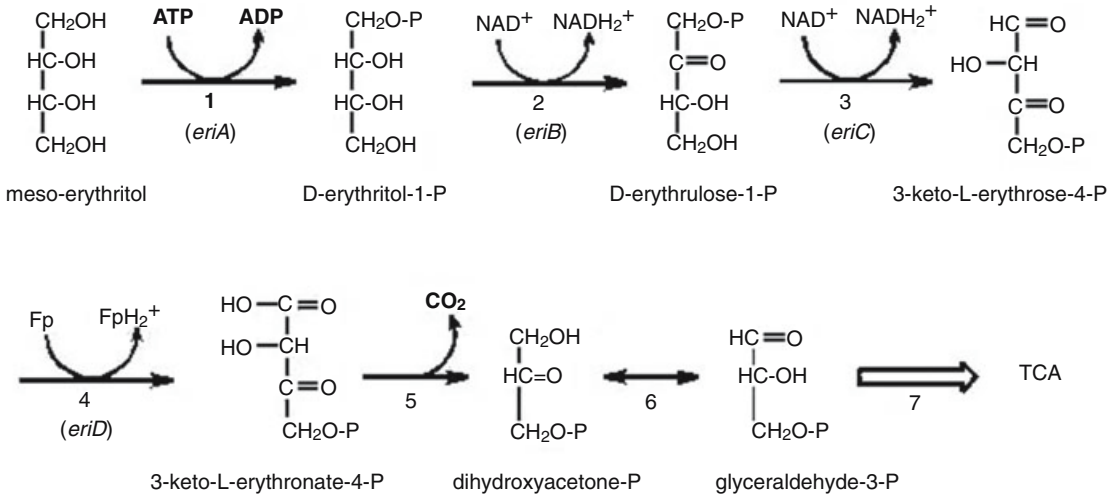


Fig. 26. The erythritol pathway in *B. abortus*. 1) erythritol kinase; 2) erythritol-1-phosphate dehydrogenase; 3) erythrulose-1-phosphate dehydrogenase; 4) 3-keto-erythrose-4-phosphate dehydrogenase; 5) 3-keto-erythronate-4-phosphate decarboxylase; 6) phosphotriose isomerase; and 7) terminal reactions of the glycolytic pathway and pyruvate dehydrogenase leading to the tricarboxylic acid (TCA) cycle. Putative genes are indicated in parenthesis.

by means of a dehydrogenase (*Acetobacter suboxydans* and *Alcaligenes faecalis*) or split L-erythrulose-1-phosphate directly into formaldehyde plus dihydroxyacetone-phosphate (*Propionibacterium pentosaceum*). However, it has to be noted that the ability to use erythritol is also present in many bacteria of the α-2 subgroup of the Proteobacteria including strains of *Rhizobium tropicii*, *Rhizobium loti*, *Sinorhizobium meliloti*, *Sinorhizobium fredii*, *Agrobacterium* biovar 2 and all known species of the genus *Ochrobactrum* (De Lajudie et al., 1994; Lebhun et al., 2000). None of these bacteria are primarily associated with animals, and although the erythritol pathway has not been elucidated in them, it should not be different from that of *Brucella*. Therefore, it seems likely that the erythritol pathway of *Brucella* has not resulted from the adaptation to pathogenicity in ruminants, but represents an ancestral metabolic pathway for the α-2 Proteobacteria (Genome Evolution).

In the study of over 100 strains of *B. abortus*, it was noted that erythritol not only did not promote growth of strain US19, but actually inhibited it (Jones et al., 1965). The US19, the current *B. abortus* 19 vaccine, and the B19, the British 19, are possibly related but whereas the former is unable to grow on erythritol which is, in fact, toxic for it, the latter catabolizes erythritol (Identification of Vaccine Strains). This peculiarity of the vaccine was explained in the course of the studies on erythritol metabolism. It was found that *B. abortus* US19 lacks D-erythrulose-1-phosphate dehydrogenase activity and has a 20% reduced 3-keto-erythrose-4-phosphate dehydrogenase activity (Sperry and Robertson, 1975). Presumably the build-up of intermediary metabolites becomes toxic for strain 19 and prevents growth when erythritol is present. This defect can be traced to a 702-bp deletion in the *ery* region of strain 19 (Sangari et al., 1994b) (Identification and Typing). This region is known to contain at least four genes of which presumably *eryA* codes for the erythritol kinase, *eryB* for the erythritol-1-phosphate dehydrogenase, *eryC* for the erythrulose-1-phosphate dehydrogenase and *eryD* for a regulator of *ery* expression (Sangari et al., 2000). The 702-bp deletion in strain 19 affects both *eryC* and *eryD*, thus creating the described phenotype. In contrast, B19 carries no deletion (Sangari, 1993), and "revertants" of US19 for which erythritol is not toxic probably carry compensatory mutations elsewhere in the chromosome.

Erythritol analogues have been studied for their in vitro and in vivo anti-*Brucella* activity. Fluoro-mesyl and bromo-derivatives of erythritol and threitol inhibit the growth of *B. abortus*, *B. melitensis* and *B. suis* in vitro and in bovine phagocytes (typical concentrations causing a 50% inhibition of *B. abortus* range from 5 µg/ml [for 1,2-3,4-dianhydro-DL-threitol] to 100 µg/ml [for 1,4-dimesylerythritol]). The therapeutic value of the analogues in *B. abortus*-infected guinea pigs is, however, very limited (Smith et al., 1965).

It has been shown that primers taken from the *ery* region amplify DNA of the expected size from *B. ovis* and *B. canis* (Sangari, 1993). These species do not or poorly oxidize erythritol (Table

2), but this substrate is not toxic for them. Thus, their characteristics are different from those of *B. abortus* US19. There is a need for comparative studies to understand these differences.

Oxidation of Amino Acids In a study of the ability of *B. abortus* 19 to grow on amino acids and related compounds, it was found that only glutamate, asparagine and histidine were used as the sole nitrogen source (Gerhardt et al., 1950b), and that the rate of oxygen uptake with glutamate was considerably greater than with any of the other substrates (Gerhardt et al., 1950a). It was also found that a simple medium containing glutamate as the nitrogen, carbon and energy source, plus accessory factors, supported growth (Table 9). The data obtained in subsequent metabolic studies (Marr et al., 1953) are consistent with a very active pathway through which glutamate is converted first to ketoglutarate and then metabolized in the tricarboxylic acid cycle to yield energy and reduced coenzymes plus intermediary metabolites for biosynthesis, including pyruvate. Oxidation of glutamate was also shown to be coupled to the urea cycle (The Urea Cycle and Urease Activity). Interest in the metabolism of glutamate was spurred by the observation that, at least in *B. abortus* and *B. suis*, its oxidative rate was lower in strains of high virulence than in strains of low virulence (Wilson and Dasinger, 1960). However, cell free studies showed no differences in the pathway of glutamate oxidation or in enzymatic activity associated with virulence, and it was concluded that unequal rates of permeability (Properties of the Outer Membrane) to the substrate could account for the observations (Dasinger and Wilson, 1962).

The Urea Cycle and Urease Activity *Brucella abortus* and *B. melitensis* resting cells slowly metabolize glutamate with transient accumulation of arginine, ornithine and citrulline as metabolites, and at least in *B. abortus*, aspartate is also metabolized to ornithine (Cameron et al., 1952). Moreover, arginase activity (direct conversion of arginine into ornithine) exists in *B. abortus*, *B. melitensis* and *B. suis* (Cameron et al., 1952; Cameron et al., 1954). This enzymatic activity is considered the landmark of the urea cycle, and therefore, these results strongly suggest that at least these three *Brucella* species should have this metabolic cycle. The linkage with the metabolism of glutamate shown by the preceding observations could occur through the tricarboxylic acid cycle plus an aspartic-glutamic transaminase, since this enzymatic activity has been shown in at least *B. abortus* (Altenbern and Housewright, 1951). Moreover, *Brucella* contains ornithine lipids (Figs. 13 and 14) in the cell envelope and the cycle should furnish this amino acid for acylation and further modification.

All classical *Brucella* smooth species, *B. canis* and some *B. ovis* strains (Corbel and Hendry, 1985) show a characteristic urease activity as do the *Brucella* marine isolates, and the enzyme is located in the cytoplasmic fraction (Brun and Descous, 1984). It has been proposed that urease is available in *Brucella* because of the occurrence of the urea as an intracellular metabolite (Cameron et al., 1952). Removal of urea would be necessary when the bacteria are growing with amino acids as the main source of carbon and energy. The role of urease in virulence is discussed elsewhere in the chapter (Metabolism), and these aspects of the metabolism of *Brucella* are summarized in Fig. 27.

Physiology of CO_2 Requirements The biochemical basis of the CO_2-dependence of some *Brucella* species and biotypes is not known. CO_2 cannot be replaced by cell extracts, complex nutrients, tricarboxylic acid cycle intermediates, purines or pyrimidines, and the major products of CO_2 assimilation in complex media are in the protein fraction as alanine and glycine and in the nucleic acid fraction as pyrimidines (Newton et al., 1954b; Newton and Wilson, 1954a). In keep-

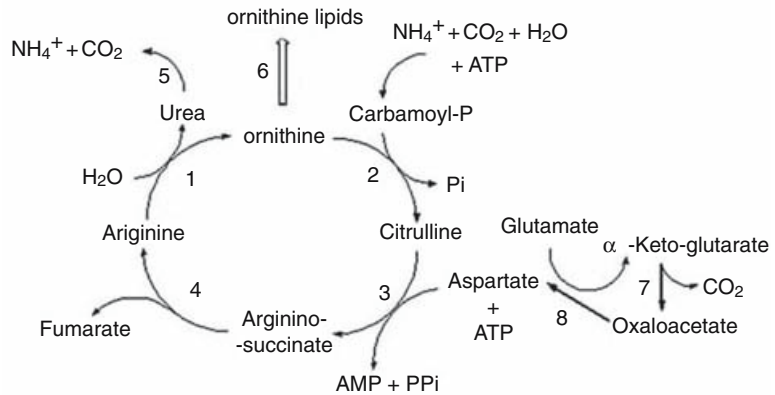


Fig. 27. The urea cycle and associated reactions in *B. abortus*. 1) arginase; 2) ornithine transcarbamoylase; 3) argininosuccinate synthetase; 4) argininosuccinase; 5) urease; 6) synthesis of membrane ornithine lipids; 7) tricarboxylic acid cycle reactions; and 8) glutamate-aspartate transaminase.

ing with the strong respiratory activity of the *Brucella* (Physicochemical Requirements and Atmosphere), these observations demonstrate that CO_2 is not required to reduce oxygen tension, but rather that it is needed as a nutrient per se. In bacteria, CO_2 is used in the synthesis of purines, pyrimidines, carbamoyl-phosphate and fatty acids. In some cases studied (Burns et al., 1995), the CO_2 requirement is due at least in part to the high *Km* of one of the carboxylases involved. The rate of appearance of CO_2-independent phenotypes in the CO_2-dependent strains has been estimated in approximately 3×10^{-10} per cell division (Marr and Wilson, 1950). Therefore, if this *Brucella* phenotype is dependent on the affinity of carboxylases for CO_2, few of them are involved. This is consistent with the observation that a CO_2-dependent strain and its CO_2-independent derivative did not differ in the distribution of fixed $^{14}CO_2$ and showed the same basic nutritional requirements (Newton et al., 1954a; Newton and Wilson, 1954b).

ATP Synthesis and Respiratory Chain Because glucose is mostly oxidized by the hexose monophosphate pathway, the substrate-level phosphorylation contribution to ATP synthesis should be minimal (provided glyceraldehyde 3-phosphate is only an intermediary to the tricarboxylic acid cycle), and *Brucella* should obtain ATP from glucose or erythritol (and presumably other sugars) mostly by coupling coenzyme oxidation to the electron transport system (Rest and Robertson, 1975). In functional studies performed with the envelope fraction of *B. abortus* 19, $NADH_2^+$ dehydrogenase activity and also other primary dehydrogenases using L-lactate and succinate as substrates were observed by spectral analyses and polarographic determination of oxygen consumption. $NADH_2^+$ dehydrogenase and succinate dehydrogenase have also been demonstrated in the characterization of the cell envelope fraction of other *B. abortus* strains and in *B. melitensis* (Moriyón and Berman, 1982). Moreover, at least in *B. abortus*, oxidation of erythritol 1-phosphate is also directly coupled to the electron transport chain (Rest and Robertson, 1975) through the 3-keto-l-erythrose 4-phosphate dehydrogenase (Sperry and Robertson, 1975; Fig. 26). Analysis of the free lipid fraction of *B. melitensis* and *B. abortus* (Thiele and Schwinn, 1969; 1973) shows the characteristic e^-/H^+ transporter ubiquinone Q_{10}, and the polarographic data obtained with B. abortus 19 (Rest and Robertson, 1975) are consistent with the location of the primary dehydrogenases early in the respiratory chain. The spectrophotometrical analyses also provided evidence for the presence of flavoproteins and several cytochromes which were different in exponentially growing bacteria from those in the late exponential phase of growth, suggesting the ability to adapt the terminal elements of the electron transfer to different oxygen tensions (Rest and Robertson, 1975).

According to the predictions of the gemomic analysis, in *B. melitensis* a terminal cytochrome o ubiquinol oxidase acts under normal oxygen concentration and a cytochrome cbb3 oxidase acts under low oxygen concentration. During microaerobic conditions, *B. melitensis* can use the cytochrome bd quinol oxidase for electron transfer to oxygen from the membrane quinone pool. Complete sets of genes for all of the subunits for complex I (NADH-quinone oxidoreductase), complex II (succinate dehydrogenase), and complex III (ubiquinol-cytochrome c reductase) and for the soluble electron-transporting cytochromes c2 and c552 have also been identified as well as those of several primary dehydrogenases (D- and L-lactate dehydrogenases, flavoprotein-quinone oxidoreductase, glycerol-3-phosphate dehydrogenase [$NAD(P)^+$], and malate:quinone oxidoreductase). These enzymes supply electrons and protons for the aerobic respiratory chain and oxidative phosphorylation pathways. Genes coding for H^+-coupled ATP synthase, NAD(P) trans-hydrogenase, and Na^+/H^+ and K^+/H^+ antiporters are present and the cognate proteins should be involved in oxidative phosphorylation and the proton gradient maintenance.

Nitrate-nitrite reduction is observed in all *Brucella* classical species but *B. ovis*. Nitrate reductase activity was detected in the course of the above-described studies on the characterization of the electron transport system of *B. abortus* 19 (Rest and Robertson, 1975). Moreover, in the studies on the catabolism of erythritol, it was shown that nitrate was almost as effective as oxygen in stimulating the breakdown of erythritol, whereas nitrite and hydroxylamine had no effect (Sperry and Robertson, 1975). Apparently, *B. abortus* (and possibly the rest of the *Brucella* species reducing nitrate to nitrite in a dissimilative way) has a terminal nitrate reductase coupled to the electron transport chain via flavoproteins and cytochromes and this is consistent with the genomic analyses. At least in *B. melitensis*, the respiratory nitrate reductase complex consists of four subunits encoded by a single operon (DelVecchio et al., 2002).

Response to Environmental Stress Aerobic organisms are endowed with enzymes that detoxify the reactive by-products of oxygen, which are generated mostly during the operation of the respiratory chain. Two types of superoxide dismutase (Sod) have been found in *Brucella abortus*, a cytosolic Mn^{++}-Sod and a Cu^{++}/Zn^{++}-Sod

(Bricker et al., 1990) located in the periplasmic space (Stabel et al., 1994). By Western-blotting with antisera to the homologous *E. coli* protein, the periplasmic Sod has been demonstrated in all *Brucella* species and biovars tested except in *B. neotomae* and *B. suis* biovar 2 (Bricker et al., 1990). Moreover, catalase activity can be demonstrated in all *Brucella* species in the standard H_2O_2 assay, and according to manometric studies performed with *B. abortus*, *B. melitensis* and *B. suis*, the latter species displays the strongest catalase activity, which is also slightly higher in *B. melitensis* than in *B. abortus* (Huddleson and Stahl, 1943). A periplasmic catalase has been purified and cloned from *Brucella abortus* and characterized as a tetrameric protein (MW of the monomer 55,000) lacking a typical N-terminal export signal sequence (Sha et al., 1994).

The periplasmic location of catalase and Cu^{++}/Zn^{++}-Sod suggests that they protect the cell from external sources of oxidative damage (Stabel et al., 1994), such as those generated by phagocytes upon ingestion of bacteria (Neutralization of Oxygen and Nitrogen Intermediates). In fact, the periplasmic catalase behaves as an oxidative stress protein, and exposure of *B. abortus* cells to sublethal concentrations of H_2O_2 increases both their resistance and the expression of the enzyme. Although the magnitude of the increase is lower, oxidative stress also increases expression of the periplasmic Cu^{++}/Zn^{++}-Sod (Table 15). Because Sod activity leads to the production of H_2O_2, this different level of response is consistent with the respective roles of these enzymes in protecting the cell from oxidative damage (Kim et al., 2000).

Homologues to the *htrA*, *groEL* and *dnaK* genes have been identified in *Brucella*, and the latter has been cloned and shown to express a functionally active product in a thermosensitive *E. coli dnaK* mutant (Cellier et al., 1992; Elzer et al., 1994; Lin et al., 1992; Roop et al., 1994; Tatum et al, 1994). As expected, the synthesis of the two cytoplasmic molecular chaperones (GroEL and DnaK) and the periplasmic protease HtrA is stimulated under heat stress (42°C), and impaired growth has been shown for an *htrA*-defective mutant under these conditions. Moreover, an increased expression of these three genes is also observed in acidified media (Table 15).

In addition to the above-described proteins, heat, oxidative, acid and nutritional stresses cause an increase in the synthesis of over 100 proteins, among which many are not expressed under normal conditions. Conversely, many proteins expressed in normal media become repressed under these conditions. A small fraction of these proteins has been identified by proteomic methods (Table 15), and some elicit antibodies in naturally infected animals, demonstrative of their expression in the host. It is noteworthy that the profile of proteins induced and repressed when *B. abortus* grows in cultured macrophages does not correspond to a simple sum of the proteins observed under stress conditions in vitro (Lin and Ficht, 1995; Rafie-Kolpin et al., 1996).

Genetics

The *Brucella* Genome

The genome of a bacterium includes the chromosome plus any extrachromosomal DNA, such as prophages and plasmids carrying nonessential genes useful only under some environmental conditions. Bacterial chromosomes can be linear and bacteria can have more than just a single chromosome (Kolst, 1999). Interestingly, of the bacteria currently known to have a large part of their essential genes in replicons outside the larger chromosome (i.e., to have one or more additional chromosomes of different size), many belong, like *Brucella*, to the α Proteobacteria (Jumas-Bilak, 1998b; Kolst, 1999; Moreno, 1998). Moreover, some members of the α Proteobacteria are known to have a linear chromosome in addition to the major circular one (Jumas-Bilak et al., 1998b) and many also have plasmids (Moreno, 1998). In this taxonomic context, the brucellae are characterized by the presence of two or, in *B. suis* biovar 3, one circular chromosome and by the absence of plasmids (Fig. 1). The results of *B. melitensis* genomic sequence (DelVecchio et al., 2002), a survey carried out in *B. abortus* (Sánchez et al., 2001) and the genome sequence of *B. suis* (Paulsen et al., 2002) represent a wealth of genetic details yet to be evaluated. The general genetic characteristics of the *Brucella* genome are summarized here, and their evolutive and taxonomical meaning is discussed elsewhere in the chapter (Biosafety).

Brucella Chromosomes

Number and Species Differences The distribution of some essential genes (ribosomal *rrn* and genes coding for heat shock proteins), combined with pulse-field gel electrophoresis, shows that the reference strains of all *B. melitensis* and *B. abortus* biovars, *B. suis* biovar 1, *B. ovis*, *B. canis*, and *B. neotomae* carry two circular chromosomes of about 2.1 and 1.15 Mb (Michaux-Charachon et al., 1993; Jumas-Bilak, 1998a; Jumas-Bilak, 1998b), and the reference strains of *B. suis* biovar 2 and biovar 4 also have two circular chromosomes but of 1.85 and 1.15 Mb (Jumas-Bilak, 1998a). On the other hand, the reference strain of *B. suis* biovar 3 has a single

Table 15. Some proteins of *Brucella* that are regulated by heat, oxidative and pH stress.

Stress	Regulation	Protein	Characteristics[a]	Immunoresponse in natural hosts	References
Heat	Induced	DnaK	Molecular chaperone (DNAK_BRUOV)	+++	Teixeira-Gomes et al., 1997, 2000; Cellier et al., 1992
		GroEL	Molecular chaperone (CH60_BRUAB)	+++	Lin et al., 1992; Kittelberger, 1995a; Lin and Ficht 1996; Teixeira-Gomes et al., 1997, 2000
		HtrA	Periplasmic serine protease (DEGP_BRUAB)	++	Roop et al., 1994; Elzer et al., 1994; Rafie-Kolpin et al., 1996
		AapJ	Amino acid-binding periplasmic protein (85.7%; *Rhizobium leguminosarum*; AAPJ_RHILV)	++	Teixeira-Gomes et al., 1997, 2000
		RRF	Ribosome releasing factor (RRF_BRUME)	+	Vizcaino et al., 1996; Teixeira-Gomes et al., 1997, 2000
		Fe and/or Mn SOD	Superoxide dismutase (73.1%; *Salmonella*; SODF_SALTY)		Teixeira-Gomes et al., 1997, 2000
	Repressed	α-ETF	Electron transfer flavoprotein α-subunit (80%; *Bradirhizobium japonicum*; ETFA_BRAJA)		Teixeira-Gomes et al., 1997, 2000
		ClpP	ATP-dependent Clp protease (heat-shock protein) (85.7%; *E. coli*; CLPP_ECOLI)	+	Teixeira-Gomes et al., 1997, 2000
		Bacterioferritin	Iron storage protein (BFR_BRUME)	+	Denoel et al., 1997
		BvrR	Regulator element of a two-component system involved in virulence (O67996)		Teixeira-Gomes et al., 1997, 2000
		IalB	Homologous to an invasion-associated protein (60%; *Bartonella bacilliformis*; IALB_BARBA)		Teixeira-Gomes et al., 1997, 2000
		Pyruvate dehydrogenase E1 component β-subunit	(66.7%; *Mycoplasma genitalium*; ODPB_MYCGE)		Teixeira-Gomes et al., 1997, 2000
Oxidative	Induced	Catalase	Periplasmic enzyme (CATA_BRUAB)		Kim et al., 2000
		Cu-Zn SOD	Periplasmic enzyme (SODC_BRUAB)	++	Teixeira-Gomes et al., 1997, 2000; Tabatabai and Hennager, 1994; Kim et al., 2000
	Repressed	30S ribosomal protein S1	mRNA binding protein (64.3%; *Rhizobium meliloti*; RS1_RHIME)		Teixeira-Gomes et al., 1997, 2000
pH 5.5	Induced	DnaK	Also increased by heat stress (see above)	+++	Teixeira-Gomes et al., 1997, 2000
		GroEL	Also increased by heat stress (see above)	+++	Rafie-Kolpin et al., 1996
		HtrA	Also increased by heat stress (see above)	++	Rafie-Kolpin et al., 1996
	Repressed	Malate dehydrogenase	Krebs cycle enzyme (100%; *Rhizobium leguminosarum*; MDH_RHILV)	+	Teixeira-Gomes et al., 1997; 2000

Symbols: +, mild, ++, moderate, and +++, strong immunoresponse(s).

[a]Where appropriate the % identity, bacteria, and/or ID in SWALL protein database is included in parentheses.

circular chromosome of 3.10 Mb (Jumas-Bilak, 1998a; Fig. 1). *Brucella* marine strains also possess two chromosomes close to the size of *B. abortus* (D. O'Callaghan, personal communication). For the species carrying the 2.1- plus 1.15-Mb chromosomes, the physical maps demonstrate a high conservation of restriction sites, although with differences due to small insertions and deletions of 1 to 32 Kb (Michaux-Charachon et al., 1997), accounting for the species-specific restriction patterns (Table 4). On the other hand, there are major chromosomal rearrangements affecting the 1.85- and 1.15-Mb chromosomes of *B. suis* biovar 2 and biovar 4, since sequences of the 2.1 Mb chromosome of *B. suis* biovar 1 are found in the 1.15 Mb chromosome of these biotypes and also in the single chromosome of *B. suis* biovar 3. Similar to what occurs in other bacteria, these differences in chromosome size and number can be explained by rearrangements caused by homologous recombination at the redundant *rrn* loci (Jumas-Bilak, 1998a) or insertion sequences (reviewed by Moreno, 1998). Taking into account the great homogeneity of the genus *Brucella* (Ancestor-descendent Relationships), the variations observed in *B. suis* are remarkable. For hypotheses on the origin of the two chromosomes, see Genome Evolution.

Size and Percentage of Guanine Plus Cytosine The data summarized in Table 1 (Number and Species Differences) show that the size of the genome varies between the 3.25 Mb of most *Brucella* spp. and the 3.0 Mb of *B. suis* biovar 3. This is significantly less than the 5.29 Mb (2.70 and 1.90 Mb chromosomes, plus two 0.59- and 0.10-Mb plasmids) of the genome of the closest known relative of *Brucella*, the genus *Ochrobactrum*, and the differences are due to both the smaller size of the chromosomes and the absence of plasmids in *Brucella*. Similar or even larger differences in genome size exist with other taxonomic neighbors, such as *Agrobacterium*, *Rhizobium*, *Phyllobacterium* and *Bradyrhizobium*, and the average G+C content of *Brucella* spp. (57%) is somewhat lower than that of these bacteria (Table 1). On the other hand, a relatively reduced genome size and a comparatively low G+C content are also characteristic of other pathogenic α Proteobacteria (Fig. 1) and probably relate to their adaptation to an intracellular or pericellular animal environment (Genome Evolution).

Methylation and Control of Chromosome Duplication and Cell Cycle It is not known how segregation of the genome is controlled in *Brucella* or in other bacteria carrying more than one chromosome. However, it is known that *Brucella* has genes whose products are involved in the control of the cell cycle. In bacteria, DNA methylation not only is involved in restriction-modification systems but also plays regulatory roles in the cell. The Dam methyl transferases of the γ Proteobacteria control the timing of initiation of chromosome duplication and the expression of some genes, including virulence genes, and are involved in methyl-directed DNA mismatch repair (Reisenauer et al., 1999). The counterpart of Dam in the α Proteobacteria seems to be CcrM (cell-cycle-regulated methyl transferase). This enzyme was originally described in *Caulobacter crescentus*, a bacterium that presents a morphologically defined cell cycle Caulobacter and of which synchronic cultures are relatively easy to obtain. The CcrM catalyzes the methylation of the adenine residue in GANTC sequences, and like Dam, uses *S*-adenosylmethionine as a donor and has no known cognate restriction enzymes. CcrM (but not Dam) is essential for cell viability and its activity is controlled during the cell cycle by tightly regulating the activation of *ccrM* expression (by the global regulator CtrA [see below] and quick constitutive degradation by Lon proteases). When *ccrM* is transcribed constitutively and the chromosomes are fully methylated, most *Caulobacter* cells abnormally increase the number of chromosomes from 1 or 2 to 3, and methylation seems to be particularly frequent in close proximity to DnaA boxes. On these bases, it has been postulated that CcrM plays a key role in the regulation of the chromosome replication (Reisenauer et al., 1999). *B. abortus* carries a CcrM homologue (78% similarity) that has been shown to be essential for viability, and when the number of *ccrM* copies is artificially increased over a given number, a dramatic effect in cell shape and a relaxation in the control of DNA are observed; cells appear highly branched and contain up to five genome equivalents (Robertson et al., 2000b). Moreover, *B. abortus* constructs overexpressing *ccrM* to levels not inducing aberrant morphologies or altered chromosome number still present reduced rates of intracellular multiplication (Robertson et al., 2000b). This indicates that other cell functions related to intracellular survival are likely to be regulated by CcrM. The attenuation of virulence caused by these alterations in CcrM is discussed elsewhere (Auxotrophic and Cell Cycle Genes During Intracellular Life).

Insertion Sequences (*IS*) and Other Genetic Elements All classical *Brucella* spp. examined carry a characteristic insertion sequence (IS*711* or IS*6501*) that is also present in the marine mammal *Brucella* isolates examined so far (Clavareau et al., 1998; Halling and Zehr, 1990;

Halling et al., 1993; Ouahrani et al., 1993). In addition, there are other IS elements scattered in the genome of those brucellae that have been sequenced (DelVecchio et al., 2002; Paulsen et al., 2002). The insertion sequence IS*711* is bound by two 20-bp imperfect inverted repeats, has a G+C content similar to that of the genome and is of 842–836 bp in length. It shows significant similarity (53.4%) with IS*427* of *A. tumefaciens*, suggesting a common ancestral sequence, and like some other IS, has overlapping open reading frames rather than one long open-reading-frame extending over the length of the element. A long open-reading-frame of 708-bp encodes a putative protein sharing sequence identity with the hypothetical protein 1 of *A. tumefaciens* and with a transposase of *Mycobacterium tuberculosis*. Characterization of the flanking sequences suggests that some IS*711* copies may have resulted from mechanisms other than transposition and also that there are hot spots for insertion. Two repeated palindromic DNA elements (Bru-RS1 and Bru-RS2) that are present in more than 35 copies in brucellae act as such insertion hot-spots (Halling and Bricker, 1994). The number of IS*711* copies per genome varies among *Brucella* species: there are 20–35 copies in *B. ovis* and 5–15 copies in other species. The copies present in a given strain may have minor differences in nucleotide sequence, and variations in position and number among species and biotypes result in genetic polymorphism (Table 4). At least in one case, the position of IS*711* causes phenotypically demonstrated differences in protein expression between *B. ovis* and the other classical *Brucella* species (Halling and Zehr, 1990), suggesting that this insertion sequence is a significant source of natural genetic variation in *Brucella* (Genome Evolution). This hypothesis is also supported by the fact that the rough phenotype of *B. abortus* RB51 is due to the spontaneous insertion of IS*711* in the *wboA* LPS gene (*B- abortus* RB51). The movement and transposition of this IS may be one of the sources for gradual microevolution, generating strains and eventually new species (Genome Evolution).

Regulation of Gene Expression

The few examples of known regulatory systems of *Brucella* that have been partially characterized are briefly discussed here.

THE BVRR-BVRS SYSTEM This is a two-component regulatory system. The sensor protein kinase BvrS is homologous to sensor proteins of the histidine protein kinase family, and the response regulator BvrR shows significant similarity to response regulators of the OmpR subfamily. The BvrS protein bears the characteristics of an intrinsic cytoplasmic membrane protein with a periplasmic domain that, in all likelihood, carries the region sensing an unidentified environmental stimulus. This protein crossreacts with ExoS sensor protein of *Rhizobium meliloti* (C. Guzmán-Verri et al., unpublished observations). Thus, it is presumed that, under some environmental circumstances, BvrS catalyzes its own phosphorylation and then transfers the phosphoryl group to BvrR, which, in turn, coordinately activates or represses the expression of genes that, although not identified as of yet, have been demonstrated to be essential for virulence and intracellular multiplication (Sola-Landa et al., 1998; Mechanisms of Entry to Host Cells). Dysfunction of the BvrS-BvrR system is known to alter the outer-membrane permeability, the expression of several Omp3 proteins (among the most conspicuous Omp3a [Omp25] and Omp3b) (Outer Membrane Proteins), and the pattern of lipid A acylation (C. Guzmán-Verri et al., unpublished results), but the underlying genetic mechanisms are yet to be unraveled (Fig. 28; The *Brucella* Genome).

An important feature of the BvrS-BvrR system is that it is highly conserved in other pericellular pathogens or endosymbionts of the α-2 Proteobacteria; it is homologous to the ChvI-

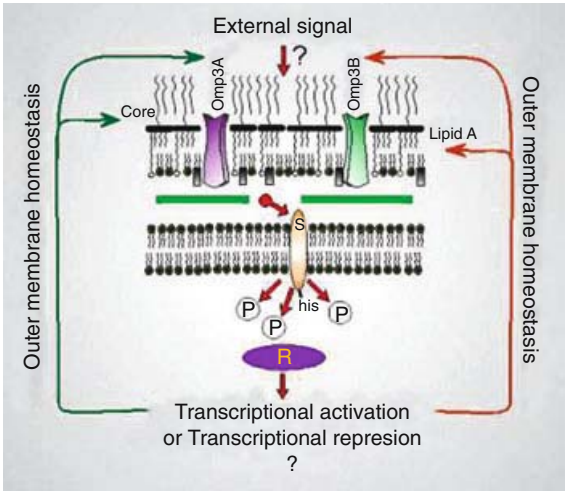


Fig. 28. Hypothetical model of the BvrS-BvrR regulatory system. Accordingly, an external unknown signal stimulates the sensor region of the BvrS protein, translocating the signal to a histidine kinase domain, which in turn promotes its own phosphorylation to phosphorylate the BvrR regulatory protein. The phosphorylated BvrR protein induces or represses transcription of genes not yet identified. By this system, the structure of the lipid A and the expression of Omp3a (Omp25) and Omp3b as well as the presence of other proteins at the *Brucella* outer membrane are regulated. The consequence of mutating either of these two genes in *Brucella* is an altered sensitivity to cationic peptides and hindrance to the cell invasion and intracellular replication.

ChvG (response regulator and sensor histidine protein kinase) of *A. tumefaciens* and to the ExoS of *R. meliloti* (Derivation of Ancestral Preexisting Structures for Virulence) as well as to genes found in *Bartonella* spp.

THE FEUP-FEUQ SYSTEM A response regulator sequence with 96% similarity to *R. leguminosarum* FeuP has been identified in *Brucella*. In *R. leguminosarum*, the FeuP is part of a two-component sensory regulatory system (FeuPQ) operating as described for the BvrS-BvrR system and known to take part in the regulation of iron uptake. Its precise role in *Brucella* remains to be determined (Dorrell et al., 1998).

THE NTRBC SYSTEM This is also a two-component sensory-regulatory system, with a sensor histidine-kinase protein (NtrB) and a regulatory transcriptional activator (NtrC) that has been identified in *B. melitensis* and *B. suis* (Dorrell et al., 1999). NtrC is a member of a family of bacterial proteins that enhance transcription of promoters recognized by the RNA polymerase carrying the alternative σ^{54} factor. The NtrCB system is involved in the regulation of the metabolism of nitrogen in several bacteria. When the NtrC element becomes growth-limiting, the genes necessary to use a variety of nitrogen sources become activated by NtrC. Consistent with this, *B. suis* NtrC mutants have increased metabolic activity in the presence of L-asparagine and L-alanine and decreased activity in the presence of other amino acids, such as glutamic, arginine, and lysine, and several sugars (Dorrell et al., 1999).

THE CTRA REGULATOR The CtrA regulator is a member of the response regulator family whose members, upon phosphorylation by a kinase, act as gene transcription activators. *Brucella abortus* carries a *C. crescentus* CtrA homologue (80% similarity) and the purified *C. crescentus* protein recognizes sequences upstream of the transcription starting site of the *Brucella ccrM* promoter, further stressing the similarity of the two methylating systems (Robertson et al., 2000b). By indirectly controlling the level of DNA methylation, CtrA should also be involved in the regulation of *Brucella* genes other than *ccrM*, but the kinase acting on CtrA has not been identified.

Genetic Exchange, Plasmids and Lysogenic Phages

No naturally occurring system for genetic exchange has ever been demonstrated for any *Brucella* spp. Moreover, the weight of the evidence shows that, in contrast to their phylogenetic neighbors, the brucellae do not harbor plasmids. They have not been found in an extensive search carried out with over 600 *Brucella* strains by a variety of methods (Meyer, 1990) and have never been occasionally observed. Likewise, no firm evidence has ever been provided on the existence of lysogenic phages (The Brucellaphages). Undoubtedly, these genetic characteristics relate to the peculiar intracellular habitat and to the genetic homogeneity of the group (Derivation of Ancestral Preexisting Structures for Virulence), and in all likelihood, the absence of plasmids is one factor contributing to the stability of the antibiotic sensitivity pattern of *Brucella* (Treatment). On the other hand, even with the limited information examined so far, there is evidence for gene acquisition by horizontal transfer. This is at least the case of the genes of the *wbk* region of *B. melitensis* 16M (the *gmd*, *perA*, *wxm*, *wzt*, *wbkA*, *wbkB* and *wbkC* LPS biosynthetic genes [Table 5]), the G+C content of which (44–49 mol%) significantly differs from that of the whole genome (57%) and suggests a shared origin with LPS genes of bacteria showing structurally related *O*-polysaccharides (Table 6; Acquisition of Ancestral Genes by Horizontal Transference).

Epidemiology

Brucella is the causative agent of brucellosis, undulant fever, contagious abortion and Malta fever, all names that describe an important malady of animals and humans. When affecting domestic animals, primarily bovines, caprines, ovines, and swines, the disease acquires a great economical importance. If the bacterium is transmitted from animals to humans, then an important zoonosis is established and public health interest arises, mainly due to the fact that the disease causes disability and requires long and expensive treatment (Treatment).

Since the time the bacterium "*Micrococcus melitensis*" (*Brucella melitensis*) was recognized more that 100 years ago in Malta as the causative agent of brucellosis, the epidemiology of this disease has received much attention. Many of the investigations carried out for already more than ten decades have been crucial for the control and eradication of brucellosis in several parts of the world. However, brucellosis remains as a relevant disease of domestic animals and as an important zoonosis, mainly in low-income countries (Geographical Distribution). Furthermore, brucellosis is an important infection of various wild life animals and a threat if some of the strains are utilized as biological weapons. In this respect, much controversy has come out in how to control and eradicate *Brucella* from these nat-

ural reservoirs and how to neutralize the bacterium if used in a confrontation (*Brucella* as a Biological Weapon). Because of these, the epidemiology and vaccination of brucellosis still capture the attention of many investigators in both developed and low-income countries (Vaccines and Vaccination). For extensive reviews in the epidemiology of animal brucellosis, the publications of Crawford and Hidalgo (1977) and Nielsen and Duncan (1990b) are recommended. For a comprehensive analysis on human brucellosis, the reviews of Spink (1956) and Flores-Castro and Baer (1979) are recommended.

Geographical Distribution

Although *Brucella* organisms infect a broad range of animals, the bacterial species and strains have strong tendency to remain in their preferred hosts (Biological Attributes in the Context of *Brucella* Species Definition). When infecting a secondary host (i.e., *B. melitensis* in cows), the removal of the source of infection (i.e., *B. melitensis*-infected goats) results in the disappearance of the bacteria from the secondary host population (Crawford and Hidalgo, 1977; Nielsen and Duncan, 1990b). In this respect, *Brucella* species and biovars follow the geographical distribution of their natural hosts and not the secondary or the accidental hosts, such as wild animals, humans or horses (Fig. 29). Since in many cases where domestic animals reside borders wildlife habitats, wild animals frequently become infected after contact with domestic livestock or their products, such as fetuses or infected tissues (Crespo-León, 1994). In wild animal accidental hosts (i.e., coyotes), the disease is limited to the infected individual;

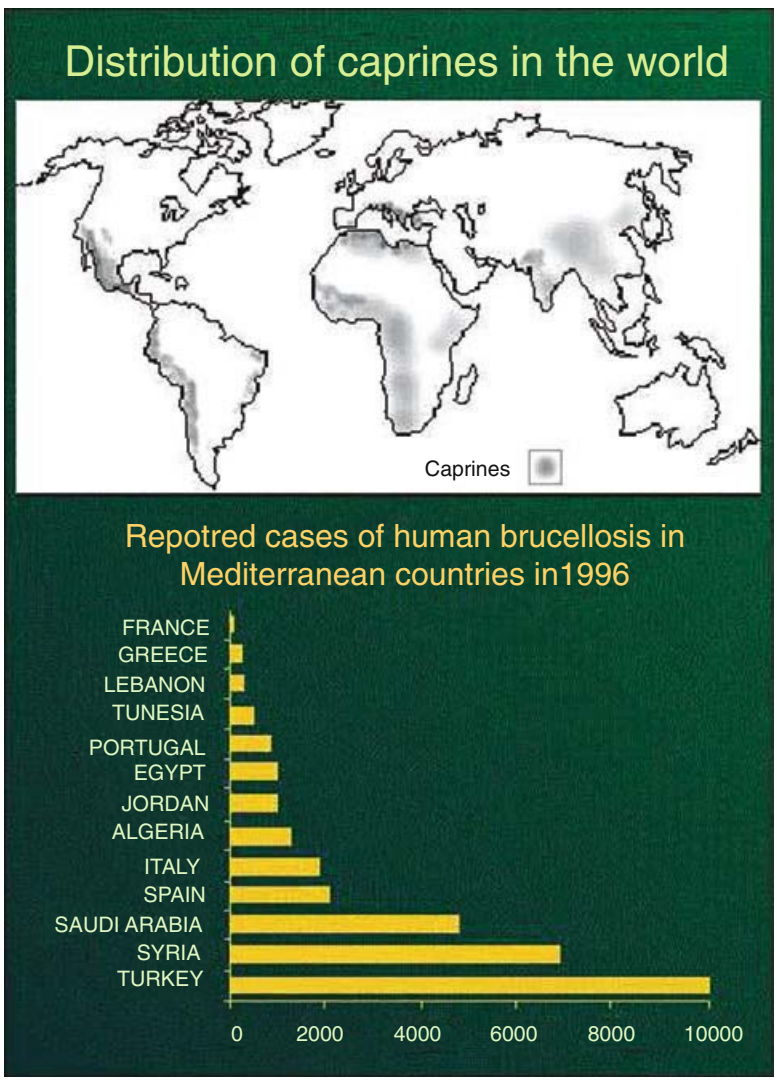


Fig. 29. Geographic distribution of domestic caprines in the world, and human brucellosis in Mediterranean countries for the year 1996. Human and animal brucellosis caused by *B. melitensis* follows the distribution of goats, the natural host for this bacterium. From the United Nations Food and Agriculture Organization/World Health Organization/International Office of Epizootic, Animal Health Year Books.

however, when wild ungulates are involved (i.e., bison), the bacteria may remain for prolonged periods in the population. In this last case, the wild animals may be considered reservoirs of the disease (Davis, 1990; Hayes, 1977). Then, a double hazard occurs: one for wildlife and the other for the domestic animals. Consequently, a conflict between environmentalists and producers arises (McCorquodale and DiGiacomo, 1985). This pattern is more critical in developing countries, mainly in Africa and Asia, since there are not efficient procedures for the control and eradication of *Brucella* in wild animal populations.

Bovine, ovine, caprine and swine brucellosis has also been eradicated from domestic livestock in Canada, Central Europe, Scandinavian countries, Japan and in the northern and central regions of the United States (Crawford et al., 1990; Crespo-Leń, 1994; Plommet, 1992). In Australia and New Zealand, the prevalence of smooth *Brucella* pathogens in bovine, porcine, ovine and caprine herds is close to zero. Although at a low prevalence, *B. ovis* remains in ovine herds in some of those countries with extensive wool production (Crespo-Leń, 1994). In Southeast Asia, bovine and caprine brucellosis is not common, although porcine brucellosis shows a relatively higher prevalence. Bovine, caprine and swine brucellosis also has been eradicated in Uruguay and in some regions of Argentina and Chile. However, ovine and caprine brucellosis caused by *B. ovis* and *B. melitensis* remains in some isolated herds in the latter two countries. In some of the "brucellosis-free" countries and regions, *B. abortus* biovar 1, *B. canis*, *B. suis* biovars 2 and 4 and *B. neotomae* prevail in wildlife hosts, mainly in bison, dogs, wild boars, reindeer and wood rats, respectively, animals native to these countries. In addition, marine *Brucella* strains infect seals, dolphins, porpoises and whales that live in the ocean limits and coasts of many of these "brucellosis-free" countries. In this sense, it could be said that with a few exceptions, *Brucella* pathogens are found worldwide (Jahans et al., 1997).

Brucellosis caused by *B. melitensis* is endemic in Mexico and the principal cause of recurrent infections in caprine and ovine herds as well as the main zoonosis (Crespo-Leń, 1994). In this North American country, *B. melitensis* is also a significant cause of bovine brucellosis due to the breeding of bovines close to goats and sheep. *Brucella abortus*, *B. suis* and *B. canis* are also prevalent as the main cause of brucellosis in bovines, swines and canines, respectively, in populations where goats are excluded. *Brucella suis* may be the second cause of occupational zoonosis in Mexico. In Central America, *B. abortus* (biovar 1) is the leading cause of brucellosis in bovines. *Brucella melitensis* is less frequently found in these animals because of the fewer goats and sheep found in these countries (Crespo-Leń, 1994). *Brucella abortus* (biovar 1) is also the main cause of bovine brucellosis in most South American countries. Peru and Bolivia possess a large number of goats infected with *B. melitensis*, and the domestic livestock is the source of infection for American camelids (llamas and alpacas). *Brucella suis* has been sporadically reported in some areas of Brazil, Uruguay, Argentina and Central America; however, the prevalence is low because of the introduction of intensive management and short-term recycling of swines in these countries.

In the European Mediterranean countries, *B. melitensis* and *B. ovis* are more prevalent than other species of *Brucella* (Plommet, 1992). The northern regions of Portugal, Spain, and Italy, as well as Southern France, have strict control programs for eradication of *B. abortus* bovine brucellosis. In these countries, *B. suis* is detected sporadically and *B. canis* is not currently isolated and may have been eradicated. Among the Mediterranean countries in Europe, Turkey, Greece, Italy, Portugal and Spain have a higher incidence of brucellosis, which is largely caused by *B. melitensis*. In the Eastern European countries and countries of the former Soviet Union, mainly in the Balkanic areas, *B. abortus* and *B. suis* seem to be highly prevalent, although accurate numbers are not available. In some of these countries, in particular those located in the Southern Euroasiatic region in which caprine and ovine herds are maintained, *B. melitensis* remains an important cause of infection. *Brucella suis* biovar 4 is prevalent in reindeer and caribou living in the northern latitudes and are predominantly limited to Alaska, Canada and Siberia.

Brucella melitensis is endemic in the Middle East, Arab countries, Indian Peninsula and southern Asia, mainly due to the population of caprines in these regions. *Brucella abortus* is also present in most of these countries and seems to be more prevalent in Central and Northern Asia (Crespo-Leń, 1994). In these countries, *B. suis* seems to be sporadic, possibly owing to the fact that the population of swines remains small because of religious practices. *Brucella suis* biovar 3 is prevalent in Southeast Asia, the Pacific Islands, Singapore, the Philippines and southern China, and Taiwan, whereas biovar 1 is more frequently found in Indonesia. Although it is known that the major pathogenic *Brucella* species are present in Africa, their prevalence and exact distribution are not known (FAO/WHO, 1986). The prevalence of *B. melitensis* in Africa seems to coincide with the distribution of the large number of caprines existing in many countries of this continent. *Brucella abortus* has been detected in bovine herds and in African wildlife, mainly in

ungulates; however, other mammals, such as elephants, giraffes and camels, have been infected, mainly as a result of contact with domestic livestock. *Brucella suis* also has been detected in feral swines in several African countries.

Although *B. canis* has been detected in widely separated areas, such as Mexico, Madagascar and Japan, the prevalence and exact distribution in the world are not known. It is likely that *B. canis* attains high prevalence in countries possessing a large population of dogs, mainly in developing countries where most dogs freely roam around urban and suburban areas (Carmichael, 1990).

Infecting the Host

It is generally accepted that the most frequent route of infection is through the digestive tract. In goats and probably in other animals as well, the ingested bacteria are engulfed and transferred to the submucosa by intestinal M cells (*Brucella* Invades Healthy Hosts), but the ability to penetrate through the oral and pharyngeal mucosa has also been documented (Enright, 1990b). The conjunctival route seems to be an alternative path for natural infection in herds where intensive management of animals is a common practice (Plommet, 1977). The tissues of the aborted fetuses are massively contaminated with *Brucella* organisms (up to 10^{10} organisms per cm^3 of tissue or allantoic fluid) and therefore are the main source of infection for susceptible hosts. Vaginal secretions are also an important source of infection, mainly for those animals under intensive management and when conditions of close contact among animals prevail. The udder of infected cows, goats and ewes frequently harbors a large number of *Brucella* organisms and the milk is a frequent source of infection. Although calves have been estimated to be more resistant to brucellosis than are adult animals (Russell, 1977), they can be infected by ingesting milk from infected cows, mostly after abortion or parturition. A similar situation may occur with kids, lambs and piglets. In ungulates, the infection is normally acquired through the habit of licking and smelling the aborted products and other infected animals. The congenital route is of epidemiological relevance because animals born from infected mothers may remain latently infected and without symptoms or serological response until pregnancy (Plommet, 1977; Fig. 9). *Brucella* organisms are also transmitted by the venereal route. This channel is more important in swine, ovine and canine brucellosis. Artificial insemination of contaminated semen with *Brucella* organisms is a risk factor for iatrogenic transmission (Manthei et al., 1950).

Although it has been demonstrated that the bacteria can survive in water, soil and manure for limited periods of time and that transmission may occur by penetration of the bacteria through broken skin or by insect bites, these probably are more fortuitous than regular routes and are considered of minor epidemiological importance (Meyer, 1977). Rodents and carnivores do not play a relevant role in the direct transmission; however, they may be important in the indirect transmission of brucellosis. For instance, wild scavengers and dogs may spread contamination by carrying aborted fetuses from infected animals to various sites (Davis, 1990). Accidental hosts, such as humans and horses are frequently infected through the ingestion or inopportune contacts with contaminated raw tissues or milk. From the epidemiological point of view, these accidental hosts are irrelevant for the transmission of *Brucella* organisms. Very little is known regarding the transmission of *Brucella* among marine mammals. It is thought that newborns may acquire the bacteria congenitally or through milk from infected mothers. Nematodes of seals have been proposed to play a role in the transmission of *Brucella* in these animals (Garner et al., 1997).

Vaccines and Vaccination

Long experience has shown that vaccination is an essential tool in the eradication of animal brucellosis (Alton, 1977). Since humans contract the disease from animals and are themselves not a source of contagion, animal vaccination is also decisive in eradicating human brucellosis. Although attempts to vaccinate human beings with some of the living animal vaccines or with subcellular vaccines have been made, these practices have been abandoned. Access to the information on human vaccination with live vaccines is difficult to obtain, but it seems that where implemented, vaccination was discontinued because of the lack of attenuation of the vaccine and/or its noxious side effects. There is information on a subcellular vaccine based on the phenol-insoluble fraction of smooth *Brucella* (Bentejac et al., 1984), but its actual value in humans has never been rigorously tested, and it does not induce protection in suitable animal models (De Bolpe and Garcia-Carrillo, 1991). Although there is a growing interest in developing attenuated strains suitable for human vaccination, none has been tested except in laboratory animals (Crawford et al., 1996; Drazek et al., 1995; Hoover et al., 1999).

Protective immunity achieved by *Brucella* vaccines depends upon several factors, such as animal species, age, gender, administration route, and vaccine dose, type and challenge. Vaccination has been extensively used in cattle, sheep, and goats but not in other domestic

animals, either because the breeding conditions do not make it necessary, or because attempts have been discouraging. None of the existing vaccines against animal brucellosis induce lifelong 100% protection against the challenge encountered in heavily infected flocks, and the most effective vaccines interfere in the serological diagnosis of brucellosis. To solve these problems, subcellular vaccines, adjuvant formulations, and different vaccine strains have been investigated. Moreover, for the classical live vaccines (i.e., *B. abortus* strain 19 and *B. melitensis* strain Rev. 1), variations in the dose, route, age of the animals at vaccination and tests used in the serological follow-up have been studied. So far, only these live vaccines have clearly proven prophylactic efficacy in the natural hosts. The use of dead vaccines in adjuvant is generally considered much less effective, and little research on subcellular vaccines has progressed beyond the laboratory animal models (Blasco, 1990; Nicoletti, 1990a). Attenuated mutants derived from the identification of genes related to virulence (Vaccines and Vaccination; *Brucella* Virulence Mechanisms) have been rarely tested in the natural hosts and either the studies have not been continued (Cheville et al., 1993) or the results are inferior to those obtained with the classical vaccines (Elzer et al., 1998).

Animals are generally immunized with the vaccine strain homologous to the *Brucella* spp. for the corresponding host range (i.e., *B. melitensis* vaccines are used in sheep and goats and *B. abortus* in cattle). There have been attempts to use *B. abortus* strain 19 in small ruminants but the results have been less satisfactory than those obtained with the homologous vaccine. Although preliminary reports are encouraging (Garcia-Carrillo, 1980; Horwell and Van Drimmelen, 1971), *B. melitensis* Rev. 1 has not been used in cattle and its safety in this host is unknown.

B. melitensis Rev 1 Attenuated *B. melitensis* biotype 1 Rev. 1 (revision one) vaccine has been efficiently used for more that 40 years in the prevention of caprine and ovine brucellosis, including *B. ovis* infections (Blasco et al., 1984b; Blasco et al., 1987; Marín et al., 1999). Although it is not exempt of problems (Origin, Characteristics and Attenuation of Rev 1), vaccination of sheep with the standard dose by the conjunctival route is the most practical means to rapidly reduce the rate of *B. melitensis* infection in both sheep and humans (Garin-Bastuji et al., 1998). This smooth-strain vaccine displays some residual virulence inducing abortions and testicular problems when administrated to sexually mature caprines and ovines, and it is also capable of infecting humans (Crespo-León, 1994). Some female animals vaccinated with Rev. 1 may release the bacteria in milk for several weeks or months.

Origin, Characteristics and Attenuation of Rev 1 *B. melitensis* Rev. 1 is strain number 1 of a set of revertants obtained from a streptomycin-dependent strain by Elberg and his colleagues at the University of California in the mid-1950s (Alton and Elberg, 1967, 1981). This strain has a smooth phenotype (Response to Environmental Stress), but also has some traits that allow its differentiation from field strains (Identification of Vaccine Strains), an essential property in a live vaccine against brucellosis. Its rate of smooth-to-rough dissociation (with the ensuing over-attenuation) is relatively high and calls for strict vaccine quality control. Moreover, the biological properties of Rev. 1 seeds conserved in different laboratories seem to have shifted through the years, so that the bacteria in different commercial vaccines may vary in critical properties. There are demonstrated cases in which the particular vaccine used clearly lacked attenuation (Blasco, 1997), whereas in other cases the vaccine varied to the rough phenotype (E. Díaz-Aparicio, personal communication). Biological quality control procedures, which should be implemented at least for periodic seed stock control, have been developed to measure Rev. 1 residual virulence and immunogenicity (Bosseray, 1991; Grilló et al., 2000).

The original Rev. 1 strain is attenuated in mice and guinea pigs, where it develops an infection milder than that caused by virulent strains and elicits protective immunity. It is also attenuated in goats and sheep where it induces a generalized infection, which is practically eliminated from most animals by the fourth month post vaccination (Alton and Elberg, 1967, 1981). The cause(s) of the attenuation is not known but the dye sensitivity pattern suggests that at least some properties of the outer-membrane are altered. It is important to stress that, despite its attenuation in animals, Rev. 1 is clearly infectious for humans (Alton and Elberg, 1967, 1981) and resistant to streptomycin (Human Brucellosis). Although it is rarely excreted in sheep milk, goats are reported to excrete Rev. 1 more often and for longer times, thus posing a hazard for farmers (Alton, 1990a). Moreover, veterinarians applying the vaccine and workers restraining the animals during this process are also at risk.

Protection of Rev 1 The vaccine can be administered either subcutaneously or conjunctivally with no differences in the degree of protection obtained. In controlled experiments, it has been demonstrated that at the optimal (or standard) dose (about 10^9 colony forming units [CFUs]) Rev. 1 protects 70–85% of the sheep against a

challenge (about 10^8 CFU of a virulent *B. melitensis* strain) infecting 75–100% of unvaccinated controls (Blasco, 1997; Garin-Bastuji et al., 1998). Under field conditions, the immunity obtained in sheep and goats is satisfactory for eradication purposes when vaccination is combined with the removal of infected animals detected in the most specific serological tests (Alton, 1990a; Garin-Bastuji et al., 1998). Controlled studies in goats show that immunity induced by Rev. 1 vaccination may last longer than four years. In sheep, the experimental evidence shows a high level of immunity for at least two and a half years (Alton, 1990a). However, it is not known whether vaccination of young animals with the standard dose induces lifelong immunity. Obviously, adult vaccination would be desirable for eradication and in fact must be applied when programs start and prevalence is high (Garin-Bastuji et al., 1998), but this increases the side effects of vaccination. Also, Rev 1 is the best vaccine available against *B. ovis* ram epididymitis, and the conditions of use are the same as in ewes (Blasco, 1990; Marń et al., 1990).

Interference in the Serological Diagnosis and Other Side Effects of Rev 1 Because it is a smooth strain, Rev. 1 induces antibodies interfering in the smooth-LPS serological tests and this complicates the diagnosis (Immunological Diagnosis of Animal Brucellosis Caused by Smooth *Brucella*). The duration of this response depends on the age of the animals at vaccination. The duration is longer as they approach or reach sexual maturity. It is significantly more protracted when the subcutaneous route is used and, in this case, many animals show positive serological results for years. When using the best strategy (i.e., the conjunctival route and less than 6-month-old animals), the great majority of the animals become serologically negative in the most specific serological test in less than a year (Alton, 1990a; Fensterbank, 1987; Garin-Bastuji et al., 1998; Jiménez de Bagüs et al., 1989; Plommet and Fensterbank, 1984; Zundel et al., 1992).

Rev. 1 is very abortifacient and rates of vaccine-induced abortions can be as high as 80% if animals are vaccinated in the second to third month of pregnancy (Alton, 1990a; Alton and Elberg, 1967; Blasco, 1997; Garin-Bastuji et al., 1998). The risk of inducing abortions is substantially reduced but not eliminated when the conjunctival route of vaccination is used (Blasco, 1997; Jiménez de Bagüs et al., 1989; Zundel et al., 1992). Alternatively, the use of reduced doses (from 10^5 to 10^7 CFU) has been proposed but abortions are not totally avoided, and there is clear evidence that the immunity achieved is not adequate (Blasco, 1997).

B. ABORTUS STRAIN 19 *B. abortus* strain 19 vaccine has been extensively used in cattle. With a few exceptions, most of the successful control and eradication programs have been carried out with this strain. It is noteworthy that this vaccine was developed more than 70 years ago and that, although several alternative attenuated strains have been developed subsequently and studied, none has such a wide application as strain 19.

Origin, Characteristics and Attenuation of S19 Strain 19 is derived from the nineteenth stock culture made by Buck from a milk isolate in the early twenties. The culture was maintained at room temperature for over a year and this resulted in attenuation in the guinea pig. Strain 19 has a smooth phenotype (Response to Environmental Stress), and since occasionally it dissociates to yield rough mutants unsuitable for vaccination (Colonies and Dissociation), quality control of at least this property is necessary. It carries some traits that allow its typing and differentiation from field strains (Identification of Vaccine Strains), including inhibition by erythritol (Erythritol).

As compared to field isolates, strain 19 is also markedly attenuated in cattle and only induces a transient infection in heifers that is usually cleared in a few months and seldom lasts for more than a year (Nicoletti, 1990a). When used in adults, however, about 2% of the animals develop udder infections and shed the vaccine in the milk for several years. Although the precise reasons for the attenuation are not known, strain 19 clearly shows altered intracellular trafficking (Association with the Autophagic Machinery; Interleukins in Brucellosis). The deletion in the *eri* region carried by this vaccine and the alteration of outer-membrane properties suggested by its antibiotic and dye sensitivity pattern may be related to the loss of virulence. Although not as dangerous as *B. melitensis* Rev. 1, *B. abortus* strain 19 also has been a demonstrated cause of human infections (Meyer, 1985).

Protection of S19 In controlled experiments, 40–75% of heifers vaccinated subcutaneously with the standard dose (10^{11} CFU) are protected against conjunctival challenges infecting 100% (about 10^7 CFU) to 87% (about 10^6 CFU) of the unvaccinated controls (Nicoletti, 1990a), and the level of protection is no different when the conjunctival route is used (Nicoletti et al., 1978; Plommet and Fensterbank, 1984). Although these experiments also show that the immunity obtained can be overcome by a heavy challenge, it has been repeatedly shown under field conditions that strain 19 vaccination reduces the prevalence to very low levels and that eradication is possible when the vaccine is combined with the

removal of the reactors in serological tests of the adequate specificity (Immunological Diagnosis of Animal Brucellosis Caused by Smooth *Brucella*). Protection is reported to last for more than five pregnancies, and it is not clear whether revaccination increases immunity (Nicoletti, 1990a). A single dose has proved to be enough to achieve eradication in test and slaughter programs applied to herds varying from medium to small size. In these cases, the problems created by revaccination in the interpretation of the serological tests (Interference in the Serological Diagnosis and Other Side Effects of S19) would outweigh the potential benefits. On the other hand, revaccination may be necessary to control the disease in large herds, or under conditions in which obligatory slaughtering is not economically viable, as is the case in low income countries (Nicoletti, 1990a). This strategy is the most practical one in those cases where diagnosis is not routinely carried out as part of a control program and where abortion is causing the major losses. Revaccination in these cases slows the abortion rate and decreases the level of infection by lowering the bacterial inocula. From this perspective, revaccination must be considered an emergency strategy. Some experiments have shown that the best protection is obtained by subcutaneous vaccination of calves followed by a conjunctivally administered booster dose (Plommet and Fensterbank, 1984).

Interference in the Serological Diagnosis and Other Side Effects of S19 Although the level of protection induced by strain 19 is good, the presence of residual antibodies against the *O*-polysaccharide complicates the differentiation between vaccinated and infected individuals. To solve this problem, four major strategies have been proposed (Hidalgo et al., 1977): (1) to develop sensitive and specific serological tests capable of differentiating infected from vaccinated animals, (2) to limit vaccination to calves, (3) to reduce the vaccine dose, and (4) to administer the vaccine by the conjunctival route. Each strategy has its benefits and drawbacks.

Although the combination of serological tests under defined circumstances may achieve sensitivity and specificity close to 100% (Immunological Diagnosis of Human Brucellosis Caused by Smooth *Brucella*), the specificity and sensitivity of these assays considerably decrease under conditions in which infection of some vaccinated individuals occurs or when revaccination is used. Calf vaccination shortens the time lapse during which there are significant levels of antibodies against the *O*-polysaccharide, but the level of protection is lower than that obtained in adult animals (Nicoletti, 1977). The optimal age of calf vaccination is between 3 and 6 months, and under these conditions, most animals are negative in the most specific tests a year later. On the other hand, if vaccinated when they are 6 months to one year old, up to 20% of the animals may be serologically positive 18 months later (Nicoletti, 1990a; J. M. Blasco, personal communication). As discussed above, there are a number of situations that make vaccination of adults necessary but the vaccine persists in adults for a longer time than it does in calves and induces a more protracted serological response. Conjunctival vaccination (two doses of 5×10^9 CFU at a 4–6 month interval) dramatically reduces the serological response without compromising the level of immunity obtained (Nicoletti, 1977; Nicoletti et al., 1978; Plommet and Fensterbank, 1984), and reducing the subcutaneous dose (from 5×10^9 to 5×10^8 CFU) also reduces the serological response. In a large field study, no differences in prevalence rates were found between groups of animals that had received the standard or the reduced dose, and the usefulness of the latter has been confirmed in other studies (Nicoletti, 1990a). However, reduced doses have to be used with care and always considering the age of the animals and particular conditions of the herds as other studies have shown that both younger and old heifers are not adequately protected by strain 19 at a dose of 1×0^9 to 9×10^6 CFU (Huber et al., 1990).

B. abortus strain 19 is abortifacient when applied to pregnant cattle but the rate of abortions is relatively low (about 5%). As mentioned (Origin, Characteristics and Attenuation of S19), adults may develop udder infections and shed the vaccine in their milk.

B. suis Strain 2 An attenuated strain (strain 2 or S2) was obtained in China after serial transfer of a virulent *B. suis* biotype 1 strain on culture media for years. *Brucella suis* strain 2 is of smooth type, shows the same level of attenuation as strain 19 shows in guinea pigs, and does not revert to the virulent state after repeated passaging through guinea pigs, sheep, goats or boars. It is reported to protect cattle, goats, sheep and pigs when administered orally. Implementation of vaccination programs in semiarid grasslands where sheep and goat breeding is extensive is necessarily difficult. Since in these areas the only source of drinking water is from wells, *B. suis* strain 2 has been administered orally in the drinking water. By this route, the S2 vaccine has been widely used for prevention of animal brucellosis in China (Xin, 1986). Good results were also obtained with sheep in a trial in Libya (Mustafa and Abusowa, 1993). However, in controlled experiments performed in France (Verger et al., 1995) and Spain (Blasco et al., 1993b), *B. suis* strain 2 administered subcutaneously did not

protect sheep against a *B. melitensis* or a *B. ovis* challenge under conditions in which the standard *B. melitensis* Rev. 1 was effective. The different breeds employed, differences in the challenging strains used, the climatic and nutritional conditions and the difficulties in obtaining reliable data under field conditions may account for the discrepancies.

ROUGH VACCINES The use of rough vaccines has been proposed on the premise that protective immunity in bovine brucellosis is carried exclusively by cells and that the generation of antibodies against the *O*-polysaccharide is unnecessary and undesirable, since it complicates the differential diagnosis (Cunningham, 1977a).

B. abortus 45/20 A killed vaccine prepared with the rough strain 45/20 suspended in a water-in-mineral oil emulsion has been used in the past (Cunningham, 1977a). This vaccine was developed to overcome the disadvantages of the interference in the serological tests caused by strain 19, which limited its use to calves before the development of more specific serological tests and the demonstration of the usefulness of reduced doses and the conjunctival route in adult cattle. However, strain 45/20 was shown to revert to smooth pathogenic forms when injected into cattle, and this limited its use to a killed in adjuvant vaccine (Adams, 1990; Chukwu, 1985). Although vaccine 45/20 induces some protection in adults, calves of less than six months old may not be well protected (Nicoletti, 1990a). Also, some commercial 45/20 vaccines undoubtedly induced antibodies to the smooth-LPS (Díaz and Jones, 1973b), thus reducing the only advantage of this vaccine. In countries where the conditions were favorable (low prevalence and intensive breeding with good animal management practices), vaccine 45/20 was found to be useful in providing herd resistance (Roerink, 1969) but other experiences have been less favorable (Plenderleith, 1970; Ray, 1976).

B. abortus RB51 In recent years, a great deal of attention has been placed on the possibilities that *B. abortus* strain RB51 offers as a live vaccine (Schurig et al., 1991). Strain RB51 is a spontaneous rough mutant selected after repeated in vitro passage of *B. abortus* 2308 (United States Department of Agriculture challenge strain) in the presence of rifampin and penicillin (Schurig et al., 1991). Selection was performed using the crystal violet and acriflavine tests (Colonies and Dissociation) and a considerable effort has been dedicated to the genetic characterization of RB51. The RB51 strain carries an IS*711* spontaneously inserted in the *wboA* gene (Table 5), which is undoubtedly related to its phenotype and attenuation (Vemulapalli et al., 1999). However, *B. abortus* 2308 *wboA* mutants obtained by transposon mutagenesis are not equivalent to RB51, which shows that the latter strain carries additional and unknown defects (McQuiston et al., 1999). The strain is stable and, although it should show very little or no virulence in humans, there is little experience on this point. RB51 infections cannot be diagnosed in the standard serological test, as this vaccine lacks the O-polysaccharide (see Immunological Tests for Brucellosis), and it has to be stressed that it is resistant to rifampin (Treatment).

Being a rough strain, RB51, although it produces small quantities of *N*-formylperosamine polysaccharides (G. G. Schurig, personal communication), does not induce antibodies interfering in the smooth-LPS serological tests (Stevens et al., 1994b) even when used as a booster in adult animals vaccinated with strain 19 during calfhood (Uza et al., 2000).

This vaccine has been reported not to be abortifacient in cattle (Edmonds et al., 1999; Palmer et al., 1997; Uza et al., 2000). However, animals vaccinated at pregnancy excrete it in the milk (Uza et al., 2000) and full doses of RB51 (1×10^{11} CFU) administered intravenously induce severe placentitis and placental infection (Palmer et al., 1996). Moreover, recent evaluations of some field experiences have demonstrated high numbers of RB51 isolates in aborted cattle (Lopetegui, 1999). When using the reduced dose of this vaccine (1×10^{9} CFU), no abortions or placentitis lesions are produced in subcutaneously vaccinated cattle (Palmer et al., 1997). However, this reduced dose does not protect against *B. abortus* (Olsen, 2000).

The data on the level of protection afforded by RB51 in cattle are contradictory. In field studies carried out in Venezuela, it was concluded that RB51 was superior to strain 19 (both used at a dose of 5×10^{9} CFU) in protecting against the natural challenge existing in infected herds (Lord et al., 1998b). On the other hand, in a controlled experiment, RB51 protected 26 of 29 vaccinated animals and strain 19 protected 20 of 22 (both used at a dose of 10^{10} CFU), but only a moderate challenge was used in the experiment (12 of 20 nonvaccinated controls were infected) (Cheville et al., 1996b). More recently, the results of a study in which 3-month-old heifers were given RB51 at doses ranging from 10^{9} to 1.4×10^{11} have been presented (Olsen, 2000). Four of eight nonvaccinates, two of four 10^{9} CFU vaccinates, and two of fourteen 1.4×10^{11} CFU vaccinates aborted. In a separate study conducted with 6-month-old heifers, three of seven nonvaccinates and one of eighteen 10^{10} CFU vaccinates aborted. The author concluded that RB51 was efficacious in preventing abortion and fetal infec-

tions, but the data also show that the incidence of maternal infection with *Brucella* at necropsy did not differ among RB51 vaccinates and nonvaccinated controls (Olsen, 2000). Additional comparative experiments with strain 19 under controlled conditions are necessary to define the level of protection achieved with this vaccine in cattle, and it is still premature to draw conclusions on its ability to protect under field conditions. Up to now, RB51 vaccine seems to prevent brucellosis under conditions of low prevalence; however, under conditions of high prevalence, this vaccine has not been satisfactorily tested. Our own experience is that under field conditions, strain 19 performs better than RB51, at least in circumstances where high inocula of virulent *Brucella* organisms (high incidence in the herd) are likely to occur (E. Moreno et al., unpublished observations).

B. abortus RB51 does not seem useful in sheep. In a stringent experiment (100% of infection in unvaccinated controls), RB51 afforded no protection against *B. ovis* under conditions in which Rev. 1 fully protected 40% of the animals (Jiménez de Bagüés et al., 1995). Moreover, controlled evaluations against *B. melitensis* challenges are also unsatisfactory (el-Idrissi et al., 2001). There is also one study in pigs, although conducted under field conditions. In these animals, the vaccine was reported to induce 100% protection regardless of the dose (10^6 or 10^9), the number of injections (1 or 3) and the administration route (oral and intramuscular) (Lord et al., 1998a). Controlled experiments would be necessary for a further evaluation of the vaccine in pigs.

Control, Eradication and Prevention

Control and subsequent eradication of brucellosis can be consummated by the simple, costly and sometimes impractical procedure of totally eliminating the primary hosts from the designated area, with or without replacement of the population with clean animals. Although this method has been attempted by some, most countries that have achieved eradication of brucellosis from bovine, ovine and caprine herds have followed a stepwise strategy (Boyd, 1977; Flowers, 1977). The first initiative towards the control of brucellosis in a specific population usually consists of the serological examination of the animals (Immunological Tests for Brucellosis). Under circumstances where the animal population has not been vaccinated, a simple sensitive test (i.e., card or rose bengal) is enough for identification of the infected animals. However, when strain 19 vaccination is carried out, a combination of sensitive and highly specific assays (Immunological Diagnosis of Animal Brucellosis Caused by Smooth *Brucella*) for the differentiation of infected and vaccinated animals (i.e., complement fixation, radial immunodiffusion [RID], enzyme-linked immunoabsorbent assay [ELISA] or competitive ELISA) is required. The second initiative is the vaccination of the population at risk followed by removal and slaughtering of the serologically positive animals. During this phase, brucellosis-free farms and regions with or without vaccination could be declared. The final step is to stop vaccination. Constant serological surveys are necessary, mainly when potential foci of contamination are colliding with brucellosis-free areas. Only after several years of continuous surveillance, brucellosis may be considered eradicated from these nonvaccinated serologically negative herds. Up to now, only strain 19 and Rev. 1 vaccines have demonstrated to control and eradicate brucellosis under conditions where control programs have been implemented (Blasco, 1997; Nicoletti, 1990a).

One very important aspect in the prevention of brucellosis outbreaks is to limit and strictly control the import of animals from regions where brucellosis has not been eradicated. Similar to this is the introduction of susceptible hosts into areas where brucellosis may remain latent in the population. This is especially important in cases involving wildlife hosts, such as the introduction of bovines, goats or ovines in regions where bisons, reindeer and perhaps marine mammals remain as potential reservoirs of *Brucella* organisms.

Zoonosis

Human brucellosis is a highly contagious bacterial zoonosis, which follows the distribution of the natural animal hosts (Fig. 29). Removal of the infected hosts, therefore, eliminates the contagion risk. Human brucellosis is most frequently found in third world countries, mainly due to the high prevalence of *Brucella* infections in domestic herds and to the ingestion of unpasteurized dairy products. The disease in humans is also known as "undulant fever" or "Malta fever," since the first isolation of the causative bacterium was made in the Mediterranean island of Malta from spleens of British soldiers who became infected as a consequence of drinking contaminated caprine milk. The most common *Brucella* species infecting humans are *B. melitensis*, *B. abortus* and *B. suis*. Among these, *B. melitensis* and *B. suis* are the most aggressive species for humans. These bacteria cause a severe syndrome, which if not treated may lead to death. *B. canis* has been reported sporadically to infect humans. *B. ovis*, *B. neotomae* and the marine *Brucella* strains have not been detected in humans. The marine

strains may represent, however, an authentic hazard for human communities that hunt and gain subsistence from whales and seals, as well as for workers that are in close contact with marine mammals (Garner et al., 1997; Higgins, 2000).

Contamination of humans depends upon interaction with infected animals, their products, or their secretions. Because of the wide acceptance of pasteurization, brucellosis is regarded in many countries as an occupational disease of sporadic occurrence among livestock handlers, veterinarians, vaccinators, laboratory workers, and meat-processing and slaughter plant personnel. In many third world countries the consumption of raw milk and unpasteurized dairy products, such as butter, fresh cheese, cream or curd, as well as fresh raw viscera and blood is still an important source of infection. The infection of domestic animals, frequently accompanied by slaughtering, and the possibility of contamination of food products with *Brucella* add important restrictions to the marketing of foodstuffs. In consequence, incomes for many families are reduced.

The diagnosis of human brucellosis is well established (Antibody Detecting Tests in Infected Patients). It depends mainly on the detection of antibodies in the serum of patients against *Brucella* organisms by immunological techniques. The confirmation of infection is achieved by the isolation of the etiological agent in the blood of infected patients. Although pasteurization is the mandatory public health procedure, examination of foodstuffs, presumed to be the source of contagion, may occasionally be necessary. The demonstration of antibodies in milk is suggestive of herd infection and of the possible presence of *Brucella* spp. in dairy products, but it is not a specific method of diagnosis because revaccination or adult vaccination can cause secretion of antibodies in milk. Shedding of the vaccine strains by the immunized animals may also occur. Since vaccine strains are virulent for humans, their presence in dairy products constitutes a zoonotic risk. Isolation of *Brucella* organisms can be achieved from milk, but it is more difficult when processed dairy products are concerned, complicating matters even more. Culture on selective media follows the same procedures described for animal diagnosis by bacteriological culture (Molecular Tests for Humans; Molecular Tests for Animals). Molecular methods, such as PCR, may be useful and could be used to detect some of the vaccine strains (Molecular Tests for Humans; Molecular Tests for Animals), but there are few reported studies on their application to processed foodstuffs.

Disease

Brucellosis is a disease of the reticuloendothelial system, the reproductive organs and of the fetus, and then the pathological signs are perceptible accordingly (Pathology). In the primary host the main manifestation of the disease is abortion or epididymitis. In humans the syndrome is in extreme complex and could be evidenced in many forms, recurrent fever being one of the main manifestations. From a gross perspective, the pathobiology of brucellosis is in several respects similar to that of other intracellular bacteria; however, it possesses many idiosyncratic manifestations and a unique intracellular life cycle (Pathobiology). The infection is controlled by an efficient immune cellular response (Control of the Infection), although antibiotic therapy is mandatory in humans (Treatment). Diagnosis is mainly based in determining the immune response of the infected host and in the isolation of the bacteria from infected tissues and blood (Diagnosis).

Pathology

The pathology of brucellosis is complex and its study requires biosafety conditions (Biosafety) due to the fact that the bacteria are primary pathogens of animals (Brucellosis in the Host Animal) and humans (Brucellosis in Humans) and then are a risk for contamination. For comprehensive reviews on the pathology of brucellosis, the publications of Blasco (1990), Enright (1990a) and Spink (1956) are recommended.

The Disease Named Brucellosis

Considering the close phylogenetic relationship among the different members of the genus, it is not surprising that the pathologies induced by the various *Brucella* species in their host animals are very similar. Likewise, the gross physiopathological events that occur in accidental or experimental infections in secondary hosts, such as humans, mice and horses, have many things in common. Although some strains are more virulent than others, the different pathologies observed seem to be related more to idiosyncratic differences of the hosts than to individual virulence properties of the *Brucella* strains. This means that once *Brucella* organisms have gained access into the tissues, they proceed in a very similar manner. Control of the infection depends mostly on the confrontation between the host defenses, on one side, and the ability of *Brucella* to reproduce and gain access to various tissues without being destroyed, on the other (Control of the Infection).

Most of the pathobiological studies have been performed in experimental animals, such as mice

and guinea pigs, as well as with a variety of cell lines. In the natural hosts, studies have been conducted with bovines, caprines and ovines and with strains of *B. abortus*, *B. melitensis* and *B. suis*. Although the data are more limited, similar pathologies have been observed with the marine strains *B.* "*maris*," *B. canis* and *B. ovis* in their natural hosts. This last species, however, demonstrates preference for male ovine reproductive organs, with low pathogenicity for ewes (Blasco, 1990). Similar to *B. ovis*, the wood rat parasite *B. neotomae* seems to display low virulence, even though production of highly aggressive strains generating similar events to other *Brucella* species has been reported (Gibby and Gibby, 1965). Finally, the study of human brucellosis has contributed to the understanding of the pathology and pathobiology from various perspectives.

Brucellosis in the Host Animal In general terms, brucellosis can be viewed as a chronic infection, which in nontreated cases may cure or persist for life, mainly within cells of the reticuloendothelial system (Blasco, 1990; Enright, 1990b). The incubation period in the preferred animal host under natural conditions depends upon the *Brucella* strain, inoculum size, as well as upon host factors. In the natural host, incubation times range from two to five weeks, although longer periods are not exceptional. In the accidental host, the incubation period is more variable and generally longer than in the natural host animal, often lasting for several months. The onset of symptoms in the natural hosts normally courses with recurrent fever and enlarged lymph nodes, spleen and liver. Arthritis with gastrointestinal and nervous symptoms may be observed. Bacteremia normally coincides with the fever events. In the natural pregnant host, the infection frequently results in abortion in the last trimester of gestation and in contagion to other members of the herd. In males, invasion of the reproductive organs is frequently observed, facilitating venereal transmission and inducing partial or total sterility. Orchitis and epididymitis are two prevailing signs. In the immunocompromised host, invasion of many organs, including the central nervous system, may take place followed by death. Reproductive impairment in females may occur upon placental retention or for other causes; however, this event is exceptional rather than a common outcome in brucellosis.

The most frequent ports of *Brucella* entrance are the mucous membranes of the oral, respiratory and gastrointestinal tracts, as well as those membranes covering the conjunctiva, vagina and the prepuces (Enright, 1990b). Very likely bacterial penetration takes place through the epithelial cells lining the mucous membranes (*Brucella* Invades Healthy Hosts). At the site of lodgment, the organisms are ingested by resident phagocytic cells and then carried via the lymphatics to the regional lymph nodes. Once ingested, the bacteria travel to a specialized intracellular compartment where they multiply. Some of the bacteria and cells die during the confrontation, and the released *Brucella* debris stimulate a local immune response with the concomitant activation and proliferation of inflammatory and mononuclear cells (Anderson et al., 1986b; Anderson et al., 1986c; Cheville et al., 1992; Tobias, 1993). The result of this confrontation determines whether the infection is contained or transgresses to other organs. In the immunized host, the memory and activated cells of the immune system normally are capable of dominating the infection (Control of the Infection). If not, polymorphonuclear leukocytes and macrophages carrying the bacteria reach the blood, transporting the microorganisms to the reticuloendothelial system, mammary glands and reproductive organs (Enright, 1990b). The histopathological signs of the colonized lymph nodes are typical of infections by intracellular bacteria, demonstrating hyperplasia with mononuclear infiltration, local hemorrhage, presence of granulomas with histiocytes, epithelioid cells and gigantic Langerhans cells, edema and extramedullary hematopoiesis. Severe paracortical lymphocyte depletion, destruction of germinal centers and serofibrous lymphadenitis with effacement of lymph node architecture are observed during the first weeks of infection. In most cases, dead and alive intracellular bacteria can be detected in large numbers within phagocytic cells and in lesser proportion within lymphocytes (Fig. 30).

The Fetus, the Most Susceptible Host In pregnant animals, such as bovines, caprines, ovines, swine and very probably marine mammals, the bacteria reach the gravid uterus, infecting the cotyledons (Anderson et al., 1986b; Anderson et al., 1986c). Once installed in this site, the bacteria replicate within the endoplasmic reticulum of erythrophagocytic trophoblasts followed by extensive replication within chorioallantoic trophoblasts (Fig. 30). This action generates necrosis and subsequent ulceration of the chorioallantoic membrane with production of anti-inflammatory exudates composed of phagocytic cells and cellular debris (Fig. 31). At this point, *Brucella* can be observed within the uterine lumen and in the placental capillaries disseminating through the chorionic villi. As a consequence, placentomal and fetal infection with concomitant inflammation proceeds. Interestingly, *Brucella* organisms are not found within placentomal trophoblasts, cells of the

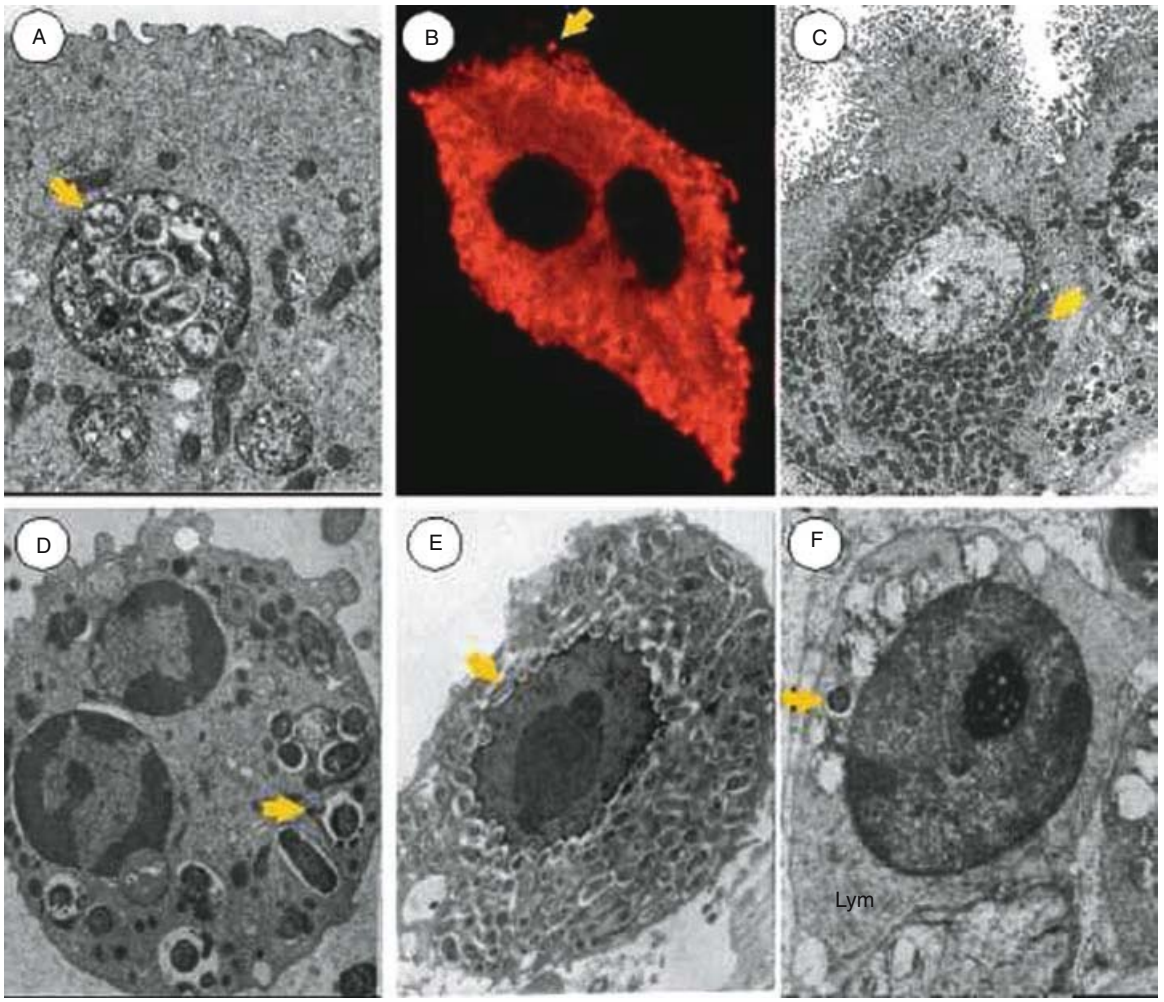


Fig. 30. Intracellular parasitism of *Brucella* in different host cells. Transit of *B. abortus* in caprine M (lymphoepithelial) cells (A); dividing HeLa cell with replicating *B. abortus* (in red) within the endoplasmic reticulum (ER) (immunofluorescence) (B); caprine trophoblast with replicating *Brucella* within the ER (C); human polymorphonuclear leukocyte with intracellular *B. abortus* in phagosomes and phagolysosomes (D); murine macrophage with replicating *B. abortus* within cisterna resembling endoplasmic reticulum (E); and caprine lymphocyte with intravacuolar *B. melitensis* (F). In (A), extracellular material was co-ingested with *Brucella* organisms. Notice in (B, C and E) that the cell nucleus is constrained but not invaded by the replicating brucellae. Notice in (B) that karyokinesis is not inhibited by the replicating brucellae. Notice in (D) that several azurophil granules fuse with phagosomes containing *Brucella*. In spite of the large number of intracellular bacteria the cells do not present signs of apoptosis or necrosis. The arrows indicate a single bacterium within a vacuolar compartment. Panel (A) is from Ackermann et al. (1988), panel (C) from Anderson et al. (1986b), panel (D) from Riley and Robertson (1984a), panel (E) from Jiang and Baldwin (1993b), and panel (F) from Cheville at al. (1996a), all with permission.

endometrium or syncytial epithelium (Fig. 32). Abortion without significant compromise and bacterial invasion of the endometrium or lamina propia are characteristic of this phase. Extensive necrosis promotes diffusion of *Brucella* into different adjacent tissues. Vasculitis with separation of the maternal epithelium promotes fetal death and abortion. Ulcerative endometritis and metritis may occur as a consequence of postparturient infections with opportunistic bacteria. From the clinical point of view, abortion due to brucellosis resembles other infectious diseases, although retention of placental tissues or endometritis seldom occurs in the natural hosts.

Following *Brucella* invasion and depending on the age of the fetus, leukocytosis with significant numbers of neutrophils develops. In ruminants, the infected fetuses demonstrate lymphoreticular hyperplasia of secondary lymphatic organs and extensive granulomatosus centers composed of macrophages, histiocytes, epithelioid cells, gigantic Langerhans cells, extramedullary hematopoiesis and a variable number of polymorphonuclear granulocytes. Liquefaction and

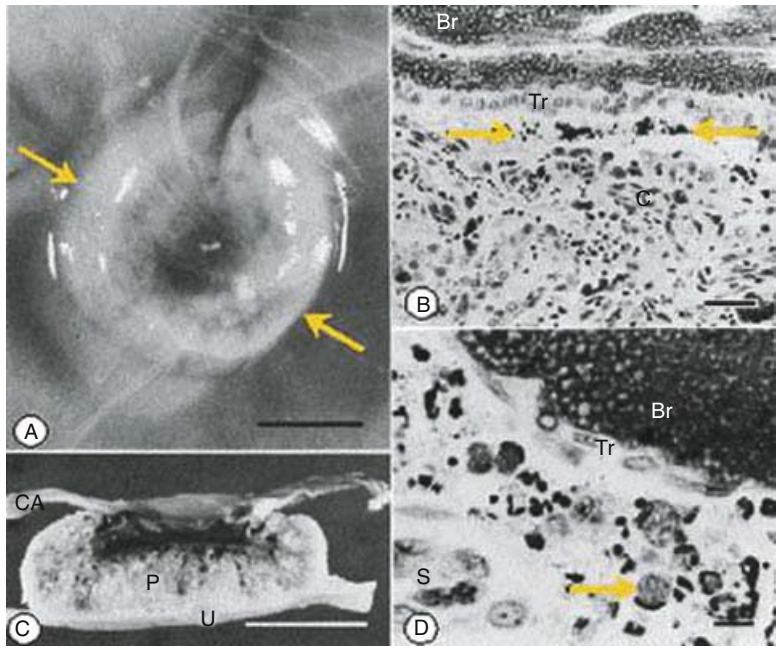


Fig. 31. Pathological examination of placentoma from a pregnant goat infected with *B. abortus*. A) Diffuse chorioallantoic edema (arrows) obscuring view of the placentoma in a goat given 10^9 *B. abortus* cells intravenously and necropsied on postinoculation day 19. White periplacentomal exudate is predominant (arrows). Bar = 1 cm. B) Multifocal abscesses and bacterial colonies of *B. abortus* in the placentoma are observed in the connective tissue of chorionic villi and rimmed by intact trophoblastic epithelium (tr). Exudate (arrows) separates maternal septa (s) and capillaries (c) from the bacteria-filled chorionic villus. Bar = 30 µm. C) Placentoma from a goat that was inoculated in a middle uterine artery with 10^9 *B. abortus* cells and euthanized while aborting on postinoculation day 14. Multiple abscesses and hemorrhages are present in placentomal cross-sections. Uterine wall (u), placentoma (p), chorionic membrane (ca). Bar = 1 cm. Multifocal abscesses and bacterial colonies of *B. abortus* are observed in the placentoma. D) Bacteria-filled phagocytes (arrow) and necrotic cell debris separating maternal septa (s) from trophoblast (tr). Brown-Hopps Gram stain. Bar = 20 µm. From Anderson et al. (1986a), with permission.

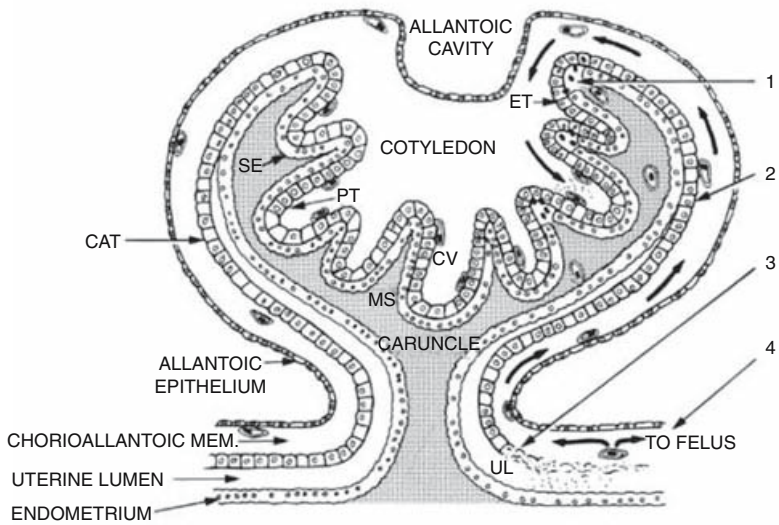


Fig. 32. Diagram summarizing the pathogenesis of placental and fetal infection in a caprine placentoma, as determined in this study. 1) *B. abortus* first seen in phagosomes of erythrophagocytic trophoblasts (ET). 2) Subsequently, *B. abortus* replicates in the rough endoplasmic reticulum of chorioallantoic trophoblasts (CAT). 3) After trophoblast infection, necrosis of chorioallantoic trophoblasts, spewing of *B. abortus* into the uterine lumen (UL), and ulceration of the chorioallantoic membrane occur. 4) Coincidentally, intravascular *B. abortus*, present in placental capillaries, spreads to chorionic villi (CV) and fetal viscera. *Brucella abortus* is not present in placentomal trophoblast (PT), maternal septa (MS), syncytial epithelium (SE), or endometrium. Erythrophagocytic, placentomal, and chorioallantoic trophoblasts form the continuous epithelial sheet that covers chorionic villi of the cotyledon and the chorioallantoic membrane. They are differentiated here, owing to differences in anatomic location, function, and role in *Brucella placentitis*. From Anderson et al. (1986b), with permission.

mineralization of the necrotic zones may occur. Commonly, these reactions are also observed in a variety of fetal organs, such as the liver, lungs and kidney. *Brucella* invasion of the central nervous system is a frequent outcome.

Male Infections In male ruminants, bilateral acute orchitis with extensive irreversible damage is a frequent event (Blasco, 1990; Enright, 1990b). After invasion and bacterial replication, the tunics become distended, and necrotic foci are evident within the testicular parenchyma, with hemorrhagic foci and purulent exudate. Focal necrotizing epididymitis and spermatic granulomas are frequently observed after the initial inflammatory reactions. Seminal vesiculitis and prostatitis are not uncommon. After these episodes caused by *Brucella* infection of the male reproductive tract, partial or total sterility remains as a consequence of the chronic infection. Perivascular edema and infiltration of mononuclear cells and polymorphonuclear leukocytes accompany the initial localization into the tubular tissue. Subsequently, the inflamed tubular epithelium develops papillary hyperplasia and local hydropic degeneration and intraepithelial cysts. Eventual destruction of the epithelium, either by bacteria or by inflammatory reaction, leads to extravasation of the spermatozoa. Complete blockage of the epididymis and testicular degeneration with fibrosis represent the outcome of the chronic infection. Damage of epidydimis and prostate epithelial cells acts as a trigger for the production of autoantibodies to spermatozoa, contributing to the general pathology observed in male organs and one of the mechanisms for causing sterility (Serikawa et al., 1984). *Brucella ovis* is an important cause of epididymitis in rams, and it is less frequently associated with abortions in ewes. Although female ovines are more resistant to the infection than males are, they are important sources of passive venereal transmission to the copulating males. The target organ of *B. ovis* is the epididymis and accessory sexual glands. The pathological signs caused in ram epididymis are similar to those described above.

Brucellosis in Humans Although many of the pathological signs of *Brucella* infection in the natural hosts are reproduced in humans, idiosyncratic differences are found (Spink, 1956; Young, 1983). The outcome of the disease in humans depends on host factors, inoculum size and the *Brucella* strain. In general terms, the most aggressive species for humans is *B. melitensis*, followed by *B. suis* and *B. abortus*, although this tendency is not strictly lined up. For instance, *B. abortus* biotype 5 and *B. suis* biotype 2 seem to harbor low pathogenicity for man. Limited infections have been described with *B. canis*. No human infections caused by *B. ovis*, *B. neotomae* or *B. maris* have been reported. However, human brucellosis caused by the marine strains has been barely explored, owing to the fact that these strains were recently described (Higgins, 2000; Jahans et al., 1997).

The evolution of brucellosis in humans has the tendency to be more dramatic than in the animal reservoir, with no pathognomonic signs or symptoms. The incubation period is highly variable, with the most common interval between one and three weeks. However, longer periods are not uncommon. After invasion of host tissues, the organism multiplies in regional lymph nodes draining the site of entry and gives rise to a bacteremia, which may vary from short duration to intermittent or prolonged times. Normally this event is accompanied by symptoms that resemble in some aspects a strong cold, with clinical features that include undulant fever, chills, weakness, malaise, sweating, headache, backache, muscle joint pain and anorexia. After this period, and following colonization of the reticuloendothelial system, more severe symptoms may arise which cause weight loss, nausea, cough, vomiting, diarrhea, epistaxis and rash. After this, lymphoadenopathy, hepatomegaly, splenomegaly, osteomyelitis, thrombosis and valvular endocarditis may arise. In many cases compromise of the central and autonomous nervous systems may appear as well as compromise of the urogenital organs. Abortion due to *Brucella* infections, with invasion of maternal and fetal tissues, has been reported in a limited number of cases. If nontreated, complications due to *Brucella* occur in about 10% of the cases. These generally involve suppurative abscesses in different organs and tissues. Mortality usually is less than 2%, mainly reduced by antibiotic therapy (Animal Brucellosis). Chronicity with sporadic appearance of symptoms may remain for years. Allergic hypersensitivity is a common consequence of human and animal brucellosis (Díaz and Oyeledum, 1977; Spink, 1956). This is especially important in laboratory workers or medical personnel who may be exposed to *Brucella* organisms or their products (antigens) on a routine basis.

Brucellosis in Experimental Animals In addition to the natural hosts, a number of experimental animal models have been tested including chick embryos, rabbits, rats, hamsters, gerbils, guinea pigs and mice (Garcia-Carrillo, 1990). For many years guinea pigs remained as the preferred model, mainly due to their high susceptibility to *Brucella* infections and the resemblance of the disease with humans and also because these animals were originally used for

isolating *Brucella* organisms from clinical samples. In the last 20 years, guinea pigs have been abandoned, and mice have been established as the preferred animal model. The reasons lay mainly in the economical feasibility of the mouse model, the extensive number of available defined strains, the amenability of mice to genetic manipulation, and the extensive knowledge of the immune system of this murine species. However, mice display a relatively strong innate resistance to brucellosis and may not be the more appropriate model for *Brucella* studies.

Brucellosis in Guinea Pigs In guinea pigs, as few as 50 intraperitoneal or intravenously injected *Brucella* colony forming units (CFU) are required to cause infections, with incubation periods that range from one to three weeks (Ne'eman and Jones, 1963). Although these rodents are susceptible to being infected by oral, conjunctival or respiratory routes, which are estimated to be the most common natural course of infection, safety has prevailed and intraperitoneal, subcutaneous and intramuscular routes are preferred. No major differences have been found between male and female or the various strains of guinea pigs. However, pregnancy seems to be a propensity factor for susceptibility to *Brucella* infection and is an experimental model to demonstrate vertical transmission and abortion. The initial stages of the infection are similar to those described for other animals, including humans (Moulton and Meyer, 1958; Sterba, 1984). After this initial outcome, splenomegaly, hepatomegaly and compromise of the lymphatic tissues are almost invariably observed. Acute and subacute inflammation occurs with exudation of fluids and accumulation of polymorphonuclear leukocytes. As the infection continues, typical granulomatous lesions with the presence of macrophages, histiocytes, epithelioid cells and giant cells surrounded by proliferating lymphocytes occur. In later stages of the infection, fibrosis is detected (Moulton and Meyer, 1958; Sterba, 1984). In males, compromise of urogenital organs can be observed. In pregnant females, compromise of placental and fetal tissue is evident. Although *Brucella* organisms have been detected in the semen and fluids of infected guinea pigs, no horizontal transmission has been demonstrated. After several weeks, the infected guinea pigs demonstrate strong delayed-type hypersensitivity reactions when tested with *Brucella* protein extracts.

Brucellosis in Mice It is estimated that, in general, mice are from 10^3 to 10^6 times more resistant than guinea pigs to *Brucella* infections (Taran and Rybasov, 1972). After intravenous inoculation, *Brucella* organisms are cleared by phagocytic cells of the reticuloendothelial system where the bacteria replicate (Cheers, 1984). In the case of intraperitoneal inoculation, *Brucella* organisms are rapidly cleared by resident phagocytic cells that transport the bacteria to the regional lymph nodes, spleen and liver. Normally during the first stages of the infection, lasting from one to two weeks, there are no manifestations of symptomatic acute brucellosis. However, depending on the size of the inoculum and virulence of the *Brucella* strain, a variable number of organisms are recovered from the spleen, liver and lymph nodes. Following this, replication of *Brucella* rapidly increases until a plateau is reached. This period commonly lasts a few days; then, the number of organisms declines until equilibrium has been established, corresponding to the development of the immune response. In spite of the fact that splenomegaly is maintained and the organisms may be isolated up to three months after the experimental infection, no severe pathological symptoms are developed. Histopathological studies during the replicating and steady phases reveal a focus of chronic inflammatory reactions in the spleen, lymph nodes and liver.

If large quantities of bacteria are inoculated ($>10^6$ per mouse), overloading the innate and immune capabilities, or immunocompromised (hormone- or mucine-treated or genetically immunodeficient) mice are used, granulomas and suppurative infections in different organs are observed, followed by death. In cases where low and moderate numbers of *Brucella* are administered, limited placental colonization and a few vertical bacterial transmissions to the newborn are observed (Bosseray, 1983; Bosseray, 1984). However, injection of large numbers of *Brucella* in pregnant mice results in preferential bacterial replication within placental giant trophoblasts and within the visceral yolk sac endoderm. Despite the anatomical differences in placentation between ruminant and rodent (epithelial versus hemochorial) placental damage, fetal infection and fetal death occurs (Tobias et al., 1993), but in contrast to what is observed in the natural hosts, pregnant mice are relatively more resistant to abortion and fetal death.

Outcome of the Disease and Self Cure In the natural host, the immune system generally gains control of the infection a few weeks after the appearance of the symptoms (Enright, 1990b; Nicoletti and Winter, 1990b). However, some bacteria may remain in reproductive organs, mammary gland, bones and joins for prolonged periods. Some of the primary infected hosts, therefore, may remain as healthy shedders and of epidemiological importance. In contrast, in the accidental hosts, the bacteria are dispersed

into different tissues, causing a severe disease with no shedding and in consequence of medical but not epidemiological importance (Alton, 1990a, 1990b; Spink, 1956). In nontreated accidental hosts, such as horses or humans, hepatic involvement in the form of acute diffuse hepatitis with focal necrosis, generation of granulomas, and the compromise of lymphatic organs is a common event of chronicity.

Both symptomatic and asymptomatic animals can transmit brucellosis, which means that many of the infected individuals shed the bacteria independently of being ill or healthy. After abortion, infection commonly recedes and self-cure proceeds, which is the most frequent outcome in the natural host (Enright, 1990a). Some bovines are naturally resistant to brucellosis, which is related to the ability of their macrophages to kill *Brucella* organisms intracellularly (Price et al., 1990; Templeton and Adams, 1990). Moreover, calves, kids and lambs may remain without clinical signs, in spite of drinking milk containing millions of *Brucella* secreted in the udder by their infected mothers. A low proportion of these infected animals at a young age may abort later in life; however, many remain infected but asymptomatic, demonstrating a good equilibrium between the *Brucella* invader and the host. Furthermore, several of the infected animals which acquired *Brucella* by vertical transmission, in addition to being asymptomatic, do not display positive serology (Fig. 9), suggesting anergic immune responses. Other individuals, mainly secondary hosts, may remain allergic for life after cured (Spink, 1956).

Pathobiology

After invasion of host tissues, *Brucella* binds and penetrates cells. Once inside, the bacterium is protected from extracellular microbicidal substances and the new life cycle begins. Intracellularly, *Brucella* traffics and replicates within vacuolar compartments, avoiding digestive mechanisms of cells by means of virulence factors that allow the bacterium to control its cellular environment for its own benefit. The complexity of these bacterial mechanisms and the cross-talk between the host cell on one side and the *Brucella* parasite on the other are just being unrevealed. Comprehensive reviews on these topics can be found elsewhere (Baldwin and Winter, 1994; Liautard et al., 1996; Pizarro-Cerdá et al., 1997; Pizarro-Cerdá et al., 1999b; Pizarro-Cerdá et al., 2000).

Brucella Invades Healthy Hosts It is known that some bacterial pathogens rapidly gain access to the internal host tissues by penetrating specialized epithelial cells. The penetration of invasive bacteria requires the interaction of specific receptors and ligands between the animal and bacterial cells that trigger the internalization and the transference of the pathogen inside the body (Van Nhieu and Sansonetti, 2000). The primary routes of *Brucella* infection are the cells of mucosal surfaces (Enright, 1990b). In goats, *B. abortus* laying in the ileum is ingested by M cells using a zipper-like mechanism but not by enterocytes (Ackermann et al., 1988; Fig. 33). In these phagocytic cells, the ingested bacterial organisms are located as single entities or in small clusters, within transient vacuoles that do not show signs of lysosomal fusion. The phagocytic vacuolar membrane does not associate with ribosomes, and some of the *Brucella*-containing compartments demonstrate coingested opaque material. The engulfed *Brucella* are eventually translocated by M cells to the gastrointestinal-associated lymphatic space, where they are taken up by macrophages and neutrophils (Ackermann et al., 1988; Fig. 33). The numbers of intracellular *Brucella* in M cells decrease with time after bacterial inoculation, indicating that microorganisms are steadily translocated. After penetrating mucosal layers, both virulent and vaccine *B. abortus* strain 19 strains induce an inflammatory response in the submucosa (Cheville et al., 1992).

It has been demonstrated that urease-negative *B. abortus* mutants are virulent when injected intraperitoneally in mice (Grillóet al., 1997). Wild type and urease-deficient mutants also show the same sensitivity to acid. However, when urease-deficient mutants are administered by the oral route, the numbers of bacteria recovered in the intestine or in the spleen are considerably lower than those of the wild-type strain. As in the case of Ureaplasma or *Helicobacter pylori*, in which urease plays a relevant role in the colonization of the host cells (Cover et al., 1991), it may be that in *Brucella* this enzyme also participates in modifying the immediate local acid environment (Salyers and Whitt, 1994; Sangari et al., 2000). This is an important aspect that must be taken into consideration because the intestine seems to be the most frequent route for invading the primary and secondary hosts through ingestion of milk and dairy products as well as through licking of the aborted fetus or contaminated secretions.

Survival Outside Host Cells *Brucella* are organisms whose ultimate goal is to propagate inside cells (Habitat). Therefore, when released to the extracellular lumen, these pathogens are deprived of their shelter and natural replicative niche and subjected to the attack of a variety of bactericidal substances present in the plasma and body fluids, against which they must defend. The blood, pulmonary secretion, tears, intestinal flu-

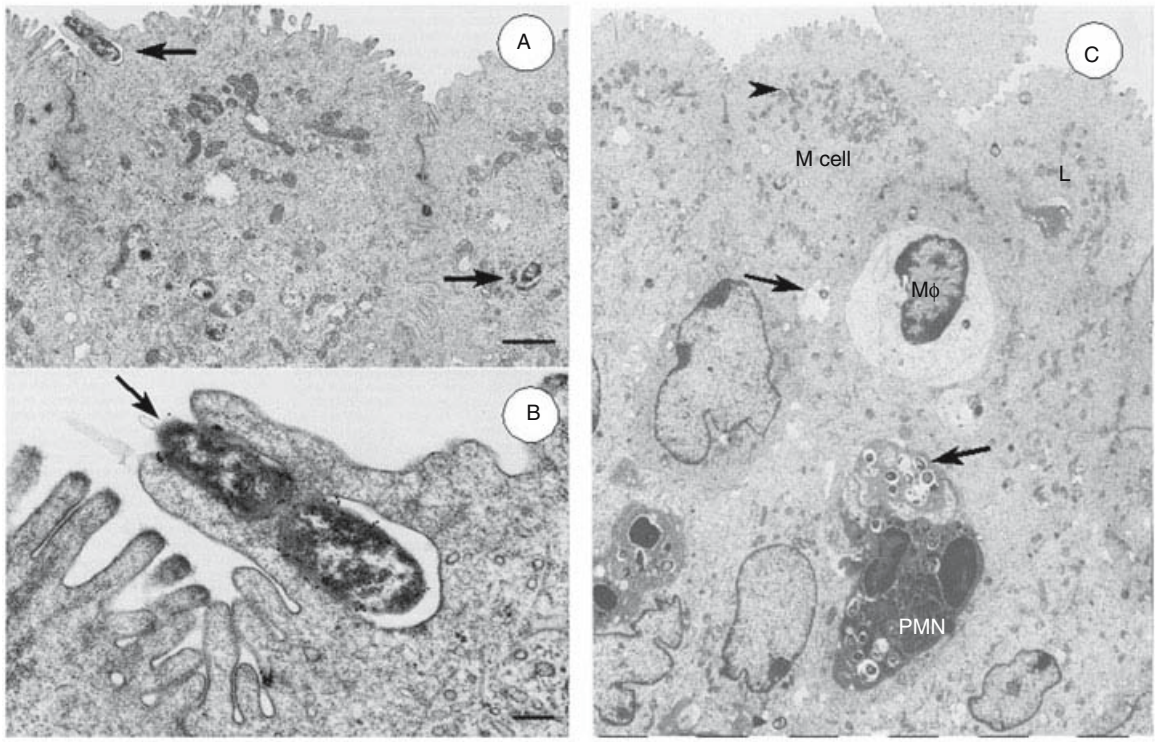


Fig. 33. *Brucella* transit in M (lymphoepithelial) cells. A) Dome lymphoepithelial cell from calf ileum, two hr postinoculation. Apical extensions engulf two *B. abortus* cells labeled with immunogold (arrows). Labeled *B. abortus* cells in phagolysosome (arrow). Bar = 1 µm. B) Immunogold-labeled *B. abortus* engulfed by lymphoepithelial cell apical extensions (image is [A] at higher magnification). Bar = 0.2 µm. C) *Brucella* is found within macrophages (M) and neutrophils (PMN) and also between the base (arrows) of M cells from ileum cells (L) at two hr postinoculation. Abundant apical mitochondria are seen in the M cells (arrow head). From Ackermann et al. (1988), with permission.

ids, milk and colostrum contain substances that participate in protecting the body against bacterial invasion (Opsonization and Complement Susceptibility). In addition, *Brucella* discharged in the inflammation site is exposed to the local attack of molecules released by leukocytes, such as those present in the azurophilic and secondary granules. In general, it has been demonstrated that *Brucella* is capable of resisting the microbicidal action of soluble substances released by cells (Modulation of Neutrophil Function and Resistance of Bactericidal Secretions). Despite that, there is not a complete picture of how this is achieved. Various properties, mainly related to the structure and function of the *Brucella* outer-membrane, have been implicated in the resistance of *Brucella* to humoral substances (Properties of the Outer Membrane).

Opsonization and Complement Susceptibility
Work carried out mostly with *B. abortus*, *B. melitensis* and *B. suis* has identified several features of these bacteria which are relevant for parasitism (*Brucella* Virulence Mechanisms). It is becoming clear that some of these bacterial properties are modulated by the interaction between antibodies and complement with the *Brucella* cell surface molecules. Indeed, opsonized bacteria stimulate the oxidative burst and the intracellular killing mechanisms in a higher proportion than that in nonopsonized *Brucella* (Caron et al., 1994b; Harmon et al., 1987; Harmon et al., 1988; Jiang and Baldwin, 1993a; Jiang et al., 1993c; Kreutzer et al., 1979b; Young et al., 1985). The *O*-polysaccharide makes *B. abortus* more resistant to complement-mediated killing in the presence of *O*-polysaccharide-specific antibody (Corbeil et al., 1988). It is feasible to hypothesize that evasion of opsonization and complement lysis constitute adaptive survival mechanisms employed by *Brucella* organisms.

Animals infected with smooth *Brucella* characteristically generate a strong antibody response against smooth LPS and NH polysaccharides (Díaz-Aparicio et al., 1993; Marín et al., 1999; Fig. 19). The NHs are LPS-independent molecules intertwined with the *O*-polysaccharide in the outer-membrane (Aragón et al., 1996b; Fig. 20). Part of their function is to increase the density of O-type polysaccharides

on the cell surface, creating a protective layer that prevents the binding of antibodies and the interaction of lytic complement with the deeper structures of the outer-membrane (Aragón, 1996a; Fig. 20). It has been demonstrated that with the exception of core and lipid A determinants, all the other epitopes of smooth LPS are present in the NH (Aragón et al., 1996b; Moreno et al., 1987). Therefore, it is not surprising that practically all infected animals with smooth *Brucella* strains develop similar levels of antibodies to NH and to the *O*-polysaccharide of the LPS; however, a proportion of the same animals do not develop detectable antibodies against core or lipid A determinants (Fig. 34). Despite this, the reaction of these molecules differs regarding the antibody-mediated complement activation. In sharp contrast with smooth LPS, the antibodies bound to NH are poor consumers of complement component C3b (E. Moreno and I. Moriyón, unpublished observations). The failure of NH molecules to activate complement may be due to subtle differences in the degree of formylation between the smooth LPS *O*-polysaccharide and NH, rather than in the absence of core and lipid A, in the latter (M. Staaf et al., unpublished observations). Indeed, absorption assays performed to remove antibodies against core and lipid determinants showed that complement activation by smooth LPS could not be due to antibodies against these moieties.

Abundant surface C3b receptors promote phagocytosis in activated macrophages (Wright and Silverstein, 1983). Reduced C3b opsonization could help to avoid ingestion by those cells in which brucellae would have a reduced chance of survival. Although NH mostly binds immunoglobulin G (IgG; Díaz-Aparicio et al., 1993), and macrophages bear Fcγ receptors, reduced C3b opsonization could be important at the onset of the infection when the predominant antibodies are IgM. At this time, *Brucella* could penetrate phagocytes by interaction of the smooth LPS and NH with the mannose-fucose receptor without triggering the production of toxic oxygen derivatives, as has been pointed out before (Campbell et al., 1994). This opsonin-independent route of entry could represent a strategy suitable for an intracellular pathogen because *B. abortus* cells either nonopsonized or opsonized with nonimmune serum fail to induce a significant oxidative burst in bovine polymorphonuclear leukocytes (Canning et al., 1988).

Modulation of Neutrophil Function and Resistance of Bactericidal Secretions Although *Brucella* organisms are moderately resistant to acid (Teixeira-Gomes et al., 2000), they display a strong resistance to bactericidal proteins normally found in secretions, such as lysozyme, lactoferrin, lytic complement, chelating substances, proteolytic enzymes, phospholipase A2, defensins and defensin-like molecules, as well as to a variety of substances released by leukocytes during inflammation (Martínez de Tejada et al., 1995; Páramo et al., 1998; Riley and Robertson, 1984a). This resistance is related to the *Brucella* outer-membrane structure (mainly the LPS and

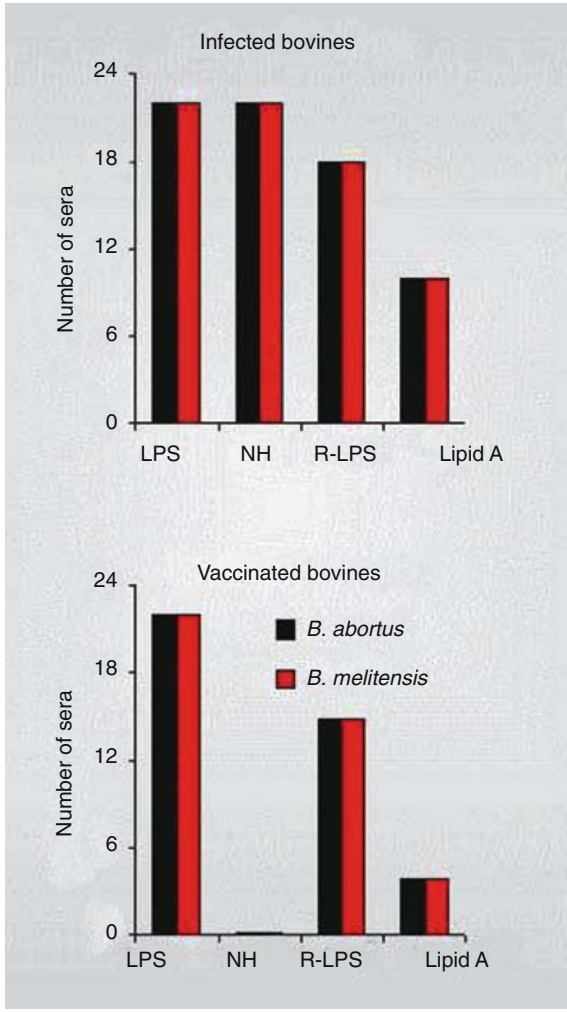


Fig. 34. ELISA and radial immunodiffusion (RID) representing the IgG reactivity of *B. abortus* S19 vaccinated or *B. abortus* (biotype 1) naturally infected cows against smooth LPSs, rough LPSs, NHs and lipid as from *B. abortus* (biovar 1) and *B. melitensis* (biovar 1). Independent of the antigen attached (*B. abortus* or *B. melitensis*) to the ELISA plate or the NH used in the RID analysis, results are the same for both types of antigens. ELISA optical density values for *B. abortus* and *B. melitensis* antigens did not vary more than 5% in three different runs. RID was qualitatively evaluated as positive or negative. Notice that vaccinated animals do not react against NH, while infected animals do. Compare to (Fig. 19). The relevant epitopes in these reactions are the overlapping common epitopes in both *B. abortus* and *B. melitensis* antigens.

polysaccharide molecules), which from the chemical and functional point of view constitutes an excellent shelter for resisting the assault of these bactericidal mediators (Kreutzer and Robertson, 1979a; Martínez de Tejada et al., 1995). In addition to this natural resistance, *Brucella* cells or extracts obtained from these organisms do not significantly induce the exocytosis of neutrophil granules (Canning et al., 1986; Kreutzer et al., 1979b), suggesting functional modulation of these cells. Indeed, *Brucella* LPS does not stimulate the degranulation, respiratory burst or lysozyme release from neutrophils (Rasool et al., 1992). Moreover, GMP- and adenine-containing *Brucella* extracts have been proposed to inhibit neutrophil degranulation (Canning et al., 1986). This last result, however, must be considered from the perspective that killed *Brucella* do not inhibit digestion by phagocytic cells.

Brucella Binds and Penetrates Host Cells

Very few clear-cut studies have addressed the question of the mechanisms by which *Brucella* binds to and penetrates cells. Similarly to other pathogens (for review, see Méresse et al., 1999; Pizarro-Cerdá et al., 1997; Pizarro-Cerdá et al., 2000), *Brucella* organisms may have developed specialized microbial structures to gain access to host cytoplasmic compartments. In nonprofessional phagocytic epithelial cells, *Brucella* may induce its own uptake, since certain mutant bacteria are poorly internalized by the inoculated monolayers (Sola-Landa et al., 1998). Compared to other intracellular Proteobacteria, such as *Salmonella*, *Shigella* or *Legionella*, surprisingly low numbers of virulent *Brucella* bind to the surface of naive professional and nonprofessional phagocytes (Pizarro-Cerdá et al., 1998b; Pizarro-Cerdá et al., 1998c; Sola-Landa et al., 1998). Despite this low attachment of virulent *Brucella*, their efficiency of penetration is close to 100%. As early as five minutes after infection, *B. abortus* is found within vacuoles of cells. Electron micrographs of *Brucella* penetrating cells have shown that phagocytosis can occur via a zipper mechanism (Ackermann et al., 1988; Pizarro-Cerdá et al., 1999a; Fig. 33) or alternatively by a slow process resembling capping with little cell membrane rearrangements (Guzmán-Verri et al., 2001).

Interaction with the Host Cell Membrane

Sequences coding for putative proteins similar to those necessary to build flagella have been identified in the *Brucella* genome (DelVecchio et al., 2002; Sánchez et al., 2001; O'Callaghan et al., 1999), but none of these structures have ever been observed in *Brucella* cells, precluding their participation as attachment molecules. Alternatively, the involvement of nonfibrillar adhesins remains open, since sequences compatible with these protein molecules have been discovered in *Brucella* (DelVecchio et al., 2002; Ugalde, 1999; http://www.genome.scranton.edu.WIT2; www.tigr.org).

Various studies have reported that nonvirulent smooth and rough *Brucella* mutants bind more readily and in larger amounts to the surface of professional and nonprofessional phagocytes than virulent strains do (Detilleux et al., 1990a; Detilleux et al., 1990b; Sola-Landa et al., 1998). However, there is not a strict direct correlation between the expression of *O*- and NH polysaccharides and binding to cells (Freer et al., 1999; Pizarro-Cerdá, 1998a). For instance, the virulent smooth *Brucella* and the "natural" rough *B. ovis* and *B. canis* (not expressing *O*-polysaccharide or NH) bind in very low number to HeLa cells. In contrast, the perosamine synthetase (perA) rough *B. abortus* mutants (not expressing *O*-polysaccharide or NH molecules) and the spontaneous rough *Brucella* mutants bind to these cells in much higher numbers than do smooth brucellae (Freer et al., 1999). Although difficult to interpret, the fact is that virulent *Brucella* cells have the tendency to attach in lower numbers to cultured epithelial cells than nonvirulent bacteria do (Fig. 21).

The host cytoplasmic membrane directly adjacent to rough *Brucella* mutants is frequently thicker than the cell membrane adjacent to smooth bacteria and occasionally forms coated-pits (Detilleux et al., 1990a). These observations, although interesting, may not reflect the real physiological interactions between virulent smooth organisms and their host cells, since coated-pits adjacent to smooth *Brucella* have not been seen during phagocytosis. In contrast, increased adherence displayed by many mutant *Brucella* seems to be the result of altered outer-membrane properties, which expose "new" sites for attaching in a nonphysiological manner (Allen et al., 1998; Corbeil et al., 1988; Detilleux et al., 1990a, 1990b; Freer et al., 1999). It is known that polysaccharide moieties in the outer-membrane hide hydrophobic and ionic charges on the surface of the bacteria (Fig. 20) that obstruct membrane domains capable of participating in the nonspecific interactions between *Brucella* and the host cells (Aragón et al., 1996b). In addition, the absence of surface *O*-polysaccharide and NH exposes core sugars to the external surface and gives more access to Omps, which may serve as ligand sites (Bowden et al., 1995; Cloeckaert et al., 1990).

Invasion of Professional Phagocytes

A scheme showing the binding, penetration and replication

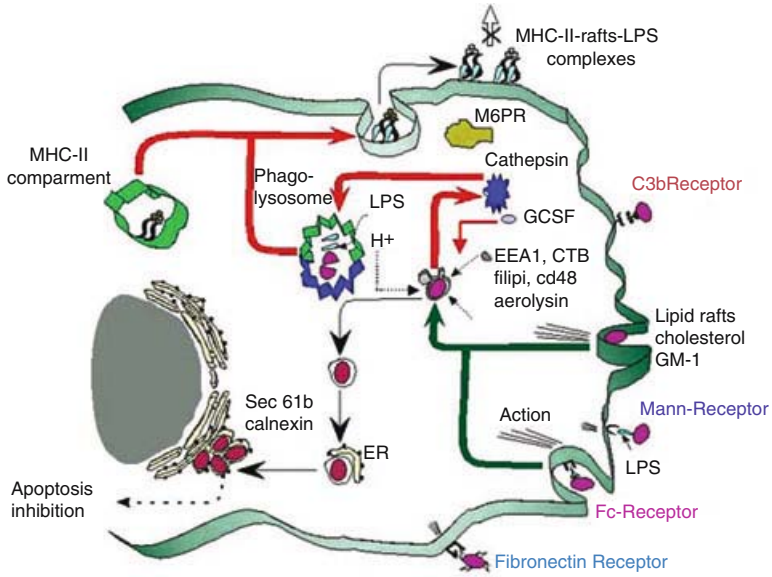


Fig. 35. Schematic model of *B. abortus* invasion and intracellular trafficking in macrophages. *Brucella* organisms bind to discrete sites via receptor molecules (FcR, C3bR, MannR, and fibronectin), some of which are unknown. *Brucella* penetrates by a zipper-like phagocytosis with moderate recruitment of actin filaments. The ingested bacterium initially follows its route (thick green arrows) to early phagocytic compartments (marked by EAA1) which may be acidified (H^+) by acquisition of specific proton pumps (dotted arrows). Granulocyte colony-stimulating factor (G-CSF) is involved in promoting fusions between endocytic vacuoles and phagosomes (dotted arrows). In macrophages, most of the brucellae are routed (thick red arrows) to fuse with lysosomes (marked by cathepsin D and LAMP1) generating phagolysosomes where they are destroyed, whereas only a few bacteria follow the route (black thin arrows) to their replicating niche inside the endoplasmic reticulum (marked by Sec61β) following the autophagocytic (marked by Sec61β and LAMP1) pathway. The released LPSs merge with MHC-II compartments and the complexes MHC-II-LPS are transported to the cell membrane, inhibiting antigen presentation (crossed white arrow). Inhibition of apoptosis is induced in both infected and noninfected phagocytic cells (broken arrow). Steps indicating endoplasmic reticulum recruitment and *Brucella* replication within the endoplasmic reticulum have been adapted from unpublished results from Jean Celli of the CIML-Marseille-Luminy, France.

cycle of smooth *Brucella* in macrophages is presented in Fig. 35. *Brucella* cells bind to and penetrate more efficiently professional than nonprofessional phagocytes. Still, the number of *Brucella* organisms associated to the former cells is comparatively much lower (from one to four logs) than the numbers observed with other intracellular Proteobacteria, such as *Salmonella*, *Shigella* or *Legionella*. There are differences in the binding and internalization of *Brucella* by phagocytic cells (Pizarro-Cerdá, 1998a). Concomitantly, opsonized *Brucella* cells attach and are more readily ingested by phagocytes than are nonopsonized cells. This phenomenon is more clearly observed with activated than with non-activated professional phagocytes (Arenas et al., 2000; Eze et al., 2000; Gross et al., 1998; Harmon et al., 1988; Young et al., 1985). As in the case of *Mycobacterium* (Schorey et al., 1997), it may be that highly opsonized *Brucella* (prozone effect) could take advantage of phagocytosis by Fc or complement receptors to invade cells expressing these receptors. However, opsonization seems to negatively affect the rate of survival and multiplication of *Brucella* within phagocytes, suggesting that Fc or complement receptor-mediated phagocytosis works in favor of the host cells rather than the bacteria (Caron et al., 1994b; Gross et al., 1998; Harmon et al., 1988; Harmon et al., 1989). *Brucella* LPS and NH do not activate the alternative complement pathway (Moreno et al., 1981; Hoffmann et al., 1984). Therefore, these polysaccharides do not serve as direct attachment of C3b on the bacterial surface. The fact that nonopsonized *Brucella* organisms bind, penetrate and reproduce in vivo and in vitro within phagocytes from nonimmunized animals (Campbell et al., 1994; Gross et al., 2000; Kuzumawati et al., 2000; Sola-Landa et al., 1998) indicates the existence of a receptor-ligand mechanism independent from the Fc and complement receptors.

The exclusion of membrane negatively charged groups on the site of *Brucella* attachment in macrophages, followed by zipper-like phagocytosis, suggests specific interactions between both types of cells (Gay et al., 1981). Lipid rafts rich in cholesterol, glycosylphosphati-

dylinositol and GM1 gangliosides seem to provide a port of for *Brucella* entry into murine macrophages under non-opsonic conditions (Naroeni and Porte, 2002; Watarai et al., 2002). *Brucella* organisms (either opsonized or nonopsonized) are rapidly internalized by murine macrophages and human monocytes after inducing recruitment of actin filaments without generating marked structural changes but a general membrane ruffling (Gay et al., 1981; Kuzumawati et al., 2000; Watarai et al., 2002). Penetration of *Brucella* into bovine mononuclear phagocytes is inhibited by bacterial cell envelopes, antibody against the α chain of the MAC-1 integrin (CD11b), *O*-polysaccharide and denatured IgG, no matter whether the cells originated from cattle naturally resistant or susceptible to brucellosis. In addition, fibronectin, mannan and antibodies against C3 also inhibit the penetration of nonopsonized bacteria to phagocytes from brucellosis-resistant cattle (Campbell et al., 1994). Since the *O*-polysaccharide of LPS and mannan inhibit the binding of *Brucella* to bovine macrophages, it is likely that lectin-like receptors on the surface of phagocytic cells participate in the *Brucella* uptake. This idea also is supported by the observation that the strong binding of *Brucella* onto murine B lymphocytes is inhibited by α-methyl mannosamine and LPS (Lee et al., 1983). In addition, it has been demonstrated that phagocytosis of *Brucella* by macrophages is accompanied by secretion of fibronectin, which is then detected inside the phagocytic vacuole (Gay et al., 1986). Since fibronectin also blocks the invasion by *Brucella*, it is likely that this fibrous matrix protein could participate in the binding and penetration of *Brucella* in macrophages. Moreover, the intravacuolar fibronectin contributes to the electron-dense material that is commonly seen surrounding the phagocytosed brucellae, suggesting that this protein is coingested with the bacteria (Gay et al., 1981; Gay et al., 1986).

Invasion of Non-Professional Phagocytes A model showing the penetration and intracellular cycle of *Brucella* within nonprofessional phagocytic HeLa cells is presented in Fig. 36. As expected, immune or native sera are not necessary for invasion of *Brucella* species into nonprofessional phagocytes (Anderson and Cheville, 1986a; Anderson and Cheville, 1986b; Anderson et al., 1986c; Detilleux et al., 1990a; Detilleux et al., 1990b; Pizarro-Cerdá et al., 1999b). In HeLa cells, *B. abortus* attaches to cellular extensions that are compatible with adhesion plaques and between cell-to-cell contacts and penetrates by a zipper-like mechanisms or by capping-like mechanisms with moderate rearrangement of the cell membrane (Guzmán-Verri et al., 2001). Some of these observations have been performed in HeLa cell monolayers previously intoxicated with *C. difficile* toxins B [TcdB] and BF [TcdBF] and then infected with *Brucella*. The TcdB-intoxicated cells retract their body, leaving cellular spikes attached to the substrate. In contrast, TcdBF induces cell rounding and retraction of cellular spikes from the substratum. *Brucella* organisms attach in normal numbers to the cellular spikes of TcdB-treated cells. On the contrary, in TcdBF-intoxicated cells, *Brucella* organisms bind only in reduced numbers, and when binding occurs, the bacteria locate on the cellular body. It has been proposed that α5β1 integrin mediates the adhesion of rough mutant *B. abortus* RB51 to bovine trophoblasts (Bress et al., 1996). However, this observation has not been reproduced in HeLa cells in spite of the highly adherent nature of mutant rough *Brucella* to cells and inert substrates (Pizarro-Cerdá, 1998a). Uptake of killed or alive *Brucella* by Vero cells is suppressed by inhibitors of energy metabolism (iodoacetate and dinitrophenol), inhibitors of receptor-mediated endocytosis (monodansylcadaverine, amantadine and methylamine) and by repressors of endosomal acidification (chloroquine, ammonium chloride and monensin). These drugs are capable of inhibiting penetration when added at the same time as the bacterial inoculum (8 h), but not when added after the inoculation period, suggesting that the infection process occurs via receptor molecules and requires energy input from the host cell (Detilleux et al., 1991).

The participation of the cytoskeleton, second messengers and GTPases in the internalization of *B. abortus* to HeLa cells has been recently investigated (Guzmán-Verri et al., 2001). Phalloidin staining has revealed a modest recruitment of actin filaments in the site of *Brucella* attachment. Inhibition of actin filaments by drugs (Detilleux et al., 1991; Pizarro-Cerdá, 1998a; Guzmán-Verri et al., 2001) or by different clostridial toxins (TcdB, TcdBF, TcdA, and TcsLT) functionally modifying the actin cytoskeleton through interaction with small GTPases of the Rho family hampers internalization but not binding to cells (Guzmán-Verri et al., 2001). Infection also is inhibited by chemicals and toxins that increase the levels of cyclic-AMP (dibutyl-cyclic-AMP and *Vibrio cholerae* enterotoxin), but it is stimulated by toxins and chemicals that increase the levels of cyclic-GMP (*Escherichia coli* enterotoxin A and dibutyl-cyclic-GMP). This suggests an inverse relationship between these two second-messengers during *Brucella* infection. Similarly, wortmanin (which inhibits the PIP3 kinase) considerably reduces the internalization of *Brucella* by HeLa cells, suggesting involvement of PIP3-kinase

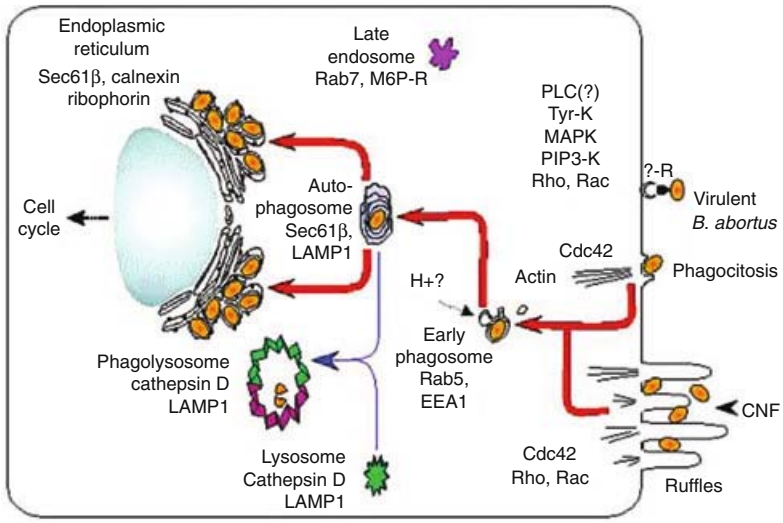


Fig. 36. Schematic model of *B. abortus* invasion and intracellular trafficking in epithelial HeLa cells. *Brucella* organisms bind to discrete sites of cells via unknown receptor molecules (???-R) and penetrate by a discrete phagocytosis with moderate recruitment of actin filaments, activation of small GTPases (Cdc42, Rac, and Rho), mainly Cdc42, and signals mediated by second messengers (Tyr-K, MAP-K, and PIP3-K). The ingested bacterium is initially routed (green arrows) to early phagocytic compartments (marked by Rab5 and EEA1) which may be acidified (H^+) by acquisition of specific proton pumps (dotted arrows). In HeLa cells most of the ingested virulent brucellae are routed (thick red arrows) to the endoplasmic reticulum by the autophagocytic route (marked by LAMP1 and Sec61β), whereas only a few bacteria are directed (thin black arrows) to phagolysosomes. Some mutants (BvrS/BvrR) are defective in penetration (crossed arrow), others cannot avoid fusion of early phagosomes with lysosomes (BvrS/BvrR, VirB1-VirB10, and *cgs*), whereas others transit from autophagosomes to phagolysosomes (S19) or from early phagosomes to the cell membrane (nonpolar VirB10; blue thick arrow). Signals for apoptosis inhibition may be released from the *Brucella* replicating niche.

phosphorylation during this process (Guzmán-Verri et al., 2001). Since the level of cyclic GMP usually increases when the inositol phospholipid pathway is activated, it is likely that binding of *Brucella* to cells also stimulates the generation of IP3 via phospholipase C activation. Other cellular kinases, such as tyrosine kinases and MAP kinases, seem to be required for physiological internalization to HeLa cells, since inhibition of these enzymes hampers bacterial penetration (Guzmán-Verri et al., 2001).

Brucella abortus attaches in larger numbers and is internalized more efficiently after intoxication of HeLa cells with the cytotoxic necrotizing factor (CNF), which deamidates the small GTPases Rho, Rac, and Cdc42 and induces ruffles and stress fiber formation (Guzmán-Verri et al., 2001). In these cells, *Brucella* organisms bind to the ruffles as well as to discrete sites and penetrate through these structures. Moreover, HeLa cells transfected with constitutive negative mutant plasmids expressing defective Rho, Rac and Cdc42 proteins are considerably less infected than control cells. On the contrary, the positive counterparts of these small GTPases expressed in HeLa cells stimulate binding and penetration of *Brucella*. Virulent *Brucella* selectively stimulates the generation of Cdc42 GTPase, reaching its maximum at 30 minutes after bacterial contact with cells. This property may be related to an Omp of group 3, more likely Omp3a (Omp25) and/or Omp3b. This is supported by the fact that the nonvirulent *B. abortus* BvrS mutant does not stimulate any of the GTPases, in spite of its binding to cells (Guzmán-Verri et al., 2001). Microtubule depolymerizing agents, such as nocodazole and colchicine, partially reduce the internalization but not the replication of *Brucella* in HeLa cells (Guzmán-Verri et al., 2001). This strategy for invading cells differs from those employed by other intracellular pathogens, such as *Salmonella* and *Shigella*. Despite the obvious differences between *Brucella* and *Listeria*, both types of pathogens seem to employ very similar strategies (Finlay and Cossart, 1997a).

Brucella Traffics and Replicates Within Host Cells

Brucella organisms have been found to survive and replicate within membrane-bound compartments of professional and non-professional phagocytes (Fig. 30). Despite the tropism of these pathogens for reproductive organs, the bacteria also localize within cells of various tissues at later stages of the infection (Enright, 1990b). In vivo, *B. abortus* has been

described within bovine, caprine and murine trophoblasts, in caprine lymphocytes, M cells, and chicken embryo fibroblasts, as well as in a number of professional phagocytes lining different tissues (Ackermann et al., 1988; Anderson and Cheville, 1986a; Anderson et al., 1986c; Anderson et al., 1986b; Cheville et al., 1992; Cheville et al., 1996a; Detilleux et al., 1988; Holland and Pickett, 1956; Richardson and Holt, 1964). In vitro, *B. abortus* has been described to replicate within vacuoles of hamster kidney cells (Hatten and Sulkin, 1966a; Hatten and Sulkin, 1966b), primary cultures of bovine adult and fetal cells (Richardson and Holt, 1964), and a number of epithelial cells and macrophages (Baldwin and Winter, 1994; Caron et al., 1994b; Detilleux et al., 1990a, 1990b; Liautard et al., 1996; Pizarro-Cerdá et al., 1998b; Pizarro-Cerdá et al., 1998c; Sola-Landa et al., 1998). Among the nonprofessional phagocytes, epithelial HeLa and fibroblastic Vero cell lines have been the most extensively used for studying *Brucella* replication and intracellular trafficking. Among professional phagocytes, murine J774, murine peritoneal macrophages, human monocytes, bovine mammary gland macrophages and human neutrophils have been the most widely used cells.

M cells, neutrophils, nonactivated macrophages from newly infected hosts, activated macrophages from immune animals and nonprofessional phagocytes all serve a different purpose during the course of *Brucella* infection. While the translocation of ingested *Brucella* organisms occurs through M cells, the first line of defense against *Brucella* invasion is secured by neutrophils, short-lived cells with a strong capacity to phagocytize (Ackermann et al., 1988). Although neutrophils are not the preferred niche for *Brucella* replication, some of these intracellular bacteria are capable of withstanding destruction inside these leukocytes (Kreutzer et al., 1979b; Young et al., 1985). In turn, this event may favor the spreading of the parasite from neutrophils to other tissues (Ackermann et al., 1988; Enright, 1990a). In the second line of defense are the macrophages, which, like neutrophils, can destroy an important proportion of the ingested *Brucella* but may also serve as substrate for *Brucella* replication as well as vehicles for transportation to other tissues. In the pregnant animal, *Brucella* invades the erythrophagocytic trophoblasts, which are the preferred replicating host cells and the site from which the bacteria spread to the fetus (Anderson et al., 1986b; Anderson et al., 1986c; Tobias et al., 1993). Generally, immune individuals are capable of controlling the infection via stimulation of the macrophagic system. Depending upon the animal species, the humoral response may serve as an important aid for phagocytosis and for conducting the intracellular route of the phagocytosed bacteria to destructive compartments.

Important findings revealing the interaction between *Brucella* parasites and their cells have been established. At the molecular level, the information is still incomplete and analysis of the bacterial factors and cellular receptors involved in the invasion process is necessary to understand the downstream steps observed during internalization and intracellular transit. Physical isolation and molecular examination of the different *Brucella*-containing compartments are necessary for the identification of the molecules that play a role in the original trafficking exhibited by this parasite.

Survival Within Polymorphonuclear Neutrophils Neutrophils are the primary line of defense during *Brucella* invasion. These cells are capable of ingesting and killing *Brucella* faster and more efficiently than other cells are (Kreutzer and Robertson, 1979a; Riley and Robertson, 1984a; Riley and Robertson, 1984b). After incubation with neutrophils, virulent *Brucella* organisms are steadily destroyed. From 50 to 80% of the ingested bacteria are killed within 2 to 5 hours (Riley and Robertson, 1984a). This microbicidal activity seems to be more efficiently performed when bacteria are opsonized (Canning et al., 1988; Young et al., 1985). Once ingested, the bacteria are found within vacuoles, some of which already demonstrate fusions with lysosome-like granules (Figs. 30 and 33). At later times, fusions of azurophilic granules increase in most but not all *Brucella*-containing vacuoles. These events proceed without extensive neutrophil degranulation (Riley and Robertson, 1984b). Exudates from lesions, milk and blood from infected animals demonstrate *Brucella* within resident neutrophils with no signs of replication (Ackermann et al., 1988; Tobias et al., 1993). It seems that some intracellular bacteria are capable of resisting for several hours or even for days the microbicidal assault displayed by neutrophils, which in time may release live bacteria on sites where they could invade more temperate host cells (Ackermann et al., 1988).

Life Within Non-professional Phagocytes The initial number of *Brucella* cells per infected epithelial Vero or HeLa cell is low, with one or two bacteria per cell (Detilleux et al., 1990a, 1990b; Pizarro-Cerdá et al., 1998c; Pizarro-Cerdá et al., 1998b; Fig. 21). Even if the bacterial inoculum is augmented in several logs, the rate of infection per cell remains low, suggesting that not all cells are permissive (Sola-Landa et al., 1998). Despite this, once the brucellae bind, the penetration efficiency is close to 100%. Immediately after phagocytosis, *Brucella* organisms localize within

single membrane compartments generally containing only one bacterium. During the first hours (1–5), no clear signs of intracellular bacterial replication are demonstrated, and the rate of cellular infection remains relatively constant for the next 5–8 hours. After 24–48 hours, the number of infected cells may decrease down to one half, depending on the concentration of antibiotic in the culture media, suggesting that at early stages phagosomes containing *Brucella* may converge with readily pinocytosed vacuoles from the constitutive endocytic route. The number of bacteria, estimated as CFU, increases several logs from the initial inoculum, indicating extensive bacterial replication within the cytoplasm of infected cells. Although many cells possess a large number of intracellular bacteria, the cell monolayers do not show signs of cytopathic effect and the individual cells remain attached to the matrix. A small proportion of cells contain a low number of *Brucella* (1–50) with no signs of bacterial degradation, while fewer of them demonstrate bacterial debris within phagolysosome-like compartments. The small number of bacteria within cells is not the result of new infections, since normally low concentrations of gentamycin (0.5 µg/ml) are capable of controlling extracellular bacteria (Pizarro-Cerdá et al., 1998c; Pizarro-Cerdá et al., 1998b). Alternatively, the low number of live bacteria within host cell vacuoles after 48 hours may be reminiscent of chronic infections in animals (C. Guzmán-Verri et al., unpublished observations). Multivesicular bodies compatible with autophagosomes are frequently present in infected cells but not in control monolayers.

The absolute increase in CFU is due to an expansion in the number of intracellular bacteria per parasitized cell rather than to an increase in the number of infected cells (Detilleux et al., 1990a, 1990b; Pizarro-Cerdá et al., 1998b; Pizarro-Cerdá et al., 1998c). Under the electron microscope, the cell cytoplasm is filled with *Brucella* within ribosome-lined cisternae, compatible with the endoplasmic reticulum (Detilleux et al., 1990a, 1990b). In the most heavily infected cells, bacteria also are seen within the perinuclear space. Although the nuclear membrane is constrained due to the large number of bacteria surrounding the nucleus, none of them invade the cell nucleus. These heavily parasitized cells do not show degeneration, do not look apoptotic or present signs of necrosis (Chaves-Olarte et al., 2002). Moreover, dividing cells with the cytoplasm packed with bacteria are frequently observed (Fig. 30). Nevertheless, beyond 48 hours cellular rupture proceeds as a consequence of *Brucella* overgrowth. The antibiotics in the tissue culture medium kill the freed bacteria, generating a rapid decrease in the number of colony forming units (Pizarro-Cerdá et al., 1998b).

Very similar events to those described in Vero and HeLa cells at 48 hours postinfection have been described in trophoblasts from experimentally infected animals (Anderson and Cheville, 1986a; Anderson et al., 1986b; Anderson et al., 1986c). Most if not all the *Brucella* cells are within the endoplasmic reticulum, and despite being filled with bacteria, infected trophoblasts do not show signs of degeneration, and the cellular junctions look normal (Fig. 30). Matching to what has been observed in cultured monolayers, bacteria remain within the endoplasmic reticulum compartments of individual cells, with no signs of horizontal movement from cell to cell. Actually, some noninfected cells are bound together with heavily infected trophoblasts, giving a clear indication that, even in vivo, *Brucella* organisms remain within vacuoles of their host cells until released. The trophoblast nucleus is never parasitized, although it is constrained by bacteria that lay very close or within cisternae that collide with the nuclear membrane. Similarly to epithelial cell monolayers, the infected trophoblasts seem to fracture at later times, owing to the large number of intracellular bacteria that eventually are released to the lumen.

Transient Interaction with the Early Endosomal Network During the first minutes after invasion, both the virulent *B. abortus* strain 2308 and the attenuated strain 19 interact with an intracellular compartment related to the early endosomal network (Fig. 36). This is confirmed by the presence of markers, such as the transferrin receptor, the small GTP-binding protein rab5, or the early endosomal antigen 1 (EEA1), in the *Brucella*-containing compartments (Pizarro-Cerdá et al., 1998). Several of these markers also have been observed in early phagosomes of other intracellular pathogens, such as *Salmonella typhimurium*, *Leishmania donovani*, *Mycobacterium tuberculosis* and *Listeria monocytogenes* (Alvárez-Domńguez and Stahl, 1999; Steele-Mortimer et al., 1999; Scianimanico et al., 1999; Via et al., 1997). All these parasites use very different strategies to associate and penetrate host cells, suggesting that there is an invariable minimal cellular machinery regularly required to accomplish the internalization steps of an external agent. This seems to be more important when the host cell is the entity that performs an active role in the process. A different phenomenon could be expected in the case of the *Brucella* close relative *Bartonella bacilliformis* or the protozoan *Toxoplasma gondii* and *Trypanosoma cruzi*, in which the motile forces of the pathogens themselves drive the invasion processes

(Doborowski et al., 1996; Tardieux et al., 1992; Scherer et al., 1993).

The association of *B. abortus* with the early endocytic network is transient, since after 10 min of internalization, the number of *Brucella*-containing compartments labeled either with rab5 or EEA1 decreases significantly, and no labeling is detected with these markers after 30 min postinoculation (Pizarro-Cerdá, 1998a). The integrity of the early endosomal system is relevant to the subsequent normal trafficking of *B. abortus* in host cells. For instance, in the cell line NIH3T3 rab5Q79L, in which the activated form of rab5 (bound to GTP) is expressed, an important fraction of the internalized parasites are unable to escape from the early *Brucella*-containing compartment supporting bacterial replication within giant vesicles labeled with rab5 (Pizarro-Cerdá, 1998a). However, after 48 h of infection, *B. abortus* proliferation is attenuated in NIH3T3 rab5Q79L cells in comparison to the wildtype NIH3T3 counterparts (Pizarro-Cerdá, 1998a). The augmentation of the endocytic activity of the mutant cells could be responsible for an increase in the delivery of gentamycin to intracellular compartments. Indeed, an important fraction of the intracellular brucellae remains associated with the early endosomal network, exposing the bacteria to the bactericidal activity of the antibiotic within this compartment. Alternatively, this unnaturally generated compartment may not be suitable for the adequate delivery of the necessary nutrients for intracellular bacterial replication.

Association with the Autophagic Machinery

After their transit through early phagosomes, neither the virulent *B. abortus* strain 2308 nor the attenuated strain 19 interacts with the late endosomal network at 30 min postinvasion (Pizarro-Cerdá et al., 1998b). In contrast, latex beads or dead bacteria-containing phagosomes interact transiently with late endocytic compartments, characterized by the presence of the small GTP-binding protein rab7 or the mannose 6-phosphate receptors. This result is confirmed after infection of NIH3T3 rab7Q67L cells (in which rab7 is expressed in its GTP-bound form) with *B. abortus* 2308. In this mutant cell line, the bacterial replication is similar to that in wildtype cells (Pizarro-Cerdá, 1998a). Although *Brucella* does not transit through the late endosome network, vacuole acidification seems to be required, since chloroquine, ammonium chloride and monensin (all substances that inhibit endosomal acidification) are capable of reducing the number of intracellular bacteria at early but not at later times after infection (Detilleux et al., 1991). Acidification step without the acquisition of lysosomal markers may be necessary for the activation of virulence genes as it occurs with other parasites (Antoine et al., 1990; Buchmeier and Heffron, 1990).

Following these initial steps, the *Brucella*-containing compartment is transformed gradually, and after 1 h of internalization, both virulent strain 2308 and attenuated strain 19 are present in an intracellular multimembranous compartment decorated with the lysosomal-associated membrane protein (LAMP) 1, but devoid of the luminal lysosomal hydrolase cathepsin D. This finding supports previous propositions in the sense that virulent *B. abortus* inhibits the fusion of its phagosome with lysosomal compartments (Frenchick et al., 1985). Several criteria permitted the identification of this late *Brucella*-containing compartment as an autophagosome (Fig. 36). First, the multimembranous nature of the LAMP-1-positive cathepsin D-negative vacuole is highly reminiscent of autophagosomes. Second, this compartment is labeled by monodansylcadaverine, a marker known to accumulate in autophagosomal bodies. Third, the endoplasmic reticulum marker sec61β is present in this *Brucella*-containing vacuole, attesting to an endoplasmic reticulum-related origin of this compartment. Fourth, modulation of the autophagocytic process regulates the intracellular fate of the internalized brucellae (Pizarro-Cerdá et al., 1998b; Pizarro-Cerdá et al., 1998c).

The presence of LAMP-1 in the late *Brucella*-containing compartment could be explained by a direct delivery of this molecule from the Golgi complex to the maturing autophagosomes. It is interesting to note that LAMP-1 has been widely associated with pathogen-containing compartments, such as the vacuoles of *S. typhimurium*, *L. donovani* and *L. monocytogenes* (Scianimanico et al., 1999; Steele-Mortimer et al., 1999; J. Pizarro-Cerdá, personal communication). This molecule could be present in these compartments accidentally as a bystander, only as an outcome of the different trafficking pathways followed by this molecule, transported in certain cases to the plasma membrane before being delivered to the lysosomes (Hunziker and Geuze, 1995). However, the actual function of the LAMP family of glycoproteins has not been clearly defined (Andrejewski et al., 1999), and it would be interesting to determine if this molecule actually plays an active role that could be relevant to the intracellular survival of all the intracellular pathogens described above. The association of an intracellular pathogen with the autophagic pathway is not unique to *B. abortus*, and it has also been observed in the case of *Legionella* (Swanson and Isberg, 1995). It is not known how these bacteria are able to interact with the autophagic cascade. An interaction between early endocytic compartments and

autophagic vacuoles has already been detected (Liou et al., 1997), indicating that a physical connection could exist between early *Brucella*-containing compartments and autophagosomes. Several scenarios could then be conceived to explain the transfer of the pathogen from one intracellular compartment to the other. First, there could be a fusion between the *Brucella*-containing compartment and an already formed autophagic vacuole. However, how the bacteria are finally found within the luminal space of a multimembranous compartment could not be directly explained by this hypothesis. A second, but highly improbable, possibility is the escape of *B. abortus* from the *Brucella*-containing compartment to the cytoplasmic space, where the bacteria could be captured by nascent autophagosomes, but free brucellae are seldom observed in the cytosol of infected cells. A third possibility would be that the autophagosomal vacuoles are formed by invagination of the endoplasmic reticulum membranes around *Brucella*-containing compartments. Nevertheless, the absence of other endoplasmic reticulum markers, such as BiP or ribophorin, in the *Brucella*-containing compartment (Pizarro-Cerdá et al., 1998b) contradicts this hypothesis. A modification of this alternative would be that only specialized regions of the endoplasmic reticulum, devoid of BiP or ribophorin, are involved in autophagosome formation.

Replication Within the Endoplasmic Reticulum In contrast to what is observed with attenuated and killed bacteria, most of the intracellular virulent *Brucella*-containing phagosomes lose the LAMP-1 labeling and never acquire lysosomal markers in nonprofessional phagocytes (Pizarro-Cerdá et al., 1998b; Pizarro-Cerdá et al., 1998c). However, this final *Brucella*-containing compartment retains the sec61β labeling and acquires other markers of the endoplasmic reticulum, such as the protein disulfide isomerase and calnexin (Pizarro-Cerdá et al., 1998b). The morphology of the final *Brucella*-containing compartment also differs from that of the autophagosomal stage: only a single membrane is detected around the replicating brucellae, and their intracellular location corresponds to the perinuclear area of the infected cells (Pizarro-Cerdá et al., 1998b; Pizarro-Cerdá et al., 1998c). All these data suggest that the virulent *B. abortus* transits from autophagosomes to the endoplasmic reticulum of host cells, where actual bacterial multiplication occurs (Fig. 36), confirming previous ultrastructural studies in trophoblasts and other mammalian cell lines (Anderson and Cheville, 1986a; Anderson and Cheville, 1986b; Detilleux et al., 1990a, 1990b). Additional evidence confirms the nature of the final niche of *B. abortus* replication in host cells. First, treatment of infected cells with brefeldin A, which normally induces the reorganization of the Golgi complex around the endoplasmic reticulum, induces the colocalization of Golgi markers around the *Brucella*-containing compartments (Pizarro-Cerdá et al., 1998b). Second, the treatment of infected cells with proaerolysin, a drug from *Aeromonas hydrophyla* that induces vacuolization of the endoplasmic reticulum in target cells, induces vacuolation of *Brucella*-containing compartment (Pizarro-Cerdá et al., 1998b). Treatment of target cells with proaerolysin before *Brucella* inoculation impairs the bacterial replication process and induces the degradation of virulent strain 2308 (Pizarro-Cerdá et al., 1998c), suggesting that the integrity of the structure of the endoplasmic reticulum is indispensable for the appropriate multiplication of *B. abortus*.

The benefits involved in the association of intracellular pathogens with the host-cell endoplasmic reticulum have not been characterized yet. Besides *B. abortus*, *L. pneumophila*, *T. gondii* and simian virus 40 multiply in this intracellular environment, revealing a path of convergent evolution in nonrelated organisms. In addition to being a strategy for avoiding lysosomal fusion during the final steps of intracellular invasion, association of *B. abortus* with the host endoplasmic reticulum could be a means of obtaining metabolites synthesized or translocated to this compartment (Sinai et al., 1997a; Stang et al., 1997; Swanson and Isberg, 1995). The strategy would be to take advantage of the biosynthetic enzymes, protein-conducting channels or peptide pores to increase the local nutrient supply (Sinai, 1997b), fulfilling the nutritional requirements for the bacterial growth. A possible prediction of this model would be that the cellular stock of short-lived molecules would decrease in *B. abortus*-infected cells due to the shortage of amino acids and peptides or to the blocking of the biosynthetic process of new proteins in the endoplasmic reticulum. However, heavily infected cells show no decrease in stock levels of short-lived molecules, such as LAMP-1 and LAMP-2 (J. Pizarro-Cerdá, personal communication). As with other intracellular parasites, such as *S. typhimurium* or *Leishmania*, it is still not clear why certain intracellular locations are preferred by different subsets of intracellular parasites to proliferate within host targets.

Trophoblasts as a Source of Growth Factors In the pregnant animal, *Brucella* organisms preferentially replicate in placental trophoblasts during the middle and late stages of gestation (Fig. 32), only after these cells actively secret steroids. The reason for this affinity and the process leading to

abortion after midgestation are not known. Infected trophoblasts produce cortisol, a steroidal hormone not normally generated by the placenta (Enright and Samartino, 1994). The increased levels of prostaglandin F2α and decreased production of progesterone, coupled with increased synthesis of estrogens and cortisol in the *B. abortus*-infected trophoblast at mid- and late-stages of gestation, are identical to the hormonal changes occurring at term in normal cattle with the initiation of parturition. Intracellular *Brucella* probably induces synthesis of steroids and modifies the metabolism of prostaglandin precursors, such as arachidonic acid, because these hormones may be used as growth factors by the bacteria. The increased hydrophobicity of the *Brucella* outer-membrane (Fig. 22), together with the preference of the bacteria for replicating within this cistern (Anderson et al., 1986b; Anderson et al., 1986c), may represent an evolutionary adaptation for using hydrophobic substances available within the endoplasmic reticulum of trophoblasts.

Life Within Macrophages Taking apart the innate differences in susceptibility among the various species and strains of mammals, as well as the different sources from which macrophages could be isolated, it is important to understand that intracellular trafficking and replication of *Brucella* within these cells could be studied from various dichotomous perspectives. Each of these dichotomies possess their own variables; for example, activated versus resting macrophages, immune versus naïve macrophages, phagocytosis of opsonized versus nonopsonized bacteria, and cell cultures in the presence or in the absence of antibiotics. It is obvious that combined models of these dichotomies are possible, complicating matters even more. Therefore, it is important to understand that each of the various scenarios may alter the intracellular trafficking and the replication of the ingested bacteria (Eze et al., 2000; Jiang and Baldwin, 1993a; Jiang et al., 1993c). Similarly to the ingestion of *Brucella*, the microbicidal activity seems to be better performed with opsonized rather than with nonopsonized bacteria and by activated rather than by nonactivated macrophages (Pomales-Lebron and Stinebring, 1957). In addition, the presence of antibiotics considerably alters the outcome of the infection, depending upon their concentration in the culture medium (Eze et al., 2000). In this sense, it is expected that bacterial opsonization and macrophage activation determine, to some extent, the outcome in the intracellular trafficking and the fusion events of vacuoles containing *Brucella*. With respect to opsonization, caution is necessary, since conflicting results have been obtained.

Structural and Morphological Changes During Macrophage Infection During the first two hours after macrophage *Brucella* infection, the bacteria localize within single phagosomes, with no signs of microbial degradation (Arenas et al., 2000; Pizarro-Cerdá, 1998a; Pizarro-Cerdá et al., 1998b; Pizarro-Cerdá et al., 1999b). The number of ingested *Brucella* depends upon the inoculum size, opsonization and macrophage activation (Kuzumawati et al., 2000). Fusions between lysosome-like granules and some *Brucella*-containing phagosomes are evident at these early times, and few intracellular bacteria localize within multimembrane compartments. After 12–15 h of ingestion, the number of *Brucella* decreases one to two logs, and more than one bacterium per vacuole is frequent (Gross et al., 1998; Gross et al., 2000; Jiang and Baldwin, 1993a; Jiang et al., 1993c; Porte et al., 1999). Microscopic observation reveals that an important proportion of *Brucella*-containing compartments have fused with lysosomal granules and many of the bacteria within look degraded, although a few appear intact, generally surrounded by a single vacuolar membrane (Arenas et al., 2000; Gay et al., 1981; Gay et al., 1986; Harmon et al., 1988). At later times (15–24 h), the number of intracellular bacteria increases in some but not all infected cells. While some macrophages are capable of controlling the infection, others become an adequate substrate for *Brucella* replication (Gay et al., 1981; Gay et al., 1986; Pomales-Lebron et al., 1957). Still, some bacteria look digested within vacuoles and bacterial debris are evident, but a proportion of nondegraded bacteria remain, generally surrounded by a single vacuolar membrane. Between 24 and 48 hours, the number of intracellular *Brucella* per cell rises, until the cytoplasm of the phagocytic cells is filled with parasitic bacteria (Jiang and Baldwin, 1993a; Pizarro-Cerdá, 1998a; Pizarro-Cerdá et al., 1999a; Pomales-Lebron et al., 1957). Similar to what has been described for nonprofessional phagocytes, the nuclear membrane looks constrained, owing to the large number of microorganisms surrounding the nucleus. However, none of the bacteria invade the cell nucleus (Fig. 30). These heavily parasitized macrophages do not look apoptotic, look vacuolated or present signs of necrosis. Nevertheless, beyond 48 h, cellular rupture seems to proceed as a consequence of *Brucella* overgrowth, and release of the bacteria occurs. The freed bacteria are normally killed by the antibiotics in the tissue culture medium, generating a rapid decrease in the number of colony forming units (Sola-Landa et al., 1998).

Trafficking and Replication Within Macrophages The fact that not all cultured infected macrophages sustain bacterial replica-

tion after 12 h is an indication of the powerful brucellacidal activity of these cells. Those bacteria that finally replicate intracellularly must escape from the digestive abilities of macrophages. It has been proposed that phagosome acidification dependent of proton ATPases, occurring at early (1 h) but not at later times (after 7 h), is essential for bacterial replication (Porte et al., 1999). Other investigators have observed that acidification of phagosomes takes place at early (0.5–2 h) as well as later (20 h) times (Arenas et al., 2000). Acidification of *Brucella*-containing phagosomes may work in two directions. One of them may just be the default outcome of phagosome-lysosome fusion with negative consequences for the bacterial survival. The second may work in favor of the parasitic bacteria. That is, the intracellular bacterium may require an acidic environment for activating genes necessary for an intracellular life cycle, such as the type IV secretion system apparatus (Alpuche-Aranda et al., 1992; Boschiroli et al., 2002). Even though lowering the pH of the phagosome may be necessary to prepare the *Brucella* replicating environment, this may not be sufficient, since phagosomes containing live bacteria or killed organisms acidify at a similar rate. Another consideration is the presence of gentamycin in the culture media. This antibiotic is readily pinocytosed by macrophages and considerably inhibits the intracellular growth of the *Brucella*, mainly when it is used in the culture medium at concentrations above 10 μg/ml (Eze et al., 2000).

The biogenesis of phagolysosomes and the ability to degrade invading microorganisms involve a regulated series of interactions between phagosomes and endocytic organelles (Desjardins et al., 1994; Finlay and Falkow, 1997b; Pitt et al., 1992). During the first stages after infection (0.5–2 h), an important proportion of phagosomes containing bacteria seem reluctant to fuse with newly internalized vesicles, with the characteristics of lysosomes (Pizarro-Cerdá et al., 1999a; Pizarro-Cerdá, 1998a). At ten minutes after inoculation, *Brucella* organisms are transiently detected in phagosomes, characterized by the presence of EEA1 (Fig. 35). Live but not dead *Brucella* exclude annexin I from early macrophage phagosomes (Harricane et al., 1996). At one hour postinoculation, bacteria are located within a compartment positive for LAMP but negative for mannose-6-phosphate receptor and cathepsin D protein, indicating that virulent *Brucella* avoids fusion with late endosomes and lysosomes (Pizarro-Cerdá et al., 1999a; Pizarro-Cerdá, 1998a). At later times (12–24 h), fusions between newly internalized vesicles and *Brucella*-containing vacuoles are common events. However, the lysosomal markers LAMP and cathepsin D are excluded from vacuoles containing replicating *Brucella* (Pizarro-Cerdá, 1998a), indicating that some bacteria have actively avoided the constitutive degradative pathway commonly followed by inert particles (Desjardins et al., 1994). Mature compartments containing live replicating *Brucella* (after 24 h) are devoid of the late endosomal marker mannose-6-phosphate receptor and lysosomal proteins (LAMP and cathepsin D) but are positive for the endoplasmic reticulum marker sec61β (Fig. 35), suggesting that at least some bacteria have reached the same replicating niche (the endoplasmic reticulum) as in nonprofessional phagocytes (Pizarro-Cerdá et al., 1999a; Pizarro-Cerdá, 1998a).

In general terms, phagocytic cells are more prone to destroy *Brucella* and control its intracellular replication than are nonprofessional phagocytes. For instance, when *Brucella* strains of low and high virulence are compared, it is clear that no strain displaying low virulence withstands the powerful destructive machinery of professional phagocytes, whereas some of these strains are capable of replicating within nonprofessional phagocytic cells (Detilleux et al., 1990a; Detilleux et al., 1990b; Harmon et al., 1988; Fig. 35). Similarly, macrophages defective in fusion events are more permissive cells not only for virulent *Brucella*, but also for the attenuated strains that are killed by healthy macrophages (Pizarro-Cerdá et al., 1999b). Moreover, microscopic examination reveals that while in nonprofessional phagocytes most of the internalized bacteria travel within intracellular compartments that do not fuse with late endosomes and lysosomes, in macrophages a relatively large proportion of the phagosomes fuse with acidic vacuoles and lysosome-like compartments (Arenas et al., 2000). In nonprofessional phagocytes, most of the ingested *Brucella* transit from early endosomes to autophagosomes and then to the endoplasmic reticulum (Fig. 35), whereas in macrophages just a small proportion of *Brucella*-containing compartments seem to reach the endoplasmic reticulum (Fig. 35).

In murine knockout macrophages deficient in fusion events, both virulent and attenuated *B. abortus* strain 19 replicate more readily than in normal macrophages (Pizarro-Cerdá et al., 1999a). Restoration of this defect re-establishes the microbicidal activity, indicating that fusion of vacuoles containing *Brucella* with newly internalized vesicles is necessary for controlling intracellular *Brucella* replication. Therefore, it seems that *Brucella* within nonfusiogenic vacuoles are more likely to reach the final intracellular replicating niche than those bacteria within vacuoles that fuse with lysosome-like compartments.

Electron microscopy of infected macrophages reveals that the replicating pattern displayed by *Brucella* within macrophages, at later times (Fig. 30), is reminiscent of the replicating pattern within the endoplasmic reticulum of trophoblasts and epithelial cells (Detilleux et al., 1990b; Anderson and Cheville, 1986a; Anderson and Cheville, 1986b).

Some investigators have proposed that in macrophages, *Brucella* organisms do not reach the endoplasmic reticulum but rather they delay maturation of phagosomes into phagolysosomes and that, during this process, acidification and subsequent modification of the compartment establish the replicating niche, which is compatible with a modified phagolysosome (Arenas et al., 2000). Others have proposed that virulent *Brucella* transit from early phagosomes to the endoplasmic reticulum in both macrophages and nonprofessional phagocytes (Pizarro-Cerdá, 1998a; Pizarro-Cerdá et al., 1999b). Although at this point these two views cannot be conciliated, it is clear from the pure microscopic point of view that a parallelism in the replication of *Brucella* within the cytoplasm of macrophages and nonprofessional phagocytes exists at later times. It may be that most of the *Brucella* organisms in macrophages are destroyed by lysosomes, while only a few bacteria arrive to the endoplasmic reticulum. However, once these few bacteria are within the endoplasmic reticulum, they are capable of replicating inside this compartment, which is nonfusiogenic with bactericidal lysosomes (Fig. 35). In nonprofessional phagocytes, the events are reversed: only a few internalized *Brucella* are destroyed within lysosomes, while most of the *Brucella* arrive to the endoplasmic reticulum, trafficking through the safer autophagocytic route (Fig. 36).

Obviously, the most economical pathway for the bacteria would be to use the same molecular machinery for intracellular trafficking and for subtracting resources in different host cells, rather than to possess a different molecular strategy for each cell type. An example of this can be seen in *Legionella* species, bacteria that use the same machinery and strategy to parasitize their free-living host amoebae and the resident macrophages in the lung of their victims (Barker et al., 1993). Thinking in this direction, the phagolysosome route establishes different constraints that must be surpassed. Similarly, the phagolysosomes and endoplasmic reticulum perform different functions and display a different set of resources for the intracellular bacteria. Therefore, it seems unlikely that *Brucella* could have evolved two different mechanisms to adapt equally well to two distinct intracellular environments as proposed by some authors (Arenas et al., 2000).

Cellular Functions During Infection Transit of virulent *Brucella* from autophagosomes to the endoplasmic reticulum requires cellular machinery that has not been identified. In CNF-intoxicated cells, the intracellular trafficking to the endoplasmic reticulum via the autophagocytic route remains intact, even though the actin cytoskeleton is functionally arrested (C. Guzmán-Verri et al., unpublished observations). Similarly, segregation of chromosomes and nuclei assembly are not defective, whereas the formation of a contractile ring for cell cytokinesis, mediated by an actin-myosin structure, is impaired in these CNF-intoxicated cells. Colchicine added during the infecting period has a modest effect on internalization, although it reduces the number of cells with intracellular replicating bacteria and has a profound effect on the morphology of the parasitized cells, generating polymicronuclei (Detilleux et al., 1991). It is possible that microtubule but not actin structures are involved in the retrograde transport of *Brucella*-containing compartment to the endoplasmic reticulum. The participation of molecular motors, such as dynein, which normally promotes the retrograde motion of membrane-bound vesicles through microtubules, is a mechanism that must be considered during the intracellular biogenesis of *Brucella*. Cycloheximide does not inhibit intracellular bacterial replication, and therefore, it is feasible to propose that de novo host protein synthesis is not required during *Brucella* parasitism (Detilleux et al., 1991).

Proteins from the coat protein I (COPI) complex, known to participate in the anterograde transport of vesicles in the Golgi apparatus, have recently been implicated in the retrograde transport of vesicles to the endoplasmic reticulum (Orci et al., 1986; Orci et al., 1997). The presence of several subunits of the COPI complex in compartments associated with the endocytic cascade (Whitney et al., 1995) suggests that these molecules could establish a link among endocytic compartments, autophagosomal vacuoles and the endoplasmic reticulum, a link that is necessary for the intracellular trafficking of *B. abortus*. Molecules from the SNARE family (SNAREs are membrane-associated proteins that play a central role in vesicle targeting and intracellular membrane fusion in eukaryotic cells) or small GTP-binding proteins of the rab family could also be implicated in the retrograde transport of the *Brucella*-containing compartments. Recently, it has been demonstrated that autophagocytosis could be blocked by GTP-γS (a nonhydrolyzable analogue of GTP), suggesting that this process requires GTP-binding proteins (Kadowaki et al., 1994). It is interesting to note that the

anterograde transport of vesicles from the endoplasmic reticulum is dependent on the small GTP-binding protein Sarpl and the COPI-like complex, COPII (D'Enfert et al., 1991). Similar or the same molecules could be implicated in autophagosomal formation and could be under *B. abortus* control to induce its retrograde transport to the endoplasmic reticulum.

Brucella Virulence Mechanisms Virulence can be understood as those bacterial mechanisms that cause cell damage or resist host defenses. Within this context, virulence factors are envisioned as bacterial products, structures or genes that, when absent or modified, alter the course of the pathogenesis without affecting the bacterial viability or the growth under regular culture conditions. There are overwhelming data demonstrating that the ability of *Brucella* organisms to produce disease relies in their capacity to invade cells of healthy immunocompetent hosts (Baldwin and Winter, 1994; Enright, 1990b; Liautard et al., 1996; Pizarro-Cerdá et al., 1999b; Pizarro-Cerdá et al., 2000). Two simple conclusions are derived from these clear cut facts: first, *Brucella* organisms are primary pathogens of mammals that do not require anomalous host conditions to establish infections; and second, the essential *Brucella* virulence strategy can be envisioned as the ability to replicate within host cells until more virulent bacteria are released. Therefore, the maintenance of these properties may be considered sine qua non for *Brucella* virulence and for successful parasitism.

In spite of the relatively large number of DNA sequences identified as potential virulence genes, very few structures and mechanisms related to pathogenesis have been described (Moreno and Moriyón, 2002; Sánchez et al., 2001; Ugalde et al., 1999). The *Brucella* mutants may be distinguished according to their replicating abilities in bacteriological media, cells and animals. Many of the attenuated mutants in cells or animals are metabolically defective strains displaying anomalous growth in vitro and requiring particular supplemented media and culture parameters. Others are impeded from making proteins necessary for confronting growth under stress conditions, whereas others have defects in repairing systems and division machinery. Although these general defects are interesting and prime for practical purposes, they are unlikely to be considered as virulence factors. Paragraphs in the sections Mechanisms of Entry to Host Cells through Modulation of the Immune Response summarize what it is known about the strategies followed by pathogenic intracellular *Brucella* and some of the genetic systems involved.

Mechanisms of Entry to Host Cells Only efficiency of invasion and intracellular replication, but not adherence to the cell surface, positively correlate to *Brucella* virulence (Fig. 21). As stated (Antitumoral and Antiviral Activity of *Brucella* Cells and Their Fractions), a number of natural or artificial engineered nonvirulent rough bacteria bind and penetrate cells in a proportion larger than that of wildtype *Brucella* (Detilleux et al., 1990a; Detielleux et al., 1990b; Freer et al., 1999; Pizarro-Cerdá, 1998a; Sola-Landa et al., 1998). Mutations in *bvrS* or *bvrR* genes (The BvrR-BvrS System) of a two-component, sensory-regulatory system (Fig. 28) hamper the penetration of smooth *B. abortus* into HeLa cells and considerably reduce the efficient invasion of murine macrophages without impeding bacterial binding (Sola-Landa et al., 1998). Dysfunction of either the *bvrR* or *bvrS* impairs the bacterial invasion, intracellular trafficking and virulence, while knockout of both *bvr* genes seems to be lethal (D. O'Callaghan, personal communication). Both of these avirulent smooth mutants have the tendency to accumulate on the surface of cells. The rough *perA* mutant (Table 5), deficient only in the expression of surface *O*-polysaccharide and NH (J. Pizarro-Cerdá et al., unpublished observations), binds to cells in larger numbers. However, the double rough/*bvrS* mutant binds to cells in the same proportion as the *bvrS* single mutant (C. Guzmán-Verri et al., unpublished observations). Therefore, the exposed elements on the surface of rough *Brucella* necessary for adherence to cells are absent in the bvrS mutant. Comparative studies reveal that *bvrS/bvrR* mutants are deficient in at least two sets of Omps (C. Guzmán-Verri et al., unpublished observations), which correspond to the group 3 of Omps present in outer-membrane blebs (Gamazo et al., 1989; Moriyón et al., 1987; Omp3a and Omp3b Family). Estimation of the Omp3a (Omp25) and Omp3b mRNA reveals low expression in both mutants, suggesting that the absence of this protein from the outer-membrane may be the result of reduced transcription. The role of Omp3a in virulence is further suggested by the fact that disruption of the *omp3a* gene attenuates *Brucella* (Elzer et al., 2000). Functional and structural analysis of the *bvr*S and *bvr*R mutants indicate that the lipid A and maybe the core, but not the *O*-polysaccharide of the LPS, have subtle but detectable changes with respect to the wildtype molecule. In this respect, the function of the BrvR-BrvS two-component regulatory system may be similar to that of other regulatory systems described in Gram-negative intracellular bacteria (Gunn and Miller, 1996). Although the BvrR-BvrS has low homology with PhoP-PhoQ, it is interesting that, among other characteristics, the PhoP-PhoQ activates the PmrA-PmrB system,

which in turn, regulates the lipid A structure. The overall conclusion is that multiple genes, several of them important for the expression of Omps and LPS synthesis, may be under the control of *bvr*R-*bvr*S, and that these elements are important for virulence. It has been proposed that the type IV secretion system coded by the *vir*B genes is involved in the penetration of *Brucella* to murine macrophages (Watarai et al., 2002); however, other investigators have indicated that the virB operon is not involved in cell invasion but rather in intracellular trafficking (Boschiroli et al., 2002; Comerci et al., 2001; Delrue et al., 2001; Sieira et al., 2000).

Controlling the Host Cell Eukaryotic cells have evolved strategies to destroy microorganisms within phagolysosomes by direct action of microbicidal substances. Intracellular survival requires the invader to be capable of escaping and resisting this cellular machinery. According to their functional roles, neutrophils and activated macrophages are the primary and ultimate defense cells against *Brucella* infections, respectively. Depending upon the initial inoculum, the bacterial strain and the host animal, it is likely that an ample number of *Brucella* infections are controlled on site by neutrophils (Fig. 33). In these cells, the engulfed *Brucella* seldom divides, but it must resist the assault of intracellular microbicidal molecules to survive. In the nonimmune host, *Brucella* replicates within naïve macrophages and nonprofessional phagocytic cells. Once the infection has been established, the activated macrophages, through a specific immune response, are the cardinal cells responsible for controlling and killing the invading *Brucella*. It seems that the difference in behavior observed among the various *Brucella*-infected cells is just a matter of degree in their killing capabilities. The survival strategy employed by *Brucella* within various cells seems to be basically the same and it can be resumed in the different but connected major episodes.

Brucella Resists Killing Mechanisms Professional phagocytes, such as polymorphonuclear leukocytes and macrophages, are adapted to engulf and destroy bacteria within phagolysosomes by direct action of bactericidal mechanisms that involve a collection of enzymes and cationic peptides and production of reactive oxygen and nitrogen intermediates. The respiratory burst involves a series of enzymatic reactions responsible for the conversion of oxygen to active metabolites, some of which exhibit microbicidal activities. It has been observed that ingestion of nonopsonized *B. abortus* by phagocytes induces very little respiratory burst and a very poor production of reactive nitrogen intermediates (Caron et al., 1994b; Jiang and Baldwin, 1993a; Jiang et al., 1993c; Kreutzer et al., 1979b). Alternatively, opsonized *B. abortus* organisms exposed to activated phagocytic cells stimulate significant levels of both superoxide-ion production and myeloperoxidase-hydrogen peroxide halide activity (Harmon et al., 1987; Harmon et al., 1988; Jiang and Baldwin, 1993a; Jiang et al., 1993c; Young et al., 1985). Lysosomal proteins and bactericidal cationic peptides fail to kill *Brucella* organisms (Kreutzer and Robertson, 1979a; Kreutzer et al., 1979b; Martínez de Tejada et al., 1995). Even attenuated *Brucella* are more resistant to the action of these molecules than are other intracellular parasitic Proteobacteria, such as polymyxin B resistant *Salmonella*, indicating by this the evolutionary adaptation of *Brucella* to intracellular life.

Outer Membrane Versus Bactericidal Substances The most conspicuous structural defect that renders *Brucella* organisms avirulent is the absence of the *O*-polysaccharide and the concomitant absence of the related NH polysaccharide molecules. In other words, a defect that results from the dissociation from smooth to rough phenotype (Allen et al., 1998; Detilleux et al., 1990a, 1990b; Freer et al., 1996; Kreutzer et al., 1979b; Martínez de Tejada et al., 1995; Riley and Robertson, 1984a; Stevens et al., 1994a). In general, smooth *Brucella* are more resistant than rough strains to the killing action of bactericidal substances of phagocytes.

The role of LPS in the permeability properties and in resistance to bactericidal substances has been definitively established in a series of experiments involving *Brucella* strains and the construction of LPS chimeras (Freer et al., 1996; Martínez de Tejada et al., 1995; Páramo et al., 1998). When the heterologous LPS inserted in the outer-membrane of susceptible bacteria corresponds to the less sensitive smooth *B. abortus*, the chimeras are more resistant to bactericidal cationic molecules. In contrast, when LPS is from the more sensitive bacteria, the chimeras are more susceptible to the action of bactericidal peptides. There is a direct correlation between the amount of heterologous smooth LPS on the surface of chimeric cells and sensitivity to bactericidal substances (Freer et al., 1996). Although this particular resistance to bactericidal molecules is related to the core and lipid A structures, there is a contribution of the *O*-polysaccharide and the associated NH, as suggested by the difference in susceptibility between the rough and smooth *Brucella* strains (Freer et al., 1996; Martínez de Tejada et al., 1995). It is worth noting that the resistance of *Brucella* LPS to cationic molecules is more conspicuous when it is integrated in its native outer-membrane. This is dem-

onstrated by the fact that *Brucella* smooth LPS micelles are partially permeabilized by the action of bactericidal peptides, whereas *Brucella* cells are not (Freer et al., 1996).

The *O*-polysaccharide and the NHs are only two of several factors necessary for virulence. Dysfunction of the BrvR-BrvS two-component, sensory regulatory system generates outer-membrane alterations manifested as increased susceptibility to bactericidal cationic substances and surfactants (Properties of the Outer Membrane). These defects are partially restored by inserting wildtype LPS in the outer-membrane of the *bvr* mutants (E. Moreno et al., unpublished observations), reinforcing the idea that, in addition to LPS, other outer-membrane molecules are also important. Because bactericidal cationic peptides are molecules devoted to cell defense against pathogenic parasites (Vaara, 1992), it is likely that some of the outer-membrane features relevant for resistance to lysosomal substances are under the control of the BvrR-BvrS system.

Stress Responses *Brucella* organisms generate a collection of proteins that theoretically could inhibit the bactericidal action induced during the respiratory burst, as well as enzymes that could neutralize the acid pH within the phagolysosome necessary for activating lysosomal hydrolytic enzymes. Significant changes in the pattern of *Brucella* proteins during intracellular growth have been recorded (Rafie-Kolpin et al., 1996). As expected, a proportion of the proteins expressed are heat-shock proteins, though others are possibly necessary to deal with other harsh conditions (Lin and Ficht, 1995; Teixeira-Gomes et al., 2000).

Neutralization of Oxygen and Nitrogen Intermediates *Brucella* organisms produce phosphomonoesterases, high concentrations of periplasmic cytochromes, Sod and catalase, all proteins that may be involved in the protection of the bacteria against free hydrogen peroxide and superoxide radicals generated by phagocytic cells (Kim et al., 2000; Saha et al., 1990; Tatum et al., 1992). In contrast to the enzyme of some intracellular parasites, such as *Legionella* or Leishmania, *Brucella* phosphomonoesterase does not block the production of superoxide anion and does not hydrolyze phosphatidylinositol diphosphate or IP3 molecules (Saha et al., 1990). A collection of *Brucella* mutants not expressing Cu^{++}/Zn^{++}-SodC or catalase proteins has been generated on the expectation that these defects would increase the sensitivity of the mutants to bactericidal action of oxidative intermediates produced during the respiratory burst (Kim et al., 2000; Tatum et al., 1992). It has been demonstrated that these two enzymes concomitantly increase after exposure of the bacteria to peroxide or superoxide ions (Kim et al., 2000), suggesting an adaptive response. In spite of their increased sensitivity to hydrogen peroxide (Kim et al., 2000), the catalase-deficient mutants replicate at the same rate as wildtype bacteria in mice, indicating that, at least in this model, catalase per se does not play a significant role in virulence (Grillǿ1997). Similarly, *Brucella* SodC-deficient mutants exhibit virulence and establish chronic infections in mice (Latimer et al., 1992). Mutants interrupted in the operon coding for cytochrome bd oxidase, which catalyzes an alternate terminal electron transport step in bacterial respiration, are highly attenuated and unable to replicate in cells. Superexpression of Cu++/Zn++ Sod and catalase in these mutants alleviates the loss of cytochrome bd oxidase (Endley et al., 2001), suggesting that these two enzymes play after all a role in virulence under certain conditions.

Role of Heat Shock Proteins Significant changes in the pattern of *Brucella* proteins during intracellular growth have been recorded. It has been shown that, in addition to variations in the expression level of 73 proteins, repression of 50 in vitro proteins and induction of 24 new proteins occur during growth of *B. abortus* within macrophages (Rafie-Kolpin et al., 1996). Acid and oxidative conditions, as well as nutritional and heat stresses, induce the synthesis of "new" bacterial proteins. However, the quantity of these "new" molecules produced in vitro is not equivalent to the amount expressed within macrophages (Lin and Ficht, 1995; Teixeira-Gomes et al., 2000).

Brucella *htr*A, which codes for the periplasmic heat shock-induced serine protease HtrA, is likely to participate in the degradation of oxidatively damaged proteins. Resembling *Salmonella* and *Yersinia* *htr*A mutants, *Brucella* *htr*A mutants demonstrate higher sensitivity to oxidative substances, reduced survival in neutrophils and defective replication in macrophages (Elzer et al., 1996; Phillips et al., 1995; Phillips et al., 1997). Comparable to other *Brucella* stress proteins, such as RecA, the mutation of which also reduces multiplication in mice, *htr*A *Brucella* mutants establish chronic infections, suggesting residual virulence (Phillips et al., 1995; Tatum et al., 1992; Tatum et al., 1993). In goats, *htr*A *Brucella* mutants demonstrate a more attenuated phenotype (Elzer et al., 1996).

Regulated expression of *dna*K under oxidative stress seems to be used by bacterial pathogens to withstand the respiratory burst of phagocytes (Caron et al., 1994a). Analysis of *Brucella* mutants carrying inactivated *dna*K and *dna*J (which code for the stress molecular chaperones DnaK and DnaJ) has led to the conclu-

sion that DnaK, but not DnaJ, is required for growth at 37°C. The *dna*K mutant survives but does not multiply within phagocytes at a temperature of 30°C, whereas the *dna*J mutant multiplies normally (Köhler et al., 1996). It has been proposed that intracellular *Brucella* organisms enter into a period of starvation that would favor their resistance to oxidative conditions found within vacuoles (Alcantara et al., 2000; Robertson and Roop, 1999; Robertson et al., 2000a). *Brucella abortus hfq* mutants (defective in the RNA chaperone host factor (HF-1)) do not replicate in macrophages, but initially they multiply in mice, suggesting that HF-1, normally required for maintenance at stationary phase, is also necessary during intracellular survival. The HF-1 protein participates, within several pathways, in the regulation of the sigma factor RpoS, required for maintenance at the stationary phase (Robertson and Roop, 1999). As a consequence, the deficient *hfq* mutant displays an impaired stress response and problems for adapting to the stationary growth phase. This mutant, impaired for long-term survival under nutrient deprivation, also demonstrates growth stage- and medium-dependent sensitivity to hydrogen peroxide and a decreased capacity to resist acidic environments, conditions likely to be found intracellularly.

Brucella lon mutants are impaired in their capacity to resist hydrogen peroxide and puromycin and display reduced survival in macrophages and significant attenuation in mice during the initial periods, but not at later times. The ATPase-dependent Lon protease is one of the principal enzymes involved in the turnover of stress-damaged proteins. The level of transcription of this protein increases in response to several environmental stresses. It has been proposed that Lon functions as a stress-response protease and is required in *Brucella* during the initial stages of infection, but it is not essential for the establishment and maintenance of chronic infections in the host (Robertson et al., 2000b). The role of other potential *Brucella* virulence genes, such as those coding for the heat shock protein GroEL and ClpATPase (Lin et al., 1996), has not been investigated in detail. *Brucella suis* null mutants for ClpATPase chaperonine behave similarly to the wild-type strain, indicating that ClpA by itself is dispensable for intracellular growth (Ekaza et al., 2000).

Role of the Urease Considering ammonium is the principal product and has a strong basic character, the decomposition of urea catalyzed by urease may serve to neutralize the acid pH within the phagolysosome. Urea is in all likelihood a product of the metabolism of *Brucella* (Response to Environmental Stress) and all *Brucella* species but *B. ovis* have a cytoplasmic urease. However, the urease-defective mutants do not display attenuation and behave similarly to the parental pathogenic strains in some virulence assays, evoking what has been observed with the catalase and SodC mutants (Jubier-Maurin et al., 2001; Grilló1997). This does not rule out a possible role for urease during the initial steps of host invasion in the penetration through the intestinal route (*Brucella* Invades Healthy Hosts).

Inhibition of Lysosome-Phagosome Function After 24 h, epithelial cells infected with virulent *B. abortus* display a significant increase in the number of intracellular bacteria distributed in the perinuclear region corresponding to the endoplasmic reticulum. In contrast, cells treated with killed *Brucella* harbor a small number of intact bacteria together with bacterial degradation products scattered throughout the cytoplasm, an action that proceeds with time. At these stages, cathepsin D, a well-known marker for lysosomes, colocalizes with vacuoles containing killed *B. abortus* and bacterial degradation products, attesting that phagosomes have fused with lysosomes (Pizarro-Cerdá et al., 1998a; Pizarro-Cerdá et al., 1998b). By contrast, intracellular virulent *Brucella* organisms seldom colocalize with cathepsin D in epithelial cells, indicating that this bacterium avoids fusion with lysosomes. In vitro fusion experiments between vacuoles containing *Brucella* and lysosomes isolated from macrophages show that although compartments containing killed bacteria fuse with lysosomes, vacuoles containing live *Brucella* do not fuse with these organelles (Naroeni et al., 2001). Moreover, intracellular *Brucella* organisms do not hamper the fusion of latex beads with lysosomes, a phenomenon that agrees with the cellular parasitism displayed by some rough attenuated *Brucella* mutants. Some rough intracellular brucellae follow the constitutive endosome-lysosome route, whereas others traffic to the endoplasmic reticulum in the same cell. The key virulence factor is not the general inhibition of lysosome-phagosome fusion in cells, but rather the active modification of the *Brucella*-containing compartment, making this organelle non-fusiogenic with lysosomes.

It has been found that phagosomes containing live *Mycobacterium* retain a host protein (TACO, a phagosomal coat protein that prevents degradation of mycobacteria in lysosomes) that prevents delivery of lysosomes into the parasite-containing phagosome (Ferrari et al., 1999). Similarly, it has been proposed that the *Leishmania* parasite inhibits phagolysosome biogenesis through insertion of a lipophosphoglycan into the phagosome membrane, precluding in this

manner the fusion of the phagosome with lysosomes (Desjardins and Descoteaux, 1997). Indirect evidence for inhibition of neutrophil "degranulation" by *B. abortus* has been obtained from studies of the effects of live or heat-killed organisms and bacterial extracts. *Brucella abortus* extracts apparently devoid of enzymes and LPS (but containing 5′-guanosine monophosphate and adenine) inhibited neutrophil degranulation (Canning et al., 1986). The same molecules seem to inhibit the myeloperoxidase-hydrogen peroxide-halide activity by specifically hampering degranulation of peroxidase-positive polymorphonuclear granules (Bertram et al., 1986). Similarly, studies conducted with *B. abortus* extracts were capable of inhibiting phagosome-lysosome fusion in macrophages (Frenchick et al., 1985). It has been observed that smooth *Brucella* LPS enhanced the intracellular survival of rough mutant bacteria in bovine neutrophils (Soto et al., 1991). Despite this, some authors have proposed that LPS does not participate in the inhibition of phagosome-lysosome fusion (Kreutzer and Robertson, 1979a; Frenchick et al., 1985). All these investigations, although valuable, need to explain the fact that only live virulent *Brucella* is capable of hampering lysosomal fusion (Pizarro-Cerdá et al., 1998c; Pizarro-Cerdá et al., 1998b; Naroeni et al., 2001).

Lipopolysaccharide Fails to Stimulate Lysosomal Activity Several studies have cited the *Brucella* LPS molecule as a virulence factor (Freer et al., 1996; Rasool et al., 1992; Velasco et al., 2000). However, in contrast to what has been proposed for other LPS, the virulence of *Brucella* LPS is not expressed in the classical form of an endotoxic active molecule (Fig. 18). It is known that LPS from most Gram-negative bacteria activates phagocytic cells by stimulating the respiratory burst, inducing the production of bactericidal nitrogen intermediates and the generation of active cytokines (Brade et al., 1988; Cline et al., 1968; Kelly et al., 1991). All these mechanisms contribute to the destruction of intracellular bacteria and promote the fusion of the ingested bacteria with lysosomes (Silverstein and Steinberg, 1990). In contrast, *Brucella* LPS practically does not stimulate the oxidative burst or stimulate the release of lysozyme in phagocytic cells and fails to generate significant amounts of bactericidal reactive oxygen intermediates (Jiang and Baldwin, 1993a; Jiang et al., 1993c; Rasool et al., 1992). Furthermore, *Brucella* LPS induces very little release of interferon gamma (IFNγ) or tumor necrosis factor (TNF), substances which are known to enhance lysosomal fusion and the microbicidal mechanisms of phagocytic cells (Goldstein et al., 1992; Keleti et al., 1974). In this respect, the low biological activity of *Brucella* LPS may be envisioned as an advantage of *Brucella* parasites to adapt to intracellular life. Within this context, the *Brucella* LPS is considered a virulent factor.

Iron Chelation and Bactericidal Action *B. abortus* organisms secrete 2,3 dihydroxybenzoic acid and the catecholic siderophore brucebactin (Denoel et al., 1997a; González-Carrero et al., 2002; Leonard et al., 1997; López-Goñet al., 1992; Uptake of Inorganic Nutrients). Hypothetically, iron-capturing molecules may compete for intracellular iron within the macrophage and inhibit iron-mediated bactericidal killing systems (Leonard et al., 1997). It has been demonstrated that iron-loaded macrophages have enhanced capability to kill or prevent the replication of intracellular *B. abortus* (Jiang and Baldwin, 1993b). This effect was demonstrated with opsonized and nonopsonized bacteria, as well as with attenuated and virulent strains of *B. abortus*. The augmented bactericidal activity in the presence of iron may be indirectly mediated by the Haber-Weiss-Fenton and related reactions (Jiang and Baldwin, 1993b). In these reactions, hydroxyl radicals are generated from hydrogen peroxide in the presence of Fe^{++}, which is oxidized to Fe^{+++}. Generation of more hydroxyl radicals and Fe^{+++} results from the reaction between superoxide and Fe^{++}. The fact that the bactericidal action could be blocked with hydroxyl scavengers supports this general idea. It is likely that, in activated macrophages, the concentration of superoxide ions and hydrogen peroxide increases within the lysosomes, thus providing the substrates for the above-summarized reactions. Similar phenomena have been observed with other Gram-negative and Gram-positive bacteria.

Although 2,3 dihydroxybenzoic acid has a comparatively moderate ability to chelate iron, it is able to block the Haber-Weiss-Fenton reaction. This compound protects killing of *Brucella* mediated by activated macrophages during the first 12 h and increases the number of intracellular brucellae recovered after 48 h of infection (Leonard et al., 1997). Mutations in *aro*C (the gene coding for chorismate synthase) block chorismate synthesis and therefore these mutants do not produce *para*-amino benzoic acid, a precursor of 2,3 dihydroxybenzoic acid. *Brucella aro*C mutants show reduced virulence, supporting a role for this molecule and/or the siderophore during intracellular survival (Foulongne et al., 2001; Hong et al., 2000). However, *aro*C mutants are pleiotropic, and Δ*entC B. abortus* mutants (which are defective in isochorismate synthase and thus do not produce 2,3 dihydroxybenzoic acid) are not attenuated in mice (Bellaire et al., 1999). Concurrent with this, *B. abortus* mutants in an *entF* homologue do not produce brucebac-

tin or excrete 2,3 dihydroxybenzoic acid but replicate normally in murine macrophages (González-Carrero et al., 2002). Therefore, neither brucebactin nor 2,3 dihydroxybenzoic acid seems to work as a virulence factor in the mouse model. However, there are reports on the attenuation of *B. abortus* Δ*entC* in pregnant goats, suggesting that the situation may be different in natural hosts (Bellaire et al., 1998). This is, however, somewhat contradictory to the fact that *Brucella* bacterioferritin deletion mutants replicate normally within human phagocytes (Denoel et al., 1997a).

Modulation of Intracellular Trafficking After phagocytosis, bacteria are found inside a membrane-bound compartment, which undergoes a maturation process that depends upon the nature of the ingested organism (Méresse et al., 1999). The "constitutive route" followed by inert particles, such as latex beads or killed organisms, is revealed by the sequential acquisition of molecules displayed on the vacuole-containing bacteria (Garin et al., 2001). Immediately after uptake, the phagocytic vacuole exhibits plasma membrane molecules, then markers of early endosomes followed by molecules distinctive of late endosomes, and finally elements characteristic of lysosome molecules. Among these molecules, small GTPases of the Rab family, involved in the regulation of fusion events along the endocytic pathway, reflect the capacity of the phagosomes to fuse with endocytic organelles. It has been proposed that phagosome maturation occurs by multiple transient fusion events, a process referred to as "kiss-and-run." Intracellular pathogens have evolved strategies to avoid this progressive transformation of their vacuole to a phagolysosome, thereby evading killing mechanisms. *Brucella* organisms, as do other intracellular pathogens, actively evade the constitutive route, promoting their migration to a compartment where they are capable of extracting nutrients, and replicate (Pizarro-Cerdá et al., 1998b). This process is commanded by the interaction of complex mechanisms with the intervention of several genes and molecules. The absence of one or more of these elements alters the intracellular biogenesis of the bacteria and breaks the natural equilibrium established between the parasite and its host cell.

Escaping from the Endocytic Pathway *Brucella bvrS-bvrR* mutants forced to penetrate CNF-treated HeLa cells follow the degradative pathway (C. Guzmán-Verri et al., unpublished observations), indicating that this two-component, sensory regulatory system is involved not only in penetration (Mechanisms of Entry to Host Cells) but also in controlling vacuole maturation. Cyclic glucans of plant pathogenic bacteria are essential factors for parasitism (Breedveld and Miller, 1994). *Brucella* possess a periplasmic cyclic-β-(1,2)-glucan, which is not osmotically regulated, in contrast to what happens in *Agrobacterium* or *Rhizobium* parasites (Briones et al., 1997). *Brucella cgs* mutants, unable to produce cyclic glucan, are avirulent in mice and incapable of avoiding fusion with lysosomes (Iñón de Iannino et al., 1998; J. P. Gorvel, personal communication). Since *Brucella cgs* mutants adhere and penetrate cells similarly to the wildtype bacteria, it is concluded that cyclic glucans are necessary for the correct biogenesis of these organisms.

Recently, it has been described that a genetic system (*virB*) encoding for a type IV secretion machinery (Boschiroli et al., 2002; Comerci et al., 2001; Delrue et al., 2001) is also involved in regulating the intracellular trafficking of *Brucella*. Type IV secretion systems are complex structures composed of several proteins, some of which concomitantly span the inner and the outer-membrane (Fig. 5). These systems are specialized in transferring molecules (proteins or DNA) from the internal to the external milieu of the bacterial cell (Christie and Covacci, 2000). The *Brucella* type IV secretion system is composed of 13 open reading frames and shows similarity with the VirB complex of other cell-associated bacteria, such as *Agrobacterium*, *Legionella* and *Rickettsia*. Polar mutations in the *vir*B1 and *vir*B10 abolish the ability of *Brucella* to replicate in mice and cells (Comerci et al., 2001). Nonpolar mutation in *vir*B8 and *vir*B10, coding for internal membrane proteins, generates attenuated bacteria. Mutations in *vir*B12 and *vir*B13 do not demonstrate defects (O'Callaghan et al., 1999; D. J. Comerci, personal communication). Mutations in *vir*B genes do not have an effect in the attachment and internalization of *Brucella* to cells (Boschiroli et al., 2002; Comerci et al., 2001; Delrue et al., 2001), although some authors claim that the VirB system is implicated in internalization (Watarai et al., 2002). *Brucella* organisms harboring either polar or nonpolar mutations in *vir*B10 are capable of penetrating cells, as is the wild-type *Brucella*, localizing in LAMP-1-positive compartments at early times of infection. However, after this period, *vir*B10 polar mutants are sorted to degradative compartments positive for lysosomal markers, whereas nonpolar *vir*B10 mutants remain within compartments devoid of lysosomal or endoplasmic reticulum proteins, but retain early endosome markers. After 12 h, a large proportion of intracellular nonpolar *vir*B10 mutant bacteria are recycled to the cell surface. Once outside, the bacteria seem to replicate adhered to the cell

envelope. These results indicate that although the *vir*B genes are not required for attachment or invasion, they are necessary for regulating the intracellular trafficking from early endosomes to the endoplasmic reticulum. In addition, the VirB10 product seems to be essential for preventing the fusion of *Brucella*-containing vacuoles with lysosomes. Therefore, the absence of VirB11 would preclude the correct assembling of the type IV secretion machinery necessary for secreting bacterial substances that prevent lysosomal fusion. It follows that the absence of VirB10 will allow a defective but partially functional secretory system competent for controlling lysosomal fusion, but incapable of releasing substances necessary for promoting retrograde *Brucella* trafficking to the endoplasmic reticulum.

The putative *vir*B10 and B11 genes seem to code for an internal transmembrane protein of unknown function (structural?) and for a cytoplasmic or inner membrane protein that has a conserved Walker A NTP-binding motif, respectively. Gene reporter analysis has revealed that the expression of the *Brucella* VirB system is activated during the internalization (from 3–12). Then the expression of the system diminishes, corresponding to the replicating time in the endoplasmic reticulum (Boschiroli et al., 2002; Comerci, personal communication). Some authors claim that *vir*B induction only occurs once the bacteria are inside the cells and phagosome acidification has occurred, being this the major signal for expression of this system (Boschiroli et al., 2002). Others, in contrast, sustain that *vir*B operon is turned on during stationary phase and that acidic conditions do not affect the expression of the VirB system (Sieira et al., 2000; Comerci, personal communication). Therefore, it seems that the type IV secretion machinery is required for controlling the brief trafficking of *Brucella* through the endocytic network until the bacterium reaches its replicating niche within the endoplasmic reticulum. Once the bacterium has reached the endoplasmic reticulum, the secretion apparatus may be turned off.

Trafficking from the endocytic network to the endoplasmic reticulum via autophagosomes requires not only live virulent bacteria, but a physiologically balanced *Brucella*-containing vacuole. For instance, in cells expressing an activated form of rab5 (bound to GTP), an important fraction of the internalized parasites are unable to escape from the early *Brucella*-containing compartment (Chaves-Olarte, 2002). Although a fraction of bacteria are capable of reaching the endoplasmic reticulum in these mutant cells, unexpectedly a few *Brucella* escape from these "early" giant vesicles to the cytoplasm (J.-P. Gorvel, personal communication). These events suggest that *Brucella* is capable of adapting to different intracellular environments by controlling its own intracellular trafficking through direct interaction with its host vacuole.

Escaping from Autophagosomes The first step in the biogenesis of autophagosomes is the acquisition of lysosomal membrane-associated proteins. Acidification of the maturating compartment occurs by inclusion of the H-ATPase, and finally delivery of acid hydrolases allows the degradation of intravacuolar isolated cytoplasmic materials (Dunn, 1994). In fact, it has been shown that nocodazole treatment causes the accumulation of acidic autophagosomes that lack acid hydrolases, supporting the concept that vacuole acidification and acquisition of hydrolytic enzymes are separate events. The presence of LAMP-1 and LAMP-2 but the absence of cathepsin D at 2 h postinoculation in *B. abortus*-containing phagosomes also supports the model of a stepwise maturation of autophagic vacuoles. Whether *B. abortus*-containing autophagosomes are able to acquire the H-ATPase remains to be established. *Brucella* phagosomes seem to acidify rapidly after infection in murine J774 macrophages (Arenas et al., 2000; Porte et al., 1999). This acidification step seems to be independent of phagosome-lysosome fusion. In Vero cells, repressors of endosome acidification, such as chloroquine and ammonium chloride, reduce the number of *Brucella* with respect to controls (Detilleux et al., 1991). Adjustment of intravacuolar pH has been shown as essential for the activation of virulence genes in certain intracellular parasites. Parasitophorous vacuoles of *Leishmania amazonensis* maintain an acidic pH in infected macrophages (Antoine et al., 1990). New sets of proteins are synthesized by *S. typhimurium* upon infection of host cells after modulation of the phagosome pH (Buchmeier and Heffron, 1990). This latter bacteria induces reduction of phagosome acidification to activate the virulence genes of the *phoP-phoQ* complex (Alpuche-Aranda et al., 1992). *Brucella* abortus is able to synthesize a new set of proteins during macrophage infection (Lin and Ficht, 1995; Rafie-Kolpin et al., 1996), suggesting that intracellular conditions (one of which could be intravacuolar pH) may contribute to the activation of genes necessary for trafficking from autophagosomes to the endoplasmic reticulum (Pizarro-Cerdá et al., 1998c). The critical difference between the attenuated vaccine strain 19 and the virulent *B. abortus* 2308 seems to be the inhibition of autophagosome maturation by the virulent strain (Fig. 36). The attenuation of strain 19 may lay in its incapacity to respond to environmental stimuli present in the autophagosome (acidification, for example) that could activate virulence

genes for the expression of proteins important to remodel the autophagosome.

Life Within the Replicating Niche In addition to a suitable source of carbon and energy, *Brucella* requires some cofactors and vitamins for growth (Nutritional Requirements). It is also able to use some amino acids as a source of carbon and energy and to eliminate the urea that could build up as a metabolite noxious for the host and the bacterium under some conditions (ATP Synthesis and Respiratory Chain; Oxidation of Amino Acids). Besides being a strategy for avoiding lysosome fusion during later steps of intracellular invasion, association of pathogenic *Brucella* with the endoplasmic reticulum could be a strategy for obtaining metabolites synthesized or translocated to this compartment (Simon and Blobel, 1991). By stimulating host cell autophagy, the bacteria would increase degradation of host proteins and availability of amino acids. However, the mechanism by which these small compounds would be delivered back to the endoplasmic reticulum and used by the bacteria should be defined. In contrast to professional phagocytes, it seems that the placental environment offers a privileged site for brucellae replication. It is becoming evident that trophoblasts are metabolically active cells capable of producing a variety of hormones and other factors which may stimulate the growth of *Brucella* organisms (Enright and Samartino, 1994). Genes coding for putative efflux pumps capable to serve as export of quorum sensing metabolites have been identified in Brucella (DelVecchio et al., 2002). Quorum sensing hydrophobic molecules such as acyl-homoserine lactone released by Brucella down regulate the expression of *vir*B genes in vitro (Taminiau et al., 2002), suggesting that this small hydrophobic molecule could play a role in the control of the type IV secretion system, necessary for the intracellular trafficking, but unnecessary and inhibitory during intracellular growth inside the endoplasmic reticulum.

Role of the Outer Membrane in Nutrient Uptake The overall higher hydrophobicity of *Brucella* cell envelopes, the close association among the macromolecules of the outer-membrane and the properties of the porins are the factors implicated in the selective penetration of nutrients inside the bacterial cell. It has been demonstrated that the absence of a barrier to hydrophobic substances is linked to the structure of the *Brucella* LPS core and lipid A (Freer et al., 1996; Martínez de Tejada and Moriyón, 1993; Velasco et al., 2000). The possible advantages of a hydrophobic envelope for intracellular α-2 subclass Proteobacteria also have come to light by the finding that *Rhizobium* LPS becomes highly hydrophobic during bacteroid development (Kannenberg and Carlson, 2001). The net result of this structural change in the LPS is that intracellular bacteroids have a more hydrophobic outer-membrane than the free-living rhizobiae have. This adaptive condition could promote the exchange of nutrients and favor intracellular life of the bacteroids. Obvious comparisons between the intracellular lifestyle of *Brucella* and *Rhizobium* emerge, since these two bacteria are phylogenetically close relatives (Moreno et al., 1990). The permeability of the *Brucella* outer-membrane to sexual hormones and siderophores is discussed elsewhere in the chapter (The Hydrophobic Pathway; Uptake of Inorganic Nutrients). As noted (Porin Proteins), *B. abortus* only expresses one of the two porin protein genes it carries, and it has been suggested that the second one may be specifically expressed during intracellular parasitism (Marquis and Ficht, 1993; Ficht et al., 1989).

Auxotrophic and Cell Cycle Genes During Intracellular Life Many of the mutations described in this section may hamper the adequate extraction of nutrients from the replicating niche. It is likely that these genes are devoted to the regulation of vital functions not directly involved in virulence, but with more general aspects of the *Brucella* physiology.

Mutants displaying reduced virulence and/or reduced intracellular survival within macrophages have been identified by signature-tagged transposon mutagenesis (Foulongne et al., 2000). Several of these attenuated mutants carry auxotrophic defects, such as those necessary for leucine, arginine or aromatic acid biosynthesis, whereas others carry deficiencies in the synthesis of chorismate for the generation of *para*-aminobenzoic acid necessary for quinone synthesis, 2,3 dihydroxybenzoic acid, brucebactin and folic acid. Mutations in genes involved in the glucose metabolism, such as in the gene coding for phosphoglucose isomerase, also attenuate *Brucella*, probably owing to several pleiotropic defects, including the synthesis of cell wall peptidoglycan. Similarly, transposon insertion in the *gpt*-like gene coding for hypoxanthine-guanine biosynthesis attenuates *Brucella*, probably owing to alterations in the nucleotide biosynthesis. In support of this are the *B. melitensis purE* (purine auxotrophic) mutants that display reduced growth in macrophages (Cheville et al., 1996a; Drazek et al., 1995). The *purE* mutation has minimal effect on internalization, but effectively blocks intracellular replication. The *purE* mutation may cause bacterial death simply as a result of starvation.

Other mutants, also identified by signature-tagged mutagenesis (Foulongne et al., 2000), have defects in regulatory systems, such as LysR transcriptional regulator, which is part of a positive regulator system of virulence genes in several bacteria. Mutants in the expression of NtrY protein (a sensor of an Ntr-related regulator; Dorrell et al., 1999) are weakly attenuated, probably as a result of a pleiotropic negative effect on the *ntr* regulon (The NtrBC System). As in other bacteria, mutations in genes involved in glutamine metabolism reduce the ability of *Brucella* to replicate in macrophages (Foulongne et al., 2000). The *Brucella ccrM* gene codifies for a CcrM DNA methyltransferase that catalyzes the methylation of the adenine in sequences GANTC (Methylation and Control of Chromosome Duplication and Cell Cycle). This gene performs important functions during cell division and it is, therefore, essential for viability (Robertson et al., 2000b; Wright et al., 1997). Increase in the *ccrM* copy number, in addition to altering the morphology of the bacterial cells, attenuates *Brucella*, indicating that controlled cell cycle and bacterial division are necessary for intracellular survival. Mutations in the *bacA* gene, which codes for a putative cytoplasmic membrane transporter, render *B. abortus* avirulent (LeVier et al., 2000). *Brucella bacA* mutants display altered transport of molecules and then deficient *Brucella* replication within cells. Auxotrophic mutants for ferrochelatase, the enzyme involved in the last step of the heme synthesis (catalyzing the incorporation of ferrous iron into the protoporphyrin molecule), are also attenuated (Almirón et al., 2001).

Maintaining the Host Cell Alive Some parasites may promote programmed cell death, whereas others are prone to prevent it, prolong cell life, or are even capable of stimulating replication of their host cells. Proteobacteria of the α subdivision, such as *Rickettsia*, *Agrobacterium*, *Rhizobium* and *Brucella*, as well as other intracellular parasites capable of establishing chronic infections (i.e., *Mycobacterium* and *Chlamydia*) prevent cell death. Heavily infected *Brucella* trophoblasts, epithelial cells or macrophages do not display signs of necrosis or apoptosis (Anderson et al., 1986b; Anderson et al., 1986c; Detilleux et al., 1990b; Jiang and Baldwin, 1993a; Tobias et al., 1993; Fig. 30). DNA synthesis, microtubule spin formation, chromosome migration, karyokinesis and cytokinesis are not inhibited by intracellular *Brucella* (Chaves-Olarte et al., 2002). As a consequence, dividing cells filled with brucellae are frequently observed in vivo and in vitro (Detielleux et al., 1990a; Detilleux et al., 1990b; Detilleux et al., 1991). In CNF-treated HeLa cells, cytokinesis is inhibited due to paralysis of actin filaments without affecting nuclear division. When these cells are infected with *Brucella*, karyokinesis proceeds without signs of degeneration, despite the large number of intracellular *Brucella* within the endoplasmic reticulum (Chaves-Olarte et al., 2002). Live but not killed *B. suis* prevent programmed cell death of infected human monocytes (Gross et al., 2000). This suggests that infection protects host cells from several cytotoxic activities generated during the immune response. Since both invaded and noninvaded cells are guarded against apoptosis, it has been suggested that this protective mechanism is mediated through soluble substances released during bacterial infection. Apoptosis inhibition is independent of LPS and requires the overexpression of the *A1* gene by infected cells, a member of the *bcl-2* family involved in the survival of blood-forming cells.

In contrast to other LPS, *Brucella* LPS practically does not display endotoxicity and is a poor inducer of cytotoxic mediators (Goldstein et al., 1992; López-Urrutia et al., 2000; Moreno et al., 1981; Rasool et al., 1992). This property shared by other intracellular animal pathogens (such as *Rickettsia*, *Legionella* and *Bartonella*) may be envisioned as an evolutive advantage of *Brucella* parasites for adaptation to intracellular life (Freer et al., 1996; Moreno, 1992; Rasool et al., 1992; Velasco et al., 2000). It is not surprising that lipid As of *L. pneumophila* and *B. abortus* parasites have coevolved to have a similar structure and, in consequence, display low endotoxicity and reduced ability to stimulate cells (Goldstein et al., 1992; López-Urrutia et al., 2000; Moreno et al., 1981; Rasool et al., 1992; Zähringer et al., 1995; Fig. 7). This property may be idiosyncratic and useful for intracellular bacteria.

Modulation of the Immune Response Once inside macrophages, pathogens might diminish or abrogate their antigen presentation capacity, thus reducing the T cell-mediated immune responses. The LPSs from different bacteria have been shown to modulate the immune responses in several systems (Cella et al., 1997; Knolle et al., 1999; Krieger et al., 1985; Uchiyama et al., 1984). *Brucella abortus* LPS molecules accumulate inside lysosomal compartments and associate with MHC-II proteins in antigen-presenting cells (Forestier et al., 1999a; Forestier et al., 1999b; Forestier et al., 2000). The intracellular LPS, which remains for long periods without being degraded, is exported to the cell surface where it forms stable macrodomains (Figs. 35 and 37). Once inside macrophages, *B. abortus* LPS impairs the MHC-II presentation pathway, but not MHC-I presentation of foreign peptide antigens (Fig. 37). This impairment is neither

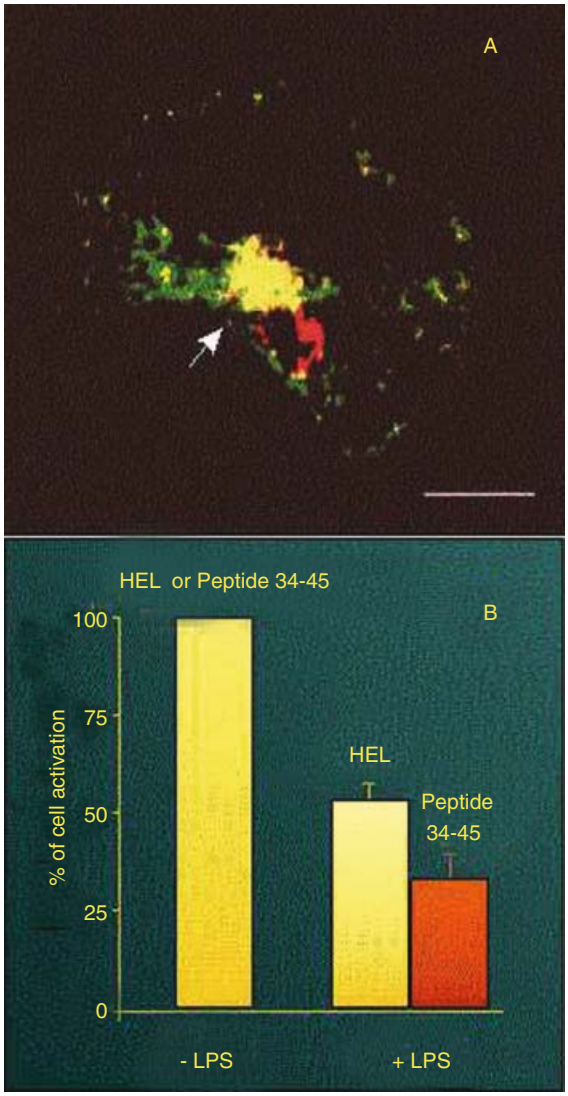


Fig. 37. *Brucella* LPS binds to MHC-II molecules and inhibits T cell responses to protein antigens. A) *Brucella* LPS (red) and MHC-II (green) colocalize (yellow) within compartments and on the surface of murine macrophages. B) Inhibition of antigen presentation of hen's egg lysozyme (HEL) peptides and derived synthetic peptide (34–45) to respective specific T CD_4^+ clones by *Brucella* LPS-treated macrophages. Colocalization was detected by double immunofluorescence and viewed in a confocal microscope. Notice that *Brucella* LPS-MHC-II complexes establish large macrodomains (white arrow points to area of yellow fluorescence) on the surface of phagocytic cells. Adapted from Forestier et al. (2000). Courtesy of Dr. J-P Gorvel, CIML, Marseille-Lunimy, France.

from a deficient uptake or catabolism of the native antigen nor from a reduced MHC-II surface expression, reduced number of B7 membranous costimulatory molecules, or defective α/β dimer formation. In addition, this inhibitory effect is not due to a direct suppressive action of LPS on T cells, independent of macrophages (Forestier et al., 2000). *Brucella* LPS macrodomains at the macrophage plasma membrane are highly enriched in MHC-II molecules (Fig. 37), suggesting that the LPS-MHC-II macrodomains may impair the appropriate recognition of protein peptide-MHC-II complexes by CD_4^+T cells (Forestier et al., 2000). The presence of such MHC-II-LPS macrodomains does not prevent the binding of antigen peptides into the groove of MHC-II molecules. Therefore, the *Brucella* LPS-induced interference on MHC-II antigen presentation is likely to occur distal to intracellular events leading to the meeting of antigenic peptides and MHC-II molecules. The LPS molecules embedded in the membrane of MHC-II-positive compartments may interact with already peptide-MHC-II forming ternary complexes, which then recycle to the plasma membrane. In one model, the LPS *O*-polysaccharide, facing the external milieu, could prevent the accessibility of MHC-II complexes to their specific T cell receptor. Another model is related to a superantigen-like function in T cell activation. It is known that superantigens modify the geometry of TCR-peptide/MHC-II complexes, which may be less critical for T cell activation than certain other factors, in particular those involved in the stability of the resulting complex (Andersen et al., 1999; Kersh et al., 1998). The serial triggering (Itoh et al., 1999; Valitutti et al., 1995; Viola and Lanzavecchia, 1996) and kinetics proofreading models (Rabinowitz et al., 1996) of T cell activation suggest that the short half-lives of TCR-peptide/MHC-II complexes are required for efficient T cell stimulation. The presence of *Brucella* LPS-MHC-II macrodomains in macrophages has been detected even after 60 days, thus highlighting the remarkable stability of these surface LPS macrodomains. In this model, LPS could downregulate T cell responses by stabilizing the MHC-II/peptide complexes at the cell surface. Consequently, in contrast to super antigens, *Brucella* LPS would be less efficient at triggering T cells because they form TCR-LPS-MHC-II complexes with a very long half-life.

The in vitro inhibition of the immune response correlates to that observed in vivo upon infection by *Brucella*. It is worth noting that chronic brucellosis is accompanied by a general immunosuppression that can be revealed by using an IL-2 detection system (Zhang, 1992; Zhang et al., 1993; Forestier et al., 1999a). Infected macrophages may exert a negative feedback, diminishing lymphocyte proliferation in response to *Brucella* antigens (Cheers et al., 1980; Riglar and Cheers, 1980). It has been proposed that chronically infected macrophages may fail to act as targets of T cells and may downregulate T lymphocyte function (Baldwin and Winter, 1994). Moreover, it is known that *Brucella* inhibits the production

of TNFα of infected phagocytes by releasing a protease-sensitive inhibitor for the expression of this cytokine (Caron et al., 1996). Therefore, it is reasonable to speculate that intracellular *Brucella* uses this strategy to regulate the activation of phagocytic cells and promote in this manner its own survival within these cells. Since LPS is also released from bacteria inside host cells (Fig. 35) and this molecule is not degraded by peritoneal macrophages, it can be hypothesized that one additional form by which *Brucella* may contribute to immunosuppression is the release of LPS inside cells (Forestier et al., 1999b). In this respect, *Brucella* LPS, together with other molecules, may play a central role in the immunosuppression observed upon brucellosis infection and may account for the presence of anergic T cells in infected patients (Renoux and Renoux, 1977). Experiments have demonstrated that *Brucella* is capable of infecting small lymphocytes in the cortical zone of lymph nodes (Cheville et al., 1996a). The infected lymphocytes harboring intracellular bacteria within vacuolar compartments with no signs of degradation look normal (Fig. 30). At the present time, the implications of lymphocyte *Brucella* parasitism are not known. The fact that *Brucella* LPS complexes with MHC-II in these cells (Forestier et al., 1999a) opens the possibility that LPS released by intracellular *Brucella* could modulate antigen presentation in lymphocytes, as it does in macrophages (Forestier et al., 2000).

Control of the Infection

Strong humoral and cellular immune responses are generated during *Brucella* infection. Although the role of cell mediated immune response has been regarded as the essential mechanism for controlling the infection, antibodies may play a more or less relevant role, depending upon the specie of the infected animal. Comprehensive reviews can be found in references (Baldwin and Winter, 1994; Liautard et al., 1996; Nicoletti and Winter, 1990b; Oliveira, et al., 1998).

Antibody Response *Brucella* infections generate an elevated production of antibodies. The most relevant molecules involved in the humoral response of animals or humans infected or vaccinated with smooth strains are by far the LPS and the NH polysaccharides (Jones et al., 1980; Marín et al., 1999; Moreno et al., 1984a; Nielsen et al., 1988; Spink, 1956). Mainly at early stages of the infection (first month), it has been estimated that close to 80% of the antibodies against *Brucella* are directed toward the *O* chain and NH determinants, with the great majority of the immunoglobulins being against the C epitopes (in smooth strains) (Fig. 34). Moreover, most of the IgG_1 passively transferred through colostrum to newborn calves are directed against these two bacterial surface molecules (Antibody Detecting Tests in Infected Patients; Smooth-lipopolysaccharide Tests in Infected Animals). Antibodies recognizing core and lipid A determinants (Fig. 34) are not detected by conventional serological methods in the sera from infected or vaccinated bovines with smooth strains, unless purified antigens are used. In contrast, individuals infected or vaccinated with rough brucellae generate a strong antibody response against LPS core and lipid A determinants as well as toward Omps (Bowden et al., 1995; Gamazo et al., 1989; Riezu-Boj et al., 1990; Rojas et al., 1994). The most immunogenic proteins are the Omps of group 3 (Omp3a and Omp3b and Omp31), some of which are strongly bound to the LPS (Gamazo and Moriyón, 1987; Kurtz and Berman, 1986; Riezu-Boj et al., 1990; Rojas et al., 1994), and stress response proteins (GroEL, DnaK, Sod, PurE and HthA) (Protein Tests in Infected Patients; Protein Tests in Infected Animals). Antibodies against various cytoplasmic soluble proteins, ribosomal proteins, periplasmic proteins, outer-membrane lipoproteins and porins, as well as against peptidoglycan, have also been detected (Fig. 38). Most of the antibodies against proteins appear at later stages (after the third week) of the infection (Protein Tests in Infected Patients).

The immunoglobulin profiles of the anti-LPS response in newly infected bovines, humans, goats and mice measured by ELISA or discriminatory agglutination are characteristic of a T-dependent cell response (Fig. 39), despite the fact that *Brucella* LPS, as well as killed organisms, has been defined as a T-independent antigen (Kurtz and Berman, 1986; Moreno and Berman, 1979; Moreno et al., 1984b; Rennick et al., 1983; Tenay and Strober, 1985). The secondary antibody responses observed in antibiotic-treated individuals who have relapsed are also typical of a T-dependent response with strong generation of IgG_1 antibodies (Antibody Detecting Tests in Infected Patients; Smooth-lipopolysaccharide Tests in Infected Animals). Under natural conditions, IgG_1 antibodies may remain for life (Castañeda, 1961; Foz et al., 1954; Spink, 1956). This may be due to the persistence of the bacteria in tissues (Enright, 1990b; Enright, 1990a; Spink, 1956) or alternatively to the permanence of *Brucella* LPS within phagocytic cells for long periods. In support of this last statement are the findings of LPS within macrophages of infected mice after several months (Modulation of the Immune Response) and the detection of large quantities of apparently intact LPS in granulomatous lesions of individuals with

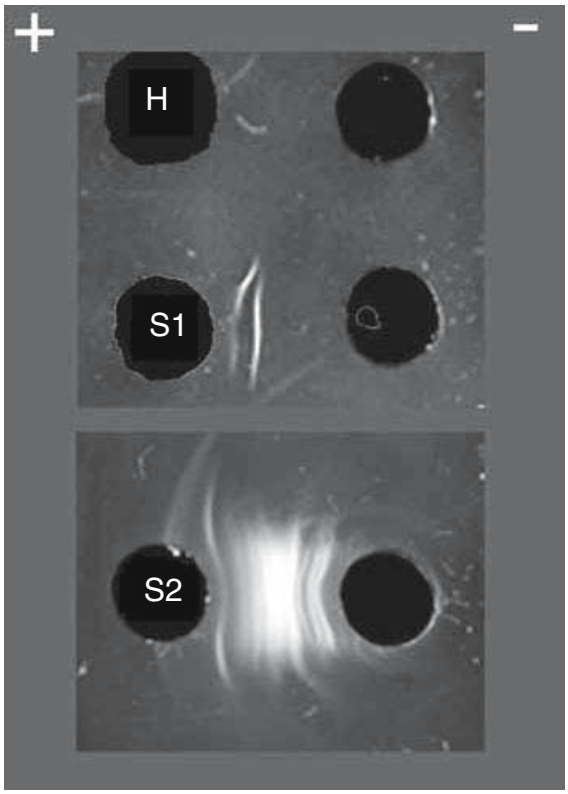


Fig. 38. Crossover immunoelectrophoresis with protein antigens for the diagnosis of brucellosis. The wells on the left contained the sera from a healthy donor (H) and from two patients after a brief (S1) or a prolonged (S2) period of infection before treatment. For reference of the antibody response to LPS see Fig. 39. Courtesy of R. Díaz, University of Navarra, Spain.

chronic brucellosis (R. Díaz, University of Navarra, Spain, personal communication).

The antibody response of mice immunized with purified LPS generates a non-typical T-independent response with relatively large quantities of IgG_3 against the *O* chain and moderate quantities of IgG_1 and IgM (Kurtz and Berman, 1986; Moreno et al., 1984b). In contrast to other types of LPS, *Brucella* LPS is immunogenic for the endotoxin-resistant C3H/HeJ mice and displays a strong IgG_1 adjuvant effect in this and other strains (Kurtz and Berman, 1986; Moreno and Berman, 1979; Moreno et al., 1984b).

The role of antibodies against *Brucella* infection has been a matter of controversy for many years. In mice, it is clear that antibodies play an important part in anti-*Brucella* immunity (Araya et al., 1989), mainly because of the exacerbation of the phagocytosis and bactericidal action mediated by professional phagocytes (Opsonization and Complement Susceptibility). Nevertheless, in other species of mammals, including humans, infections seem to course unaffected in the presence of large quantities of circulating antibodies (Ariza, 1998; Enright, 1990a; Enright, 1990b; Shuterland and Searson, 1990). However, maternal antibodies seem to play a role in protecting calves and piglets born within herds with prevalent brucellosis as demonstrated by experimental and field experience (Cunningham, 1977b; Halliday, 1968; Hoerlin, 1957; Plommet, 1977). In addition, whether antibodies to LPS, or to any other antigen, hamper the ability of the bacteria to infect animals under field conditions needs to be examined. The truth is, that despite the current opinion that antibodies do not play a role in human or ungulate infections, at the present this asseveration is not sustained by clear-cut experimental data. This doubt is not trivial and it has practical implications related to the use of vaccines derived from rough strains for controlling brucellosis (Samartino et al., 2000; Schurig et al., 1991). Finally, the role of anti-LPS immunoglobulins either as blockers of antigen recognition or as facilitators of cell invasion also needs to be re-examined in light of the recent advances in our knowledge of *Brucella* parasitism and intracellular trafficking of bacterial antigens.

T Cell Responses In a series of elegant investigations, Mackaness (1967) demonstrated more than three decades ago that acquired cellular resistance could be specifically induced by *Brucella* organisms or by other facultative intracellular parasites, but nonspecifically expressed for a given period of time. That means that, at some intervals after *B. abortus* invasion, the *Brucella*-infected mice will clear *Listeria* as though they have been immunized against this Gram-positive bacterium. The postulated cellular episodes mediated by lymphocyte cytokines capable of activating macrophages, that eventually could kill both organisms, were the basis for explaining the immunological events necessary to control *Brucella* inside macrophages (Mackaness, 1967). Passive transfer of CD_4^+, CD_8^+ cells and antibodies has clearly demonstrated the role of each of these elements in the control of murine brucellosis (Araya et al., 1989; Araya et al., 1990; Eze et al., 2000). It has been hypothesized that CD_8^+ cells, in addition to producing IFNγ, could lyse *Brucella* infected macrophages and downregulate the production of cytokines by Th2 cells, such as IL-10 and the cytokine-like CD_8^+ T cell antiviral factor (Oliveira et al., 1998). Despite the role played by antibodies and CD_8^+, it is generally accepted that the ultimate brucellacidal activity in most animals is based on CD_4^+ type-I immune response, through the generation of cytokines capable of activating macrophages (Baldwin and Winter, 1994; Liautard et al., 1996; Oliveira et al., 1998). In consequence, a more robust respiratory burst, a more powerful pro-

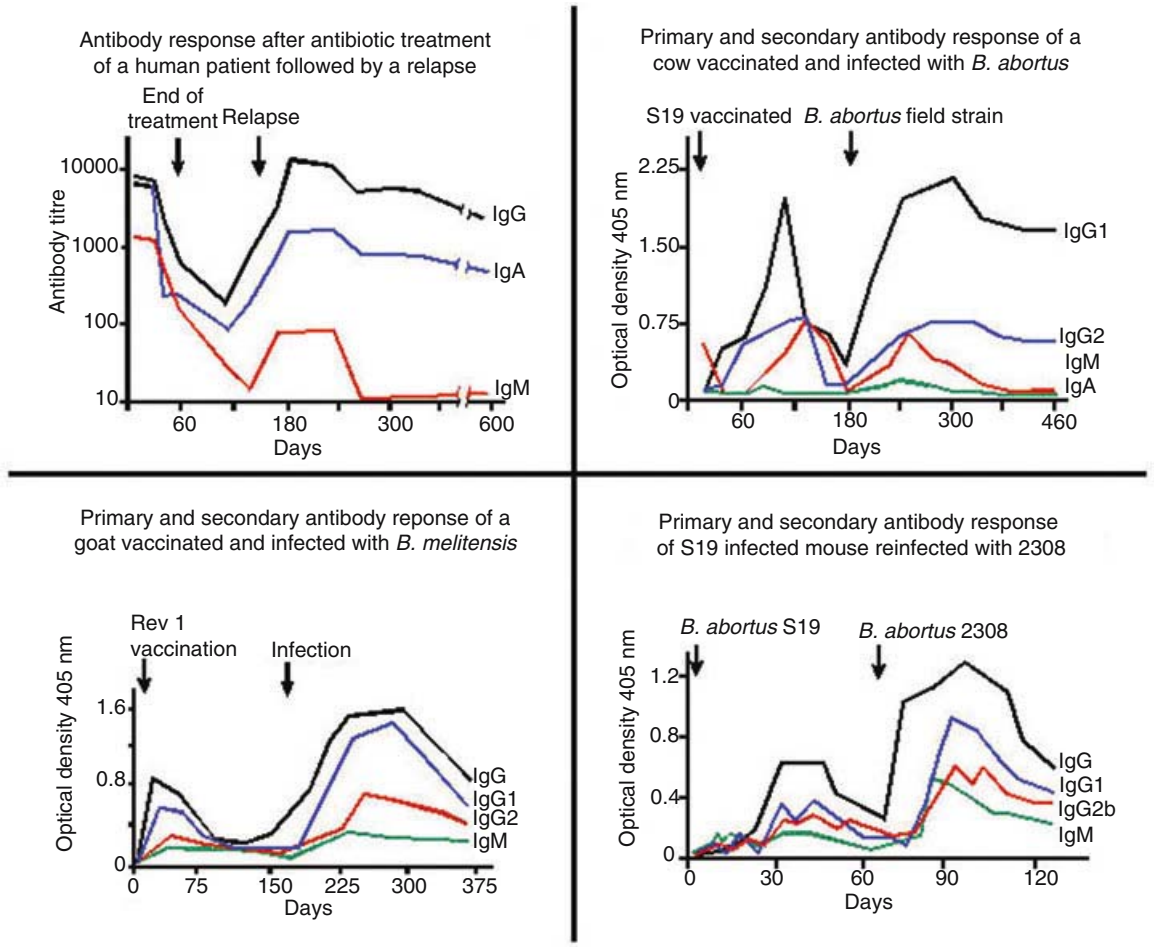


Fig. 39. ELISA immunoglobulin isotype reactivity against *B. abortus* LPS from humans and animals infected with *Brucella*. A) Antibody response (over time) of a human patient infected with *B. melitensis* after antibiotic treatment, followed by relapse. B) Primary and secondary antibody responses of a cow vaccinated with *B. abortus* S19 and infected with *B. abortus* 2308, respectively. C) Primary and secondary antibody responses of a goat vaccinated with *B. melitensis* Rev. 1 and infected with *B. abortus*, respectively. D) Primary and secondary antibody responses of a mouse infected with *B. abortus* S19 and reinfected with *B. abortus* 2308, respectively. Notice the strong IgG (IgG_1) response against *Brucella* LPS, characteristic of a type-2 T-dependent immune response. Since most of antibodies produced are directed against the common *Brucella* epitopes (Fig. 34), *B. abortus* LPS antigen bound to the ELISA plates serves its purpose, regardless of whether the individuals were infected or vaccinated with *B. abortus* or *B. melitensis*. The profile of the infected patient (panel A) was supplied by Dr. Javier Ariza, from the Infectious Disease Service, Hospital de Bellvitge, Barcelona, Spain.

duction of microbicidal reactive oxygen intermediates, and a more efficient phagosome-lysosome fusion are performed by the activated macrophages (Gross et al., 1998; Jiang and Baldwin, 1993a; Jiang and Baldwin, 1993b; Zhan and Cheers, 1995; Zhan and Cheers, 1996). As expected, the most relevant antigens involved in the T cell responses are bacterial proteins, as revealed by delayed-type hypersensitivity reactions in infected individuals with a mixture of protein extracts (Cell-mediated Immunity Tests). However, nonprotein antigens also may be involved in T cell responses (Bertotto et al., 1993; Ottones et al., 2000a; Ottones et al., 2000b; J.-P. Gorvel and E. Moreno, unpublished results).

Similar to what has been observed with other intracellular bacteria, such as *Mycobacterium*, *Listeria* and *Francisella*, the blood of humans infected with *Brucella* demonstrates an important increase in γδ T cells (Barnes et al., 1992; Bertotto et al., 1993; Jouen-Beades et al., 1997; Poquet et al., 1998). The T lymphocyte population that specifically expands is one carrying the Vγ9Vδ2 T cell receptor, suggesting that this subset of lymphocytes is generated in response to intracellular pathogens. The γδ T cell repertoire

responds to natural and synthetic low molecular weight nonpeptide ligands of diverse structure, complicating the nature of the recognition pattern carried out by this repertoire. For instance, when stimulated with isopentyl pyrophosphate from *M. tuberculosis*, Vγ9Vδ2 T cells polyclonally expand and secrete high levels of TNFα and INFγ, without stimulating αβ or Vγ1Vδ2 subsets. Similarly to other intracellular bacteria, *Brucella* stimulates the production of TNFα and INFγ by Vγ9Vδ2 T cells (Ottones et al., 2000a; Ottones et al., 2000b). It has been shown that *Brucella*-activated Vγ9Vδ2 lymphocyte subset is capable of impairing the intracellular multiplication. This effect seems to proceed by two different mechanisms. The first one is through the release of a low molecular weight soluble factor that stimulates the production of TNFα and INFγ cytokines, which activate the brucellacidal mechanisms of professional phagocytes. The second mechanism seems to take place by a direct contact-dependent cytotoxic effect on monocytic cells infected with *Brucella* organisms. The bactericidal activity mediated by this γδ lymphocyte subset during early stages of infections by intracellular bacteria, such as *Mycobacterium*, *Listeria* and *Brucella*, may explain, in part, why acquired cellular resistance could be specifically induced by one intracellular bacteria, but nonspecifically expressed for a given period of time, as indicated at the beginning of this subsection (Mackaness, 1967).

Interleukins in Brucellosis Several studies have highlighted the role of IFNγ, TNFα, interleukin (IL)-6, IL-1β, IL-10, IL-12, granulocyte macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF) in host resistance against bacterial infections (Fernández-Lago et al., 1996; Flesh et al., 1994; Flynn et al., 1995; Liautard et al., 1996; Sarmento and Appelberg, 1996; Tanaka et al., 1995). Delayed-type hypersensitivity response of *Brucella*-infected animals and humans has been used as a diagnostic parameter for brucellosis, as well as an indicator for cell-mediated immune response and release of cytokines in brucellosis (Nicoletti and Winter, 1990b). Particularly, during *Brucella*-infection IFNγ, TNFα, IL-2, IL-10, and IL-12 seem to control the intracellular growth of *Brucella* strains within macrophages, whereas IL-1α, IL-4, IL-6 and GM-CSF do not have clear effects (Fernández-Lago et al., 1996; Jiang and Baldwin, 1993a; Ottones et al., 2000a; Ottones et al., 2000b; Zhan and Cheers, 1995). Among the various cytokines, IFNγ is the most relevant for generating macrophages with strong brucellacidal activity. Moreover, IL-2, IL-10 and IL-12, cytokines that influence the acquired cellular resistance and specifically contribute to control the brucellae multiplication, seem to work via the IFNγ-dependent pathway. It is known that *Brucella* LPS is a poor inducer of cytokine production, as well as an inefficient activator of phagocytic cells (Goldstein et al., 1992; López-Urrutia et al., 2000; Rasool et al., 1992). Similar to what has been observed with *Legionella* pneumophila LPS, it seems that the CD_{14} molecule does not serve as receptor for *Brucella* LPS (Neumeister et al., 1988; E. Moreno, unpublished observations), precluding the activation of macrophages by this mechanism. This coincidence between two LPS from phylogenetically distant intracellular bacteria does not come as a surprise, since the lipid A structures of these two molecules have co-evolved to the point that they chemically resemble each other (Fig. 7). As an overall conclusion, it seems unlikely that macrophage activation and secretion of IL-12 occur via the *Brucella* LPS molecule (Huang et al., 1999).

The role of TNFα in brucellosis is not completely clear. In contrast to what has been observed in murine macrophages, *Brucella* strains do not induce TNFα in human macrophages and live *Brucella* is capable of inhibiting the production of this cytokine (Caron et al., 1994b; Caron et al., 1996). However, pretreatment of human macrophages with exogenous TNFα significantly inhibits the rate of *Brucella* intracellular replication. TNFα may not be essential for the induction of acquired cellular resistance but it is likely to directly activate effector cells by limiting the multiplication of intracellular *Brucella* (Ottones et al., 2000a; Ottones et al., 2000b; Zhan and Cheers, 1996).

It has been demonstrated that bactericidal action of activated macrophages and expression of macrophage-specific cytokines depend upon the expression of NF-IL6 (Akira et al., 1990; Katz et al., 1993; Lowenstein et al., 1993; Scott et al., 1992; Tanaka et al., 1995). Upon activation of NF-IL6 knockout macrophages by IFNγ, induction of the transcription of TNFα, IL-6, IL-1β, GM-CSF, M-CSF, IL-10, and IL-12 is comparable to that observed in normal mice (Tanaka et al., 1995). Strikingly, no induction of G-CSF expression is observed in NF-IL6 knockout mice, being this defect is restricted to macrophages and fibroblasts (Tanaka et al., 1995). Moreover, NF-IL6 knockout mice display a high susceptibility to Salmonella and Listeria infections, suggesting that NF-IL6 plays a role in controlling intracellular parasites (Tanaka et al., 1995).

Attenuated *B. abortus* strain 19 is capable of replicating in NF-IL6 knockout murine macrophages. The levels are comparable to those observed in normal macrophages infected with the virulent *Brucella* strains (Pizarro-Cerdá et al., 1998c). The role of NF-IL6 in the inhibition of intracellular bacterial replication is related to

its control of endocytosis and membrane fusion between endosomes and *Brucella*-containing phagosomes. Addition of G-CSF, where production is impaired in NF-IL6 knockout macrophages, restores both endocytosis and the morphology of endosomes, together with the bactericidal activity. During *Brucella* infection, it has been observed that endocytosis but not recycling is affected in NF-IL6 knockout macrophages, suggesting that G-CSF promotes fusion events in early phagosomes (Pizarro-Cerdá et al., 1999a; Fig. 35).

In NF-IL6 knockout macrophages, NO synthetase is not impaired (Tanaka et al., 1995). The production of reactive oxygen intermediates is, however, lower in NF-IL6 than that in wildtype macrophages (Tanaka et al., 1995), suggesting that NF-IL6 may control the expression of other elements of the respiratory burst. Indeed, it is known that G-CSF enhances the respiratory burst in phagocytes (Yuan et al., 1993). The NO synthetase was found to be associated to intracellular membrane vesicles different from lysosomes and peroxisomes in murine macrophages (Vodovotz et al., 1995). These vesicles could translocate to *Brucella*-containing phagosomes in normal macrophages and be hampered in NF-IL6-deficient macrophages due to the lack of fusion between endosomes and phagosomes. Therefore, one hypothesis is that G-CSF, by completely restoring endosome-phagosome fusion, allows elements of the respiratory burst present in endocytic compartments to reach the *Brucella*-containing phagosomes and thus to partially re-establish the bactericidal activity of macrophages (Pizarro-Cerdá et al., 1999a). Under these conditions, attenuated *Brucella* could be targeted to lysosomes and killed, whereas virulent bacteria could still replicate but to a lesser extent than in resting macrophages (Jones and Winter, 1992).

Diagnosis

Brucellosis causes a great variety of clinical symptoms in humans, and although a thorough anamnesis (by questioning feeding habits, occupation and recent animal contacts) is very informative, a definite diagnosis cannot be made solely on these bases. A similar situation happens in animal brucellosis: abortion and infertility are the main symptoms, but they are not exclusively caused by *Brucella* and do not necessarily occur in all infected animals.

The laboratory diagnosis of brucellosis logically follows clinical or epidemiological suspicion of the disease and uses direct and indirect tests. Direct tests are those that show the presence of viable bacteria by bacteriological culture or of some molecules specifically produced by the pathogen. They have the advantage that, when positive, the diagnosis is conclusive. However, they often lack sensitivity (i.e., they yield false negative results) and are cumbersome to perform. Indirect tests are those that detect the presumed effects of *Brucella* infections, which means almost always an analysis of the adaptive immune response. Depending on the tests, they can be very sensitive. However, it has been proven that a proportion of infected animals born to infected mothers do not develop a detectable immune response immediately after birth and that animals that have been infected for a long time may have no significant levels of antibody to the commonly used *Brucella* antigens. Indirect tests may also lack specificity (i.e., they may produce false positive results). This is so because a positive result of immunological tests can mean not only an active infection, but also the existence of antibodies or memory cells persisting after vaccination or recovery, contact with *Brucella* not followed by disease, or exposure to immunologically crossreacting bacteria. Thus, without a good knowledge of the circumstances outlined in the previous paragraph, the results of laboratory tests are not always meaningful.

Direct Diagnosis of Human Brucellosis

Bacteriological Culture for Humans This is by definition the most specific diagnostic test, and since in human brucellosis it is mandatory to achieve a correct diagnosis for each individual, bacteriological cultures should be performed whenever possible. Moreover, owing to the problems posed by the serological diagnosis of *B. canis* (Immunological Diagnosis of Infections Caused by *B. ovis* and *B. canis*), culture is the best diagnostic tool when an infection by this species is suspected.

Brucella has been isolated from several tissues, articular and cerebrospinal fluids, and bone marrow, but the culture of aseptically taken blood in tryptic soy broth or similar media (Table 10) in a 10% CO_2 atmosphere is the simplest and most often used procedure. This is an enrichment procedure, and subcultures on agar media are necessary to identify the bacteria. However, blood debris prevents detection of growth by the appearance of turbidity, and unless special methods are adopted, repeated blind subculture is necessary until a successful isolation happens or the blood culture is discarded as negative. Because this procedure conveys a great risk of infection (Biosafety), Ruiz-Castañeda developed a biphasic system for *Brucella* in which one of the sides of the culture flask is layered with solid medium. After inoculation, the flask is tilted every three or more days so that the broth wets the agar briefly and colonies eventually

appear. In addition, automated methods have been developed over the last decade that detect the CO_2 resulting from aerobic bacterial growth, and they also make blind subculturing unnecessary.

When using the Ruiz-Castañeda method, blood cultures are seldom positive by the fourth day of incubation, the majority are positive between the seventh and twenty-first, and only 2% are positive after the twenty-seventh day. In this method, incubations should be carried out for at least 45 days before rejecting a blood culture as negative for *Brucella* (Díaz and Moriyón, 1989). The automated CO_2-detecting systems are advantageous in that they have a shorter average period of detection (usually less than five days). However, prolonged incubation is not completely avoided as some flasks do not become positive in less than three weeks. Moreover, cultures remaining negative after the first week of incubation do not always produce CO_2 above the positive threshold level, and it is advisable to do blind subculturing for maximum sensitivity (Ariza, 1999; Casas et al., 1994; Yagupsky et al., 1997).

The low numbers of *Brucella* cells in the sample (sometimes as low as 1–2 colony forming units per ml) are in all likelihood one of the reasons for prolonged incubations, and larger samples are advantageous (Zimmerman et al., 1990). It is, therefore, advisable to inoculate 5–10 ml in duplicate flasks, as it is also advisable to perform two or three independent blood samplings at adequate intervals (Díaz and Moriyón, 1989). Some studies have found that bacteriological detection can be improved by using the leukocyte lysis-concentration procedure (Gaviria-Ruiz and Cardona-Castro, 1995; Etemadi et al., 1984).

When blood samples are taken from febrile patients, isolation is successful in the majority of cases, and much lower rates are obtained from afebrile patients (68 versus 32% in the extensive comparative study of Rodríguez-Torres et al., 1977). This difference is also found in relapsed patients. Indeed, the probability of obtaining a successful culture is greatly reduced when antibiotherapy has started. Since the percentages of positive cultures reported for *B. melitensis* (about 70-80%) are usually higher than for *B. abortus* (about 50%), it has been suggested that the infecting species influences the likelihood of a successful isolation (Dalrymple-Champneys, 1960). Unless epidemiological data are necessary, or infection with the vaccine strain US19 or Rev. 1 is suspected (Treatment), identification to genus level is enough for medical purposes (Identification and Typing).

Molecular Tests for Humans A number of molecular tests have been proposed for human brucellosis, but PCR is the only one that has deserved significant attention. So far, amplification of DNA extracted from blood with primers taken from a 31-kDa *B. abortus* soluble protein (BCSP31) is the only protocol that has been evaluated (Matar et al., 1996; Morata et al., 1999a; Queipo-Ortuño et al., 1997). This protocol has been reported to have a diagnostic sensitivity of 92% or higher (with regard to the combined clinical, bacteriological and serological evidence) and a specificity of 93%. Modifications of the original protocol have been described in further work (Morata et al., 1998; Queipo-Ortuño et al., 1999), and the protocol reported to be successful in the evaluation of recovery and in the diagnosis of relapses (Morata et al., 1999b). However, other laboratories have not been able to reproduce the results (Navarro et al., 1999). If the findings are confirmed, the method would provide medical microbiologists with an exceedingly valuable diagnostic tool.

Direct Diagnosis in Animal Brucellosis

Bacteriological Culture for Animals Bacteriological culture, by definition, is the most specific diagnostic test in animal brucellosis, and when performed exhaustively, it allows the identification of over 90% of infected cattle, sheep and also possibly of other animal species. Unfortunately, performed in this way, it is cumbersome and expensive, and the processing of samples containing *B. melitensis*, *B. abortus* or *B. suis* requires special precautions (Alton et al., 1988) (Biosafety). Its use is, therefore, restricted to some circumstances, such as the unequivocal demonstration of the disease at herd level, the identification of vaccine shedders (Identification of Vaccine Strains; Epidemiology), the assessment of the performance of serological tests and vaccines, and the diagnosis of animals of high genetic value.

Samples used include vaginal swabs, semen, aborted fetuses (stomach contents, lung and spleen) and fetal membranes. Direct examination of smears of these materials stained by Stamp's method (Cellular and Colonial Characteristics) can be very informative, although other bacteria like *Chlamydia* are also Stamp positive and a cause of abortion (Fig. 10). Blood can also be used and hemocultures are recommended as a good diagnostic test for *B. canis* in dogs. Milk (cream and sediment from the four quarters) is very useful for identifying shedders. Materials that can be obtained at the abattoir or after necropsy include the udder and the uterus of pregnant or early postparturient animals and the male reproductive organs, but the best samples are the spleen and, in particular, the lymph nodes. Although mammary nodes yield *Brucella*

most often, all major lymph nodes (up to seventeen) should be examined for an exhaustive inquiry into the infectious state of a particular animal. In addition, any tissues showing inflammatory lesions and abscesses should be cultured (Alton et al., 1988).

Most samples require maceration (in a safety cabinet). All but blood or other normally sterile fluids (which can be processed in the same way as human blood cultures) are inoculated as abundantly as possible into an appropriate selective or enrichment medium (Table 11) and incubated in a 10% CO_2 atmosphere. Cultures are seldom clearly positive by the fourth day of incubation and should be incubated for at least 15 days. The number of bacteria in the organs can vary widely and plates can contain from very few to many *Brucella* colonies. An alternative to selective media is the inoculation of guinea pigs followed by the recovery of the bacteria from the appropriate tissues at necropsy and the demonstration of seroconversion (Alton et al., 1988; Ne'eman and Jones, 1963; Brucellosis in Guinea Pigs). This method, however, is only justified when dealing with very heavily contaminated specimens and after consideration of the ethical issues involved.

Molecular Tests for Animals Tests detecting *Brucella* cells or antigens by immunological methods have been described but they have not found a practical use in the diagnosis of brucellosis, and this is also true of tests detecting directly *Brucella* DNA with labeled probes (Mayfield et al., 1990). PCR has deserved much greater attention, but so far most research has been directed to the development of protocols under laboratory conditions (Table 4), and since studies on the actual diagnostic value are scarce, there is no established PCR protocol that could replace the bacteriological culture. An assessment of the possible practical value of the existing PCR protocols is complicated by the fact that an efficient DNA extraction from samples is critical for optimal diagnostic sensitivity. There are no specific studies on this problem, but it has been noted that even in culture, *Brucella* cells are resistant to conventional DNA extraction protocols (Romero and López-Goñi, 1999). Nevertheless, the few studies that have compared the diagnostic performance of PCR with that of standard bacteriological or serological tests suggest that PCR could be a valuable diagnostic test in animal brucellosis. In the examination of tissues, organs and stomach contents of *B. abortus* infected cattle, a PCR protocol with primers taken from a putative *B. abortus* Omp showed 98% sensitivity and 96% specificity as compared to bacteriological culture (Fekete et al., 1992a; Fekete et al., 1992b). In a study carried out to assess the ability to detect *Brucella* in the stomach contents of aborted sheep fetuses, PCR with 16S rRNA primers performed closely to bacterial cultures, albeit the number of samples studied was low (Cetinkaya et al., 1999). For obvious reasons, milk would be one of the samples of choice, and in one study, conventional PCR with 16S rRNA primers was found to have 100% specificity and 87.5% sensitivity with respect to bacteriological culture (ELISA showed 100% specificity and 98.2% sensitivity in the same samples; Romero et al., 1995b). Other possible uses of PCR in the diagnosis of animal brucellosis include the direct identification of *B. abortus* strain 19 vaccine shedders by using primers taken from the *ery* region (Identification of Vaccine Strains).

Immunological Tests for Brucellosis *Brucella* evokes a cell- and antibody-mediated immune response (Control of the Infection), and accordingly, immunological tests for brucellosis can be grouped into two categories: those that detect specific antibody and those that analyze cell-mediated immunity. Mainly protein antigens trigger the cell-mediated response, but also both LPSs and proteins elicit an antibody response. Thus, broadly speaking, antibody tests for brucellosis can be subclassified into two groups corresponding to each of these antigens. Although structurally related to the LPS *O*-polysaccharide, NH or polysaccharide B is measured by a third group of antibody tests.

To understand the immunological diagnosis of brucellosis, it is necessary to keep in mind 1) the differences in surface antigens existing between smooth and rough *Brucella* species (Response to Environmental Stress); 2) the close structural similarity of the LPS *O*-polysaccharides of the *Brucella* serotypes; 3) the presence of the smooth LPS in the most effective vaccines (Vaccines and Vaccination); 4) the existence of Gram-negative bacteria that, albeit phylogenetically distant, show *O*-polysaccharides similar to those of the smooth *Brucella* species; and 5) that significant immunological crossreactivities with *Brucella* proteins have been shown only for similar antigens of phylogenetically close relatives (Fig. 3). The implications of these facts can be summarized as follows:

A. LPS tests used to diagnose infections caused by *Brucella* rough species (*B. ovis* and *B. canis*) are different from those used to diagnose *B. melitensis*, *B. abortus* and *B. suis* infections.

B. If quality control of antigens is always important, dissociation of smooth *Brucella* cultures is a constant possibility. Thus, extreme care must be taken to make sure that the antigens used in smooth LPS tests are prepared

from nondissociated cultures (Colonies and Dissociation).

C. Antibodies to the smooth-LPS can be detected with whole smooth cells (Outer Membrane Topology) or LPS extracts. It is not necessary to use bacteria or LPS of the homologous serotypes. Thus, *B. abortus* biotype 1 cells (often *B. abortus* vaccine strain 19) can be used to obtain antigens for the diagnosis of infections caused by other smooth brucellae. This has been clearly shown for *B. melitensis* and *B. abortus* biotypes serologically different from biotype 1, and it is so because polyclonal responses contain a large proportion of antibodies that, because of combinations of titer and avidity, show overlapping reactivities with the A, M and C epitopes (Fig. 15). In the diagnosis, therefore, the C epitopes are largely dominant (Fig. 34; Response to Environmental Stress).

D. Smooth LPS tests have two main sources of nonspecificity: vaccination with *B. abortus* strain 19 or *B. melitensis* Rev. 1 (Vaccines and Vaccination) and infections with Gram-negative bacteria carrying perosamine-containing *O*-polysaccharides. Among the latter, *Y. enterocolitica* O:9 is the one showing the strongest crossreactivity due to the rather close similarity existing between its *O*-polysaccharide and the *O*-polysaccharide of *B. abortus* biotype 1 (Table 6).

E. Protein tests allow a differential diagnosis of brucellosis and infections caused by Gram-negative bacteria that carry perosamine-containing *O*-polysaccharides. They are also the antigens active in tests assessing the cell-mediated immunological response.

Practically all types of immunological assays available have been investigated as potential tests for the diagnosis of brucellosis. The most relevant antigens and, where appropriate, immunoglobulins involved in some commonly used or representative tests are summarized in Table 16.

Immunological Diagnosis of Human Brucellosis Caused by Smooth Brucella *Brucella melitensis*, *B. abortus* and *B. suis* are the *Brucella* species infecting humans most often (Brucellosis in Humans). *Brucella canis* is also pathogenic for humans, but it does not pose a threat to human health of the magnitude created by the first three species, and the few reported cases are not always confirmed by bacteriological cultures (Carmichael, 1990). The immunological diagnosis of *B. canis* infection is considered in a separate section (Immunological Diagnosis of Infections Caused by *B. ovis* and *B. canis*).

Antibody Detecting Tests in Infected Patients Tests detecting cell-mediated immunity are not used nowadays for the diagnosis of human brucellosis. On the other hand, antibody-detecting tests are performed routinely and they can be performed with blood serum and, when neurobrucellosis is suspected, with cerebrospinal fluid. Since serum samples are rarely taken early enough, seroconversions are seldom observed in human brucellosis. Serological tests are then useful not only to detect anti-*Brucella* antibodies, but also to evaluate the stage of evolution of the disease through an assessment of the classes of immunoglobulins involved in a given test or specifically detected by it. Obviously, a dominant IgM response is characteristic of the early stages (acute brucellosis), whereas IgG and IgA in the absence of IgM are characteristic of a brucellosis that was acquired some time before the test was performed. To detect changes in the immunoglobulin levels also can be useful to evaluate the possibility that a relapse had occurred (Fig. 39).

Smooth-Lipopolysaccharide Tests in Infected Patients At the early stages of infection, antibodies are directed almost exclusively against the *O*-polysaccharide of the smooth-LPS, and they are also those that persist longer after recovery (Fig. 39). Thus, changes in immunoglobulin profiles are best observed in smooth-LPS tests. Moreover, these are the most sensitive tests.

Agglutination tests are inexpensive, technically simple and informative. Thus, they are recommended for the diagnosis of human brucellosis in most situations. They include the classical serum tube agglutination test used to titrate the total amount of agglutinating antibody and modified tests aimed to roughly determine the amount of IgM versus IgG plus IgA by including reagents that preferentially (but not exclusively) inactivate IgM (e.g., the β-mercaptoethanol test; Table 16). The diagnostic titer in the classical tube agglutination test may vary depending upon the prevalence and other circumstances (Díaz and Moriyón, 1989). Agglutination tests for brucellosis also include the rose bengal test (and the card test), in which the use of an acid pH makes all antibodies show their intrinsic agglutinating activity. Although it is primarily a rapid slide agglutination screening test, the rose bengal can also be used in human brucellosis with serum dilutions to obtain a rough idea of the total amount of anti-smooth-LPS antibody, which is enough for most clinical purposes. Prozones are occasionally observed in the classical tube agglutination, but not in the rose bengal, and both tests have very few false-negative results.

The serum agglutination test can be adapted to measure the nonagglutinating (low affinity) IgG and IgA (Coomb's test; Table 16), which characteristically develop in brucellosis. In acute brucellosis, Coomb's titers are usually 4–16 times

Table 16. Some tests and antigens used in the diagnosis of brucellosis.

Tests	Antigen	Antibody	Comments	References
Agglutination				
Tube agglutination (and β-mercaptoethanol and rivanol) test	S-LPS (*B. abortus* cell suspension)	IgM, IgG and IgA	It can be adapted to the diagnosis of *B. canis*. To estimate the amount of IgM, the results of the standard tests are compared with those obtained in the presence of β-mercaptoethanol (or similar reagents)[a] or rivanol[b]	Alton et al., 1988
Rose bengal (and card) tests	S-LPS (stained *B. abortus* cell suspension in lactate buffer pH 3.65)	IgM, IgG and IgA[c]	Simple and inexpensive slide-agglutination type tests in which bacteria are stained with rose bengal. Used as screening tests	Alton et al., 1988
Coomb's	S-LPS (*B. abortus* cell suspension)	IgG and IgA	Detects nonagglutinating Ig that has reacted with the bacterial surface by using an anti-Ig serum that brings about the agglutination. It can, therefore, be used to specifically detect nonagglutinating IgG or IgA with an antiserum of the appropriate specificity	Otero et al., 1982
Milk ring test	S-LPS (stained *B. abortus* cell suspension in citrate buffer pH 4.0)	IgA (also IgM and IgG)	Ig in milk reacts with hematoxylin stained bacteria, and the complex attaches to the fat that ascends to form a ring on the surface of the reaction tube. Only adequate for cow milk	Alton et al., 1988 Sutra et al., 1986
Complement consumption				
Hemolysis in agar	S-LPS coated erythrocytes	IgG[d]	Sensitized erythrocytes are included with complement in an agarose gel and a radial immunodiffusion performed	Ruckerbauer et al., 1984
Complement fixation	S-LPS (*B. abortus* cell suspension) or R-LPS plus Omps (*B. ovis* hot saline extracts)	IgG[d]	A confirmatory test for *B. abortus* or *B. melitensis* animal brucellosis; also used in the diagnosis of *B. ovis*. Cumbersome to perform unless automated; some sera show anticomplementary activity	Alton et al., 1988
Precipitation				
Double immunodiffusion	Extracts containing S-LPS, S-LPS plus NH, or R-LPS plus Omps (*B. ovis* hot saline extracts)	IgG (also IgM)[e]	Used mostly for *B. ovis* and *B. canis* diagnosis with the R-LPS plus Omps extract; usually performed in hypertonic gels	Alton et al., 1988

Table 16. *Continued*

Tests	Antigen	Antibody	Comments	References
Reverse radial immunodiffusion (RID)	NH and polysaccharide B	IgG[f]	A confirmatory test for cattle, goat and sheep (*B. melitensis*) brucellosis. Performed in hypertonic gels	Jones et al., 1980 Díaz et al., 1981
Crossover immunoelectrophoresis	Soluble proteins of rough *Brucella* mutants	IgG	Useful for the diagnosis of human brucellosis. IgM to protein antigens is not detected in this assay	Díaz et al., 1976
Immunoenzymatic				
Indirect ELISA	Purified S-LPS, S-LPS *O*-polysaccharide, S-LPS rich extracts or extracts containing R-LPS plus Omps (*B. ovis* hot saline extracts). Purified proteins	Depends on conjugate specificity	In human brucellosis, ELISAs detecting anti-S-LPS antibodies could replace classical tests if adapted to measure IgM and IgG. For animal brucellosis, ELISAs are most often adapted to detect anti-S-LPS IgG	Nielsen et al., 1996 Alonso-Urmeneta et al., 1988 Marín et al., 1998 Rosetti et al., 1996 Goldbaun et al., 1992 Letesson et al., 1997
Competitive ELISA	S-LPS, S-LPS O-polysaccharide, or S-LPS rich extracts	Presumably all Ig classes	A mouse monoclonal antibody competes with low avidity (and low titer) antibodies for the S-LPS C epitopes; the monoclonal antibody bound is then detected with an immunoconjugate specific for mouse immunoglobulins	Gall and Nielsen, 1994
Fluorescence polarization	Fluorescein-labelled *O*-polysaccharide	Presumably all Ig classes	A promising test based on the change of fluorescence of labeled antigen upon antibody binding	Nielsen et al., 1996
Delayed type hypersensitivity	Soluble proteins of rough *Brucella* mutants	None	Most often performed as a simple skin test	Jones et al., 1973 Blasco et al., 1994 Saegerman et al., 1999
Lymphocyte blastogenesis	Complex antigenic mixture; soluble and outer mebrane proteins	None	Technically demanding	Baldwin et al., 1984
γ-interferon production	Soluble proteins of rough *Brucella* mutants	None	Technically demanding	Weynants et al., 1995

[a]At least human IgA has been shown to be sensitive to the concentrations of β-mercaptoethanol used in the test.
[b]2-Ethoxy-6,9-diamino-acridine (an IgM precipitating reagent).
[c]IgA activity in the rose bengal test has been shown with human sera.
[d]There are contradictory reports on the IgM ability to activate complement. At least in cattle, IgM does not activate guinea pig complement in the absence of homologous serum components.
[e]IgM is particularly active in the immunoprecipitation of LPS extracts (this molecule forms aggregates, and the reactions mimic a microagglutination).
[f]IgM precipitating NH has been shown in some human sera. At least in cattle, IgG_1 is the major immunoglobulin precipitating NH (and polysaccharide B) and accounting for the hypertonic conditions required in the assay.

higher than tube agglutination titers, whereas at later stages they are 16–256 times higher. Thus, comparison of the serum agglutination and the Coomb's tests allows the assessment of the stage of evolution of human brucellosis. This can also be achieved by using the complement fixation test: in over 90% of the human cases, serum agglutination and complement fixation are simultaneously positive, but after the fourth or the fifth week of evolution, complement fixation titers are higher than serum agglutination titers. At the very early stages of infection, serum agglutination is positive and complement fixation negative, and the opposite is true in patients that are recovered or are developing chronic or focal forms (Díaz and Moriyón, 1989).

The indirect enzyme-linked immunosorbent assay (i-ELISA) with smooth-LPS (or with smooth cells) is both very specific and sensitive for the diagnosis of human brucellosis. Using this assay, it has been clearly proven that the response to smooth-LPS in treated patients follows a classical pattern: IgM appears and decreases faster than IgA or IgG, and in relapses, IgG and IgA, but not IgM, increase over the levels observed when the first diagnosis was made (Fig. 39). The assay also shows that IgG is the more persisting immunoglobulin and that over 80% of the patients have high titers of IgG and IgA a year and a half after clinical recovery (Ariza et al., 1992; Pellicer et al., 1988). The correlation of the IgM or IgG i-ELISA with agglutination, Coomb's and complement fixation tests is the one expected from the immunoglobulin classes involved in the latter tests. It seems, therefore, possible to replace all classical serological tests by an i-ELISA with smooth-LPS and anti-IgM and IgG conjugates, but this would require not only standardization but also the definition of the equivalence between the i-ELISA results and those of the classical tests that clinicians are used to interpreting. There is one report on the use of the competitive ELISA (c-ELISA) in human brucellosis, and the assay was found also very specific and sensitive (Lucero et al., 1999). This test could be less sensitive to interference by crossreacting bacteria, but no definite studies with human sera have been published to date.

Protein Tests in Infected Patients Both indirect evidence and studies in experimentally infected animals show that antibodies to soluble proteins develop later than antibodies to the smooth-LPS. They are characteristic of an active brucellosis, and the longer the evolution of the disease before treatment, the higher the antibody levels and the diversity of proteins to which they are directed (Fig. 38). Antibodies to *Brucella* proteins can be found not only in blood serum but also in the cerebrospinal fluid of patients afflicted with neurobrucellosis. The protein antigens that have been used include the mixture of soluble proteins present in the hypertonic *Brucella* extracts also used in the skin test (Díaz et al., 1976; Cell-mediated Immunity Tests), BP26 (Rossetti et al., 1996) and an immunocaptured cytosolic protein (lumazine synthase; Goldbaum et al., 1992; Goldbaum et al., 1999). Antibodies to Omps also develop in brucellosis but they are more cumbersome to detect and their diagnostic value is less clear (Leiva-León et al., 1991). Tests that have been used include gel precipitation (either as double gel diffusion or as crossover immunoelectrophoresis), indirect ELISA and Western blot.

By gel immunodiffusion, it has been shown that over 80% of brucellosis patients have precipitating antibodies to one or several soluble proteins in hypertonic *Brucella* extracts. In one study, in which the sera of 173 patients with acute brucellosis and 39 with brucellosis having a long evolution were examined by crossover immunoelectrophoresis, 84.9% and 91.6% had developed anti-protein antibodies, respectively (Díaz et al., 1976). Using an indirect ELISA in which the antigen had been immunocaptured with a monoclonal antibody, 94% (33 of 35) of the brucellosis patients examined showed IgG to *Brucella* lumazine synthase (an 18-kDa antigen), as well as strong anti-smooth-LPS response. By contrast, individuals classified as having a "nonactive" brucellosis seldom showed anti-lumazine synthase antibody, even though most had various degrees of anti-LPS reactivity (Goldbaum et al., 1992). All these results strongly suggest that the presence of anti-protein antibodies is a useful criterion to decide whether low levels of anti-smooth-LPS antibodies are indicative of infection or simply of contact without the development of disease. Thus, these tests deserve further attention.

Antibody Detecting Tests in Recovered Patients After recovery, serum agglutination becomes progressively negative, usually six months to two years later. The complement fixation titers also decrease but they may also persist, always for longer times than those of serum agglutination titers. The last conventional test to become negative is the Coomb's test. When the results of these three classical tests are taken together, the serology seldom becomes negative by the third month, and positive results often persist after the eighth month of evolution. In the ELISA, IgM, IgA and IgG are negative by the first year in 65, 25 and 3% of recovered patients, respectively (Ariza et al., 1992). The problem of predicting a relapse based on the results of serological tests has not been satisfac-

torily solved. It has been suggested that persisting β-mercaptoethanol-resistant antibodies (IgG) correlate with the risk of relapse, but the ELISA studies show that IgG persists for a long time in the serum of fully recovered patients.

Immunological Diagnosis of Animal Brucellosis Caused by Smooth Brucella A major difference with the immunological diagnosis in humans is that, in animals, what is most often judged is whether there has been contact with *Brucella* and not the existence of an ongoing infection. This information is enough for eradication purposes, including control of animal movements and trade, and an assessment of the immunoglobulin profile or a comparison of the results of smooth-LPS and protein tests is not necessary. However, the use of vaccines complicates the diagnosis of animal brucellosis. Vaccination induces an immunological response that closely mimics that resulting from a true infection, and since even the best vaccines available do not afford 100% protection, distinction of infected and vaccinated animals becomes necessary.

For obvious reasons, the majority of the information on the performance of the immunological tests concerns cattle, sheep, goats and swine. Positive reactions in the classical blood serum tests have been recorded for other important domestic animals, such as camels, water buffalos and reindeer, and also for a variety of wild animals (Davis, 1990). However, since these observations have not been complemented by bacteriological studies, the actual performance of the tests in detecting *Brucella* infection in all these animals is not known and the results of the immunological tests can only be taken as presumptive. The discussion is limited here to the immunological diagnosis of brucellosis in domestic ruminants and swine.

Antibody Detecting Tests in Infected Animals The tests used are often the same as those used in human brucellosis (Table 16), and in fact, some, like the rose bengal test, were first developed for the diagnosis of animal brucellosis. The presence of antibodies to *Brucella* can be investigated in blood serum but also in the whole milk or whey of dairy animals.

Smooth-Lipopolysaccharide Tests in Infected Animals Like in human brucellosis, these are the tests used most often. Their application has been studied best in cattle. There are fewer studies in small ruminants, and the number of animals included is also comparatively reduced. Moreover, for classical tests, such as the rose bengal and complement fixation, most investigations in small ruminants have been performed with the tests standardized for the diagnosis in cattle, and these conditions do not result in optimal sensitivity (Blasco et al., 1994b). This standardization problem affects only the proportion of serum to antigen and not to the particular epitopic structure of the smooth-LPS used in the assay (Immunological Tests for Brucellosis). The results of selected studies in which this difficulty is taken into account, the sensitivity defined with respect to bacteriological culture, and the specificity determined in the absence of vaccination are presented in Table 17. They show that, under those circumstances, most tests perform with a remarkable high efficiency and that the i-ELISA and the rose bengal (or card) test come close to the ideal test. There is a long practical experience in the use of the rose bengal test, but the i-ELISA has been investigated thoroughly. It has been shown that purified smooth LPS, *O*-polysaccharide or NH preparations offer no advantage over relatively crude smooth-LPS extracts (Alonso-Urmeneta et al., 1988; Nielsen et al., 1995; Wright and Nielsen, 1990b). Conjugates based on monoclonal antibodies specific for ruminant IgG_1 or on the IgG-binding Streptococcus protein G allow the diagnosis of the three common domestic ruminants with a single reagent, improving the specificity and reducing the background reactivity caused by IgM (Alonso-Urmeneta et al., 1988; Wright et al., 1990b). Peroxidase conjugates and the chromogenic substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) have been shown to produce excellent results. Thus, this assay has been well-defined and the choice between the i-ELISA and the rose bengal test depends mostly on economical and logistic reasons. With regard to swine brucellosis, studies with well-defined sera are scarce but immunoenzymatic and fluorescence polarization assays seem to outperform the classical agglutination or complement fixation tests (Table 17). The latter tests have been reported to show low sensitivity and to be useful for diagnosis only on a herd basis (Alton, 1990a).

The specificity of the smooth-LPS tests becomes compromised when vaccination with *B. abortus* 19 or *B. melitensis* Rev. 1 is implemented (Vaccines and Vaccination). To overcome this problem (and also to reduce the restrictions in the application of the vaccines), a test that is able to distinguish infected from vaccinated animals (i.e., a test showing a high specificity in the context of vaccination) would be necessary. Ideally, such a test should also have a high sensitivity and be technically simple to facilitate large-scale testing. No classical test fulfils these requirements, and therefore a combination of a simple screening test (highly sensitive) and a confirmatory test (highly specific with respect to vaccinated animals) has been traditionally used. The rose ben-

Table 17. Performance of some antibody tests for the diagnosis of infections by smooth *Brucella* spp.

Animal host	Test	Sensitivity[a] (%)	Specificity[b] (%)	No. of culture-positive animals studied	References
Cattle	Rose bengal (or card-test)	91.4–99.7	98.8–99	300 and 1,666	Huber and Nicoletti, 1986 Morgan, 1977
	Complement fixation	87.9 or 97.1[c]	93.1 or 9.8[c]	654	Nielsen et al., 1996b
	RID-NH	92	100	112 and 383	Díaz-Aparicio et al., 1993 Jones et al., 1980
	i-ELISA-LPS	100	99.7	636	Nielsen et al., 1996b
	c-ELISA-LPS	100	99.9	636	Nielsen et al., 1996b
	Fluorescence polarization	99	99.9	561	Nielsen et al., 1996a
Sheep	Rose bengal	94	100	135	Blasco et al., 1994a
	Complement fixation	88	100	135	Blasco et al., 1994a
	RID-NH	90–96	100	85 and 77	Díaz-Aparicio et al., 1993 Jimenez Bagués et al., 1992
	i-ELISA-LPS	100	100	55 and 140	Blasco et al., 1994b Marin et al., 1994
	c-ELISA-LPS	96	100	55	Marin et al., 1999
Goats	Rose bengal	100	100	55	Díaz-Aparicio et al., 1994
	Complement fixation	94	100	55	Díaz-Aparicio et al., 1994
	RID-NH	94	100	55	Díaz-Aparicio et al., 1994
	i-ELISA	100	100	55	Díaz-Aparicio et al., 1994
Swine	BPAT[d]	77	95.9	401	Nielsen et al., 1999
	Complement fixation	95.5–99.9[c]	58.1–93.2[c]	401	Nielsen et al., 1999
	i-ELISA	94	98	401	Nielsen et al., 1999
	c-ELISA	96	90	401	Nielsen et al., 1999
	Fluorescence polarization	93.5	97.2	401	Nielsen et al., 1999

Abbreviations: RID-NH, radial immunodiffusion for antibody to native hapten polysaccharide; i- and c- ELISA, indirect and competitive enzyme-linked immunosorbent assay; and BPAT, buffered antigen plate agglutination test.

[a]Sensitivity = number of test-positive sera among culture-positive animals / total number of culture positive animals.

[b]Specificity = number of test-negative sera among *Brucella*-free (unvaccinated) animals / total number of *Brucella*-free (unvaccinated) animals.

[c]Depending on whether sera with anticomplementary activity are considered as positive or negative.

[d]BPAT, buffered plate agglutination test. This is a rapid agglutination test similar to the rose bengal test, but cells are stained with a mixture of crystal violet plus brilliant green. It may be slightly more sensitive than the rose bengal test.

gal and the complement fixation, respectively, have been the tests of choice for these purposes. The former test is simple, can be automated, and is very sensitive (Table 17), but it remains positive in a large proportion of vaccinated animals long after vaccination. The complement fixation test yields negative or weak titers six months after calfhood vaccination, but it requires careful and periodical standardization, and when adult vaccination is used, some animals may have titers persisting for a long time. Despite these problems, the efficacy of the combination of these two tests with calfhood vaccination is well established (MacMillan, 1990a), and they have been the reference in the serological diagnosis of animal brucellosis for years. Although it has been accepted that their performance in sheep and goats is adequate, they lack sensitivity when standardized according to current guidelines (Blasco et al., 1994b).

Several studies have shown that the i-ELISA is highly sensitive and, when properly standardized, also specific in differentiating infected from vaccinated animals, with reported specificities as high as 95–98% for cattle (Samartino et al., 1999). In experiments performed in sheep bled periodically after vaccination, the i-ELISA was less specific than the complement fixation, an immunoprecipitation test with NH (NH [and Polysaccharide B] Tests), and a competitive ELISA (c-ELISA; Marń et al., 1999). This last type of assay has been designed to prevent reactions below a given avidity threshold level set by the concentration of a competing monoclonal antibody specific for the *O*-polysaccharide C epitope(s) (Table 16). Since there is evidence that the antibodies remaining in serum several months after vaccination are in most cases of low avidity, the c-ELISA is an interesting approach to the differential diagnosis of infected and vac-

cinated animals. An additional advantage of this method is that it does not use a conjugate specific for the immunoglobulins in the tested serum, and therefore, it can be easily adapted to detect *Brucella* infections in different animal species. Like the i-ELISA, several protocols are possible with regard to antigen, monoclonal antibody, and other conditions. In a study performed in cattle, the sensitivity of a c-ELISA with monoclonal BM-40 was 100% in animals with bacteriologically proven infection, but its specificity in vaccinated calves was only 80% (76% for the complement fixation in the same animals; Macmillan et al., 1990b). However, an improved c-ELISA with monoclonal M48 showed 100% specificity in differentiating infected from vaccinated cattle (Nielsen et al., 1995). In a large study conducted to evaluate several i-ELISA and c-ELISA protocols with sera from animals of unknown bacteriological and vaccine status, the c-ELISA with smooth LPS and monoclonal M48 was found to be the best performing assay in terms of balanced sensitivity and specificity (97.5 and 96.5%, respectively) with respect to the combined results of rose bengal and complement fixation (Gall et al., 1998). In the analysis of sera from *Brucella*-free, vaccinated, and bacteriologically positive sheep, this same c-ELISA showed slightly lower sensitivity than the i-ELISA (96 and 100%, respectively) and better specificity (about 89%) in distinguishing infected from Rev. 1 vaccinated animals (Marín et al., 1999). In this same study, the c-ELISA was more specific (87–89%) than the complement fixation in vaccinated adults and in subcutaneously vaccinated young sheep (45–66 and 78%, respectively) but not in conjunctivally vaccinated young sheep (89% for the c-ELISA versus 100% for the complement fixation). Thus, when properly standardized, the c-ELISA seems a better test than the complement fixation but the influence of the route of vaccination deserves further research.

NH (and Polysaccharide B) Tests Immunoprecipitation tests with NH or polysaccharide B are very specific when cattle are vaccinated with *B. abortus* strain 19 and can replace the complement fixation test. For optimal sensitivity (up to 92%), precipitating antibodies are detected in a reverse radial immunodiffusion (RID) system, in which the serum is allowed to diffuse in a hypertonic gel containing the polysaccharide (Díaz et al., 1979; Díaz et al., 1981), but the more simple double gel diffusion procedure is also useful (Lord et al., 1989; Pinochet et al., 1989; Fig. 19). Calves vaccinated at 3–5 months of age are negative after 2 months of vaccination, and adult cattle vaccinated 4–5 months previously with the standard dose of *B. abortus* 19 do not give positive reactions unless the animals develop vaccine infections in the mammary gland and shed the vaccine in their milk (Jones et al., 1980). In a study carried out with the sera of 1,057 cattle from infected herds in which adult vaccination was implemented, the RID test outperformed the complement fixation in specificity (80 versus 66%, respectively) and showed acceptable sensitivity (88 versus 98%, respectively) despite the fact that vaccination and infection were superimposed (Jones et al., 1980). The RID test also shows better specificity than the complement fixation test in adult and young sheep vaccinated with Rev. 1, either subcutaneously or conjunctivally (Blasco et al., 1984a; Jiménez de Bagués et al., 1992). A remarkable characteristic of the RID test is that a positive result correlates with *Brucella* shedding. In addition to the evidence mentioned above on the strain 19 shedders, this has been shown in experimentally infected cattle (Jones et al., 1980) and also in naturally infected cattle undergoing antibiotic treatment (Jiménez de Bagués et al., 1991). Thus, the combined use of rose bengal, RID and strain 19 vaccination has been proven to be useful in achieving eradication (Asarta, 1989).

The NH (and polysaccharide B) and the LPS *O*-polysaccharides are structurally very similar (Fig. 16; Response to Environmental Stress). It is thus noteworthy that whereas both antigens yield very close results when used in the i-ELISA, immunoprecipitation with smooth LPS has a much lower sensitivity than immunoprecipitation with NH (Alonso-Urmeneta et al., 1988). These differences in sensitivity can be explained by the fact that the high epitopic density and disperse state of NH (versus the bulky micelle-like state of LPS) favor its immunoprecipitation when only low titers of antibody are present. Moreover, the comparatively high threshold antibody avidity required for a positive immunoprecipitation could account for the ability of the RID with NH to discriminate infected from vaccinated animals, since it has been observed that unless udder infections develop, vaccination only transiently induces the high avidity IgG that results from an active infection (B. Alonso-Urmeneta et al., unpublished observations). Comparative studies between immunoprecipitation with NH and c-ELISA show similar sensitivity (90 and 96%, respectively) and specificity in vaccinated animals (85–100% and 87–98%, respectively; Marín et al., 1999), supporting by this manner the interpretation that the former assay detects only high avidity antibodies. It seems likely that the subtle structural and epitopic differences existing between the NH (and polysaccharide B) and the *O*-polysaccharide could contribute to the peculiar immunoprecipitating properties of the former polysaccharide. In one study in which it was com-

pared with *B. melitensis* 115 polysaccharide B, the *O*-polysaccharide obtained directly by hydrolysis of the smooth LPS of *B. abortus* strain 19 (which is poor in NH) did not produce good results (Jones et al., 1980). Although the polysaccharides obtained by acid hydrolysis of smooth virulent *B. abortus* produce satisfactory results (Cherwonogrodzky and Nielsen, 1988), these whole cell extracts contain both NH and *O*-polysaccharide, and it is not possible to determine to what extent each of these two molecules accounts for the results.

Protein Tests in Infected Animals Tests detecting antibodies to *Brucella* proteins are receiving an increased attention in the hope of improving the differential diagnosis of infected and vaccinated animals. If sensitive enough, these tests would also be very useful to avoid false positive results caused by bacteria crossreacting with smooth *Brucella* at the LPS level. Early work carried out by crossover immunoelectrophoresis showed that extracts containing a mixture of soluble proteins were promising diagnostic antigens in sheep (Trap and Gaumont, 1982). On the other hand, a gel immunodiffusion test with a purified protein (protein α-2) showed only low sensitivity (55%), even though it was highly specific (99%) in differentiating infected from vaccinated cattle (Dubray, 1984). More recently, several cloned proteins have been tested in immunoenzymatic assays. Although cattle, goats and sheep infected by smooth *Brucella* develop antibodies to group 1 (89 kDa), Omp2 (porins), Omp3a (also known as Omp25), Omp19, Omp16 and Omp10, the response is of lower intensity and frequency than that to the smooth-LPS, and these Omps used individually would have no diagnostic use (Cloeckaert et al., 1992; Letesson et al., 1997; Zygmunt et al., 1994). Cytosolic or periplasmic proteins tested include GroEL (Lin et al., 1996), Cu^{++}-Zn^{++} superoxide dismutase (Tabatabai and Hennager, 1994), and a variety of soluble proteins of 9, 15, 17, 24, 28, 32, 39, 50 and 54 kDa (Letesson et al., 1997; Salih-Alj Debbarh et al., 1995), but lumazine synthase (an 18-kDa protein; Baldi et al., 1996) and BP26 (Arese et al., 1999; Rossetti et al., 1996; Salih-Alj Debbarh et al., 1996) seem to be the most promising reagents. It seems likely that for maximal sensitivity, several of these proteins would have to be combined in a single immunoenzymatic test.

Milk Tests In dairy cattle, milk and milk serum are suitable samples either for individual diagnosis or for monitoring the status of the herd by checking pooled milk. For these purposes, the milk ring test (Table 16) was originally developed. This test uses whole milk rather than serum, has been standardized and is highly sensitive and easy to perform. Moreover, the conditions of use for testing pooled milk (i.e., sensitivity with regard to numbers of animals and total volume of sample) have been well studied (Pietz, 1977). Unfortunately, as the test depends on characteristics such as the content and type of fat in the milk, it is not applicable to small ruminants.

Several i-ELISA protocols have been evaluated as alternatives to the milk-ring test in both cattle and small ruminants. Most authors have concluded that it is an excellent test for the examination of milk from individual animals (Bercovich and Taaijke, 1990; Heck et al., 1980; Mikolon et al., 1998; Romero et al., 1995b; Thoen et al., 1979; Vanzini et al., 1998). In addition, some studies have addressed the problem of determining the conditions under which the i-ELISA could be used to detect anti-*Brucella* antibody in bulk milk. In the analysis of a large number of farms, an i-ELISA with smooth-LPS showed better sensitivity than the milk-ring test when both were applied to bulk milk (Kerkhofs et al., 1990), and the sensitivity of the assay can be improved by concentrating the antibody in the whey (Forschner et al., 1989). Poorer results have been reported by some authors possibly owing to methodological differences, but it can be concluded that when properly standardized, the i-ELISA is a suitable method for monitoring the status of herds in *Brucella*-free areas (Knosel et al., 1991).

Cell-Mediated Immunity Tests The value of the demonstration of a delayed-type hypersensitivity response for the diagnosis of animal brucellosis has been analyzed repeatedly. The test is performed by injection of a suitable protein allergen into the skin, and the demonstration of a characteristic lesion 48–72 hours later is taken as a positive result. LPS-free antigens are necessary because the *O*-polysaccharide induces an Arthus-type reaction that obscures the interpretation of the test, and the toxicity of large quantities of the LPS lipid A also induces a local reaction, which obscures (without histological examination) a true delayed-type hypersensitivity response. Most often, the antigen used is a mixture of proteins extracted with NaCl from rough *B. melitensis* 115 (brucellin or brucellergene; Bhongbhibhat et al., 1970; Jones et al., 1973), and the use of the whole cytosolic fraction or of purified proteins does not offer advantages (Blasco et al., 1994a) nor seems to improve the sensitivity (Denoel et al., 1997b). It is accepted that the skin test is less sensitive than the serological tests, but also that it may be positive in infected animals that have serologically negative responses (Nicoletti and Winter, 1990b). In a recent study per-

formed in experimentally infected cattle, a sensitivity of 78–93% was reported depending on the time lapse after infection (Saegerman et al., 1999). In a study in naturally infected sheep, the skin test showed 97% sensitivity with respect to the findings of a thorough bacteriological search, but it was also positive in many animals from which *Brucella* was not isolated (Blasco et al., 1994a). Thus, although it seems that the skin test is not reliable for detecting individually infected animals, it may be useful as a herd test. Its use does not resolve the problem of the differentiation of infected and vaccinated cattle.

Other tests that correlate with cell-mediated immunity and that have been evaluated for diagnostic purposes are the lymphocyte stimulation assays and the determination of γ-interferon (Interleukins in Brucellosis). The former tests have not been successful in distinguishing infected from vaccinated animals (Nicoletti and Winter, 1990b), and as they are expensive and technically demanding, they are not used presently. The γ-interferon assay has been evaluated preliminarily in experimentally and naturally infected heifers (Weynants et al., 1995) and reported to be more sensitive than any combination of serological tests, but its specificity has been questioned (Kittelberger et al., 1997).

Immunological Diagnosis of Infections Caused by B. ovis *and* B. canis The main differences between the tests used in the diagnosis of infections by smooth *Brucella* and *B. canis* and *B. ovis* relate to their markedly different LPS structure (Response to Environmental Stress) and outer-membrane topology. Because rough *Brucella* cells do not form stable suspensions and are prone to autoaggregate, agglutination tests produce a high proportion of false-positive results. Although this problem can be overcome in a variety of ways, the absence of the *O*-polysaccharide in the rough *Brucella* spp. comparatively reduces the importance of the antibody response to the LPS and increases that to other surface antigens. This has been clearly shown in *B. ovis* ram epididymitis where the antibody response to Omps, in particular to those of group 3 (Outer Membrane Proteins), is more intense than in the infections caused by *B. melitensis* (Riezu-Boj et al., 1990). However, neither the LPS nor the Omps by themselves are effective diagnostic antigens (Ficapal et al., 1995; Kittelberger et al., 1998; Riezu-Boj et al., 1986). This explains why serological tests for rough *Brucella* spp. are most effective when the hot-saline extract (Myers and Siniuk, 1970) is used because it is enriched in both rough LPS and group 3 Omps (Riezu-Boj et al., 1990).

A large variety of assays have been proposed for the diagnosis of *B. ovis* sheep brucellosis (reviewed by Blasco, 1990). However, only the complement fixation test, a gel immunoprecipitation assay, and some i-ELISAs have been extensively used or tested. *Brucella ovis* antigens often show anticomplementary activity, and although the effect is reduced when the hot saline or similar extracts are used, it is still significant. A sensitivity of about 90% has been estimated for the complement fixation test, similar to that found for the gel immunoprecipitation with the same antigen. The i-ELISA has been studied with respect to the antigen (purified rough LPS, hot saline extract, cytosoluble proteins) and conjugate (polyclonal of heavy and light chain IgG specificity, anti-ruminant IgG monoclonal and protein G; Ficapal et al., 1995; Marń et al., 1989b, 1998; Vigliocco et al., 1997). In addition to the demonstration that hot saline extract is the best antigen in the i-ELISA, these studies have shown that the monoclonal or protein-G based conjugates reduce the background reactivity observed with the sera of healthy animals. The sensitivity of the i-ELISA has been found to be superior (Cho and Niilo, 1987; Spencer and Burgess, 1984) or equal to that of the gel immunoprecipitation or complement fixation, and there are no objective reasons to use these methods instead of the classical tests. However, since hot saline extracts are obtained in low yields and the i-ELISA uses much less antigen, it could be more practical. *Brucella melitensis* Rev. 1 is used to vaccinate sheep against both *B. melitensis* and *B. ovis* (Vaccines and Vaccination), and it elicits a strong antibody response to the *O*-polysaccharide, interfering in the smooth LPS tests. Moreover, it also elicits antibodies to the core and lipid A sections of the LPS, and these antibodies interfere in the serological tests for the diagnosis of *B. ovis*, owing to the shared epitopes of the inner LPS sections of *B. melitensis* and *B. ovis* (The Lipooligosaccharide of Rough *Brucella* Species). This interference is reduced in the case of the complement fixation and gel immunoprecipitation tests (Blasco, 1990) and more importantly in the i-ELISA, and this is probably due to the favorable exposure of the common epitopes after adsorption to polystyrene (Marń et al., 1998).

The immunological diagnosis of *B. canis* infections has been less investigated, and existing tests may provide a large proportion of false-negative and false-positive results. In experimentally infected dogs, blood cultures were positive several weeks before a positive result was obtained in any of the agglutination and gel immunoprecipitation tests available (Carmichael et al., 1984). False-positive results relate to the nature of the antigens, since the preparation of bacterial suspensions efficient in agglutination tests is even more difficult than in the case of *B. ovis*.

Brucella canis almost always produces mucoid colonies on standard media and bacteria strongly autoaggregate at pH below neutrality, a problem also existing with the hot saline extracts obtained from mucoid bacteria (Carmichael, 1990). The problem is partially solved using growth media and buffers at pH 7.4, and *B. ovis* and a nonmucoid *B. canis* variant have been used in an attempt to develop suitable agglutination tests (Carmichael, 1990). Moreover, it has been shown that a brief treatment with β-mercaptoethanol reduces the number of false-positive results. However, even under the best conditions, the demonstration of a specific immunological agglutination using *B. ovis* and *B. canis* cell suspensions is not obvious and requires some training (Carmichael and Shin, 1996). Agglutination can be examined in tubes by the serum dilution method or in a rapid slide test, and the interpretation has to take into account the possibility (25–50%) of a false-positive result. On the other hand, the test is accurate in identifying noninfected dogs when a negative result persists after several bleedings.

As an alternative to agglutination tests, gel immunoprecipitation has been investigated. When used in this assay, hot saline or sodium deoxycholate extracts suffer from the same problems of nonspecific reactions as do agglutination tests. On the other hand, cytoplasmic protein antigens do not produce false~~ne~~gative results and the method is considered to be highly accurate in detecting antibodies. Antibodies to proteins develop late after infection, and the sensitivity is low during the first months. However, these antibodies persist in chronically infected dogs at times when the other serological tests may have become negative (Carmichael et al., 1984).

Immunological Diagnosis of Brucellosis and Cross-Reacting Bacteria Smooth-LPS tests are susceptible to yield false-positive results when infections by Gram-negative bacteria with perosamine-containing *O*-polysaccharides occur (Table 6). But for those due to *Y. enterocolitica* O:9, crossreactivities are mild, and none is a significant problem in the diagnosis of human brucellosis when the clinical symptoms and the results of the anamnesis are considered. Indeed, in addition to some of the serological tests discussed in the following paragraphs, blood cultures are 100% specific. On the other hand, *Y. enterocolitica* O:9 infections in cattle can be troublesome, particularly in countries free from *B. abortus*, since the sporadic appearance of positive serological results among unvaccinated populations calls for an immediate differential diagnosis. Although orally acquired *Y. enterocolitica* O:9 seldom induces high levels of antibodies to smooth *Brucella* LPS and the responses are transient in cattle (Garin-Bastuji et al., 1999; Kittelberger et al., 1997; Mittal et al., 1985), titers in blood serum and milk may be higher and persistent when mammary infections occur (Mittal et al., 1985). The problem can also be a significant one in swine (Wrathall et al., 1993). So far, three different approaches have been followed to develop differential tests.

The demonstration of antibodies specific for the enterobacterial common antigen (Mittal et al., 1980b), *Y. enterocolitica* flagellar antigens (Mittal and Tizard, 1979) and *Yersinia* outer proteins (yops; Kittelberger et al., 1995b; Weynants et al., 1996a; Weynants et al., 1996b; Yersinia) has been proposed to identify with precision those cases suspected of *Y. enterocolitica* O:9 infection. However, antibodies to antigens shared by enteric bacteria are not uncommon in healthy cattle and titers of anti-flagella antibody are weak possibly because *Y. enterocolitica* is nonmotile at the host body temperature. Moreover, dual infections by *Yersinia* and *Brucella* do not seem uncommon in livestock, and this reduces the usefulness of the yops (Kittelberger et al., 1995b; Mittal and Tizard, 1980a). On the other hand, yops have been found clearly useful for the differential diagnosis in humans (Schoerner et al., 1990).

An alternative approach is based on the small differences existing between the LPS of *Y. enterocolitica* O:9 and smooth *Brucella* spp. at *O*-polysaccharide (Response to Environmental Stress) and core oligosaccharide levels. Early reports suggest that whereas the antigenic determinants involved in the crossreaction are resistant to periodate oxidation and borohydride reduction, the determinants specific for *Y. enterocolitica* O:9 LPS are sensitive and on this basis an i-ELISA with both treated and untreated LPS was proposed for the differential diagnosis of human infections (Lindberg et al., 1982). The differences at LPS level were also noted in competitive immunoassays (Granfors et al., 1981). Based on the results of experimental infections in cattle, the c-ELISA has been proposed as a specific diagnostic test that is able to discriminate antibody elicited by *Y. enterocolitica* O:9 (Nielsen, 1990a). A c-ELISA with a monoclonal antibody to the C/B epitope (shared by smooth *Brucella* spp. and absent from *Y. enterocolitica* O:9) has been tested with field bovine sera and found to improve the specificity of the rose bengal and complement fixation up to 70% (Weynants et al., 1996b; Weynants et al., 1996a).

Immunological crossreactivities with proteins of taxonomically unrelated bacteria seem limited to some highly conserved proteins, such as heat shock protein-65 (Dáz and Bosseray, 1974; Spencer et al., 1994). On the other hand, they

are extensive with the proteins of taxonomically related bacteria, but although they can be shown with sera from naturally infected hosts (Drancourt et al., 1997; Velasco et al., 1997), they do not seem to represent a relevant source of nonspecificity (Velasco et al., 1997) and can be easily discriminated by using smooth LPS tests. Thus, *Brucella* protein antigens are perhaps the most useful antigens for the differential diagnosis of brucellosis and yersiniosis. Although they have been used in some antibody (Baldi et al., 1996) and lymphocyte blastogenesis tests (Chukwu, 1987), the skin test with proteins saline extracted from rough *B. melitensis* 115 (brucellin or brucellergene; Bhongbhibhat et al., 1970; Jones et al., 1973) is very useful and relatively simple to implement (Chukwu, 1987; Saegerman et al., 1999). There are contradictory reports on the value of the γ-interferon in the differential diagnosis of brucellosis and yersiniosis in cattle (Kittelberger et al., 1997; Weynants et al., 1995).

Treatment

The ability of *Brucella* organisms to replicate within cell vacuoles is a determining factor in the chronic course of the disease and in the tendency to relapse. Within the intracellular niche, *Brucella* organisms are protected from a large number of antibiotics that cannot reach the intracellular milieu. Therefore, an appropriate therapy must use antibiotics capable of killing both extracellular and intracellular *Brucella* organisms (Ariza, 1999). Unfortunately, not a single antibiotic therapy achieves complete eradication of intracellular infection. In this respect, synergistic or additive effect by the use of two or more antibiotics for prolonged periods is required to cure and to reduce the frequency of relapses (Fig. 40). The possibility of enhancing the effectiveness of antibiotics showing good in vitro activity by including them into liposomes has been studied by several authors (Dees and Schultz, 1990; Vitas et al., 1997), but attempts to use some of these formulations in animals have been discouraging (Nicoletti et al., 1989). The development of antibiotic resistance by *Brucella* has never been demonstrated. For instance, the susceptibility of strains from human patients with relapses remains identical to that of strains isolated during the initial episode (Ariza et al., 1986); similarly, *Brucella* strains isolated from animals that did not clear the infection after the first antibiotic therapy show the same antibiotic susceptibility as that of the first isolates (Guerra and Nicoletti, 1986). The absence of plasmids (Genome Evolution) should be an important factor explaining, at least in part, the observation that the in vitro susceptibility of *Brucella* strains to antibiotics remains stable over time (Bosch et al., 1986).

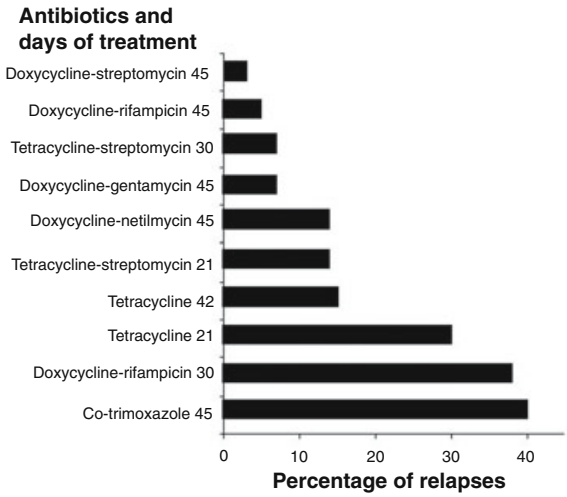


Fig. 40. Relapse rate after antibiotic therapy of human brucellosis. Patients, naturally infected with *Brucella* organisms, were treated with a combination of antibiotics for different periods (indicated on the vertical axis). The period between the end of treatment and relapse and the percentage of relapses were registered, accordingly. Bars indicate average values. Standard error was less than 10% in all cases, except in doxycycline plus rifampicin treatment for 45 days in which standard error was close to 30%. Dr. Javier Ariza, from the Infectious Disease Service, Hospital De Bellvitge, Barcelona, Spain, supplied this information.

Human Brucellosis The antibiotic treatment of human brucellosis has been recently reviewed (Ariza, 1999), and only the most significant facts are summarized here. Since there is no antibiotic therapy effective in the totality of human cases, a careful follow-up of the patients is important.

It is an established fact that tetracyclines are the most effective antibiotics in the treatment of brucellosis but also that by themselves they are not satisfactory and result in high rates (from 15–30%, depending on the duration of the treatment) of relapses. Monotherapy with other antibiotics, such as aminoglycosides, rifampicin, azithromycin, co-trimoxazol, or second generation fluoroquinolones has produced unsatisfactory results in laboratory models, human patients or both. Presently, a prolonged tetracycline-aminoglycoside combination (doxycycline 100 mg twice/day for 45 days and streptomycin 1 g/day [or 750 mg in patients older than 50 years] for 14 days) is the best treatment of the disease as judged by the comparatively low (3–5%) rates of relapse. Although aminoglycosides have low intracellular penetration, their synergy with tetracyclines is well established,

and in most cases, their effectiveness in this combination offsets the problems (parenteral administration and ototoxicity). Some aminoglycosides such as gentamycin have better in vitro activity against *Brucella* and show lower toxicity, and they are currently recommended as streptomycin substitutes. Because of its better tolerance, doxycycline is the tetracycline used. It has to be emphasized that the duration of the treatment with both antibiotics is essential to obtain the best results.

As an alternative to aminoglycosides, rifampicin can be used (900 mg in a single morning dose) along with doxycycline, both administered during 45 days. Although this treatment has no side effects and is better tolerated by the patient, its effectiveness is less than that of the doxycycline-aminoglycoside combination (the rate of relapses can be as high as 15%). It is, however, the therapy of choice when infections by *B. melitensis* Rev. 1 (Vaccines and Vaccination) occur or are suspected (Blasco and Díaz, 1993a). This sort of infection occurs most often in veterinarians and farmers, and there are also reports of accidental inhalation in vaccine-producing plants. In these cases, it is recommended that bacterial cultures be performed and the isolate typified or at least tested for streptomycin resistance.

Brucellosis in pregnant women and in children less than seven years old requires a different therapy because of the side effects of the antibiotics of the standard treatment. There is no established optimal therapy, but rifampicin for 8 weeks in the first case and rifampicin for 4–6 weeks plus gentamycin for the first 7–10 days in children are recommended. Recent reports suggest that gentamycin is less effective than streptomycin in children (Issa and Jamal, 2000). *Brucella* endocarditis is a dangerous form of the disease that also calls for a special treatment to ensure clearance of the infection. Doxycycline plus gentamycin, both for 3 weeks, plus rifampicin for at least 8 weeks is recommended.

Accidental inoculation or exposure to virulent *Brucella* in the diagnostic and research laboratories calls for a prophylactic antibiotic therapy. For this purpose, doxycycline alone is effective provided the treatment starts immediately. Although the duration of this treatment has not been determined, it is generally considered that the standard dose (doxycycline 100 mg twice/day) for 7–10 days is effective in preventing the development of the disease.

Animal Brucellosis Antibiotics have been rarely employed in animal brucellosis but they have been applied in some cases to treat animals of high genetic value or to prevent spreading of the disease in small dairy herds of highly valuable animals.

In cattle, monotherapy with either oxytetracycline or free or liposomal streptomycin (Nicoletti et al., 1985; Nicoletti et al., 1989) is very unsatisfactory. The combination of long-acting oxytetracycline (for example, 20 mg/kg administered intramuscularly every 3 days for 21 days) and streptomycin or dihydostreptomycin (for example 20 mg/kg administered intramuscularly daily for 10 days) is successful in eliminating milk shedding of *B. abortus* in 55–71% of treated animals (Jiménez de Bagüés et al., 1991; Milward et al., 1984; Nicoletti et al., 1985; Nicoletti et al., 1989). A more prolonged treatment (14 oxytetracycline and 8 streptomycin doses) accompanied by intramammary infusion with oxytetracycline for 4 days is reported to have a 100% rate of success (Radwan et al., 1993). In the noncured animals, the oxytetracycline and streptomycin therapy reduces the number of bacteria in milk or in tissues after necropsy (Jiménez de Bagüés et al., 1991; Milward et al., 1984; Nicoletti et al., 1985; Nicoletti et al., 1989) and when combined with adult vaccination with *B. abortus* 19, allows for control of the disease without eliminating the infected animals (Jiménez de Bagüés et al., 1991).

Antibiotic therapy also has been used in infections caused by *B. ovis* in rams. Like in human or cattle brucellosis, streptomycin or tetracyclines by themselves are not successful (Kuppuswamy, 1954; Marín et al., 1989a). On the other hand, combinations of aureomycin plus streptomycin (Kuppuswamy, 1954) or tetracyclines plus streptomycin have produced satisfactory results (Giauffret and Sanchis, 1974; Marín et al., 1989a). However, successful antibiotic therapy does not result in a clearance of the lesions characteristic of this disease (Marín et al., 1989a), and semen quality after treatment is reported to be poor (Kuppuswamy, 1954). Brucellosis in horses is quite severe and prolonged treatments are required. A mixture of oxytetracycline (20 mg/kg) and streptomycin (15 mg/kg) for at least 21 days is recommended.

Applications

Similarly to other intracellular parasites such as *Mycobacterium* species, *Brucella* organisms and their products have been used as adjuvants, antitumoral agents, antiviral components and immunological tools as well as vectors for delivering foreign antigens. In addition, most of the *Brucella* species are primary pathogens capable of infecting healthy hosts and in consequence candidates to be used as biological weapons.

Brucella Vaccines as Vectors for Delivering Foreign Antigens

Live bacterial vaccines are scarce and rather exceptional among useful vaccines. *Mycobacterium* BCG and attenuated *Brucella* vaccines developed more than 70 years ago are among the accepted vaccines currently in use. *Brucella* vaccines, such as *B. abortus* strain 19, *B. melitensis* Rev. 1 and *B. abortus* RB51, are the best known vaccines used in many countries for the control and eradication of bovine, caprine and ovine brucellosis. In contrast to the attenuated mycobacterial vaccine, the efficacy of which in open populations is controversial, *Brucella* vaccines have been efficiently used for many years (Vaccines and Vaccination). This recognized status makes *Brucella* vaccines good vector candidates for the expression and delivery of foreign antigens (Comerci et al., 1998; Vemulapalli et al., 2000). *Brucella abortus* strain 19 recombinant vaccine expressing a reporter repetitive protein of *Trypanosoma cruzi* induces good antibody responses against the alien protein in mice. In vitro and in vivo expression of this repetitive *Trypanosoma* antigen does not alter the *Brucella* growth pattern, nor does it generate a toxic lethal effect in the bacterium. Similarly, rough *B. abortus* RB51 vaccine has been used to express *Mycobacterium* heat shock protein 65. The foreign mycobacterial protein expressed in this vector vaccine induces a typical Th1-type immune response in mice, as indicated by the presence of IgG_2 and INFγ, without the production of IgG_1 or the secretion of IL^{-4}. The immunogenic properties and growth conditions of this recombinant vector *B. abortus* RB51 remain without change as compared to the parental strain.

The rationale for using *Brucella* vaccines as vectors for delivering foreign antigens is based on the assumption that these attenuated intracellular bacteria could successfully deliver antigens inside antigen-presenting cells (Splitter and Everlith, 1986; Splitter and Everlith, 1989). Since attenuated *Brucella* organisms follow the endocytic pathway (Escaping from the Endocytic Pathway), the prediction is that antigens delivered by this manner will follow the appropriate antigen processing route necessary for eliciting an efficient and protective immune response against foreign antigens. An important prediction would be that the expression of foreign antigens does not exclude the immune response to *Brucella*. In this respect, a double purpose vaccine would be developed.

Antitumoral and Antiviral Activity of *Brucella* Cells and Their Fractions

Live and killed bacteria and subcellular *Brucella* fractions have been used experimentally as antitumoral reagents in mice and humans (Chirigos et al., 1978; Dazord et al., 1980; Dazord et al., 1984; Schultz et al., 1978). Trials have demonstrated that live *Brucella* vaccines, killed bacterins or insoluble cellular extracts (mainly containing peptidoglycan, outer-membrane fractions and ribosomes) are capable of inducing the reduction of tumoral masses without causing unfavorable reactions. Although insoluble *Brucella* extracts exert antitumoral activity, better results are achieved when irradiated tumor cells and *Brucella* extracts are administered at the same time. The antitumoral activity mediated by *Brucella* vaccines can be understood within the context of the Mackaness effect (T Cell Responses), in which transitional acquired cellular resistance against invaders (e.g., cancer cells) could be nonspecifically induced by *Brucella* organisms or other intracellular parasites, such as *Mycobacterium*.

The logic behind the use of *Brucella*-human immunodeficiency virus (HIV) protein conjugates as vaccines is based on the property that *Brucella* bacterins have to behave as T-independent antigens (Antibody Response), which is a strategy to bypass the requirement for CD_4^+ cells in the antibody response of AIDS patients (Golding et al., 1995; Lapham et al., 1996). Killed *Brucella* bacterins conjugated with gp120 or V3 loop peptide derived from HIV induce neutralizing antibodies against HIV. *Brucella*-HIV protein conjugates mainly generate IgG_{2a} primary and secondary responses in mice, even after CD_4^+ depletion. This type of response also has been observed with other foreign "academic" antigens conjugated to *Brucella*, such as trinitrophenyl, indicating that it is a general behavior of *Brucella* bacterins (Sacks and Kilnman, 1997). Sera from mice immunized with *Brucella*-gp120 display more potent anti-HIV activity than sera from mice immunized with the *Brucella*-V3 loop peptide. In addition, killed *B. abortus* stimulates Th1-type immunity with the production of INFγ and generation of virus-specific cytotoxic T-lymphocyte response in both normal and CD_4^+-depleted mice. In addition to the T-independent properties of *Brucella* bacterin (Antibody Response), the antibody and T cell cytotoxic responses are linked to the adjuvant effect displayed by *Brucella* cells (*Brucella* Bacterins and Envelope Molecules as Adjuvants). *Brucella* also promotes the secretion of other cytokines, such as IL-12, a molecule that acts synergistically with IL-2 to induce Th1 activation and differentiation of cytotoxic cells (Huang et al., 1999). Of course, the low endotoxic activity displayed by *Brucella* LPS (Moreno et al., 1981; Rasool, 1992) is a factor that may favor the use of *Brucella* bacterin-conjugates as adjuvants.

Brucella Bacterins and Envelope Molecules as Adjuvants

In contrast to enterobacterial LPS, *Brucella* LPS behaves as a T-independent type 1 carrier that stimulates the production of high levels of IgG_2 and moderate levels of IgM in the endotoxin-resistant C3H/HeJ mice strain (Moreno and Berman, 1979; Moreno et al., 1984b; Kurtz and Berman, 1986). The primary and even the stronger secondary responses induced by the *Brucella* LPS adjuvant activity could occur without the assistance of T cells. By taking advantage of these properties, together with the low endotoxicity displayed by *Brucella* LPS (Moreno et al., 1981; Rasool, 1992), this molecule has been used as an adjuvant in foot-and-mouth disease, for enhancing the immune response against the virus (Berinstein et al., 1993; Betts et al., 1993), and to potentiate antibody production against snake venom toxins (Rucavado et al., 1996) and has been proposed as an adjuvant for HIV vaccines (Goldstein et al., 1992).

Murein-rich insoluble fractions have been experimentally used as adjuvant for generating antibodies against a variety of antigens, with variable success (Galdiero, 1995; Serre, 1982). It has been observed that these fractions induce quantitative and qualitative changes in the T and B lymphocyte population in mice, mainly with an initial increase of CD_4^+ and B cells, followed by an increase of CD_8^+ lymphocytes and a concomitant decrease of CD_4^+ and B cells.

Brucella Bacterins and Lipopolysaccharide as Immunological Tools

Brucella cells bind to human B cells and to certain leukemic cells of B origin, forming rosettes (Lee et al., 1983; Teodorescu et al., 1977). This quality has been used for diagnostic purposes in the differentiation of leukemic cells. The *Brucella* B cell receptor has been associated with the LPS, since the M serotype is more prone than the A serotype to bind to B lymphocytes (Fig. 15; O-polysaccharide). *Brucella* LPS and trinitrophenylated-*Brucella* bacterin have been extensively used as T-independent antigens in immunological studies (Betts et al., 1993; Moreno et al., 1984b; Kurtz et al., 1986; Rennick et al., 1983; Tenay and Strober, 1985). Moreover, trinitrophenylated-*Brucella* bacterin has been regarded as one of the classical T-independent type-1 antigens and used as an academic model for demonstrating this type of antibody response dependent on B lymphocytes.

Brucella as a Biological Weapon

Brucella species, particularly *B. melitensis* and *B. suis*, are potential agents of biological warfare. There are several properties that make these bacteria good candidates as biological weapons (Anonymous, 2000; Kaufmann et al., 1997). The main one is that members of the genus are primary pathogens inducing a debilitating and incapacitating severe disease that requires expensive and long-term treatment. It has been estimated that less than five bacteria of *B. melitensis* 16M are necessary to infect one human being. Alertness to suspected cases of biological terrorism with *B. melitensis* and investigations leading to integrated clinical, public health, and law enforcement against biological terrorism with *Brucella* strains have taken place in the United States. A model that compares the impact of *Brucella* melitensis released as aerosols in suburbs of a major city has been developed in this country. The model demonstrated that the economic impact of a bioterrorist attack in a populated area with *B. melitensis* could be close to $500 million per 100,000 persons. The model takes into account preventive measures, including rapid implementation of a post-attack prophylaxis program.

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Prokaryotes (2006) 5:457–466
DOI: 10.1007/0-387-30745-1_18

CHAPTER 3.1.17

Introduction to the Rickettsiales and Other Intracellular Prokaryotes

DAVID N. FREDRICKS

Introduction

In the past, any small bacterium that required an intracellular environment for growth could be classified as belonging to the order Rickettsiales (Moulder, 1974). It is now clear that bacteria from diverse phylogenetic groups have adopted the intracellular niche, an example of convergent evolution. Most eukaryotic cells have defenses that prevent prokaryotes from invading and establishing a parasitic existence. Once these defenses are breached, however, an intracellular existence has many advantages for the suitably adapted bacterium. Within a host cell, bacteria are free from competition with most other microbes and benefit from host defenses directed against external threats. Intracellular bacteria may have access to host substrates and enzymes for metabolism. Host behaviors, such as blood feeding in insects or locomotion in vertebrate hosts, may aid the spread of intracellular bacteria. Therefore, it should not be surprising that bacteria from different phylogenetic groups have evolved to take advantage of intracellular life in eukaryotic cells.

In this chapter, bacteria are organized into groups based primarily on phylogenetic inferences made from analysis of their 16S rRNA sequences (Fig. 1). Although criticized in some quarters (Pennisi, 1998), the use of 16S rRNA genes for inferring evolutionary relationships among bacteria has proven very reliable, though small subunit rRNA phylogeny may not resolve evolutionary relationships among organisms in the deepest branches of the tree of life (Woese, 2000). There is the potential for misclassification of organisms when one relies on a single gene for phylogenetic analysis. Gene insertions, deletions and transfers between organisms are possible (Nelson et al., 1999; Yap et al., 1999). However, the phylogenetic relationships suggested by analysis of 16S rRNA genes tend to be supported by phylogenetic analysis of other genes (Pace, 1997).

Bacteria that form a monophyletic group with the classical rickettsiae based on phylogenetic analysis of 16S rRNA gene sequences are listed here as belonging to the Rickettsiales. Other intracellular bacteria that have been classified traditionally as belonging to the Rickettsiales but do not cluster with this group of bacteria based on 16S rRNA analysis are listed under the Rickettsia-like prokaryotes. Figure 2 is a detailed phylogenetic tree of the bacteria described in this chapter, based on analysis of 16S rRNA. The role bacteria may play as endosymbionts of eukaryotic cells is illustrated with some examples, and the endosymbiotic hypothesis for the origin of mitochondria is discussed.

The Rickettsiales

The Rickettsiales can be organized into five major genogroups based on 16S rRNA phylogeny. These groups consist of the Rickettsiae, Ehrlichiae, Neorickettsiae, Wolbachiae and Holosporae. These bacteria form a monophyletic group within the α-proteobacteria. The traditional classification scheme for the Rickettsiales is listed in Table 1, as described in *Bergey's Manual of Systematic Bacteriology* (Weiss and Moulder, 1984). This chapter does not use the traditional classification scheme of families and tribes listed in Table 1 because the molecular phylogeny of these bacteria is at odds with that organizational structure.

The Rickettsia Group

The rickettsiae are small (0.3–0.5 × 0.8–2.0 μm), Gram-negative, aerobic, coccobacillary organisms that are obligate intracellular parasites (Saah, 2000a). They reside free in the cytoplasm or nucleus of eukaryotic cells where they divide by binary fission and metabolize glutamate via the citric acid cycle (Weiss and Moulder, 1984). They have cell walls but no flagella. The rickettsia have been propagated in the laboratory by cocultivation with eukaryotic cells, but have not been propagated using axenic media. These bacteria frequently have a close relationship with arthropod vectors that may transmit the

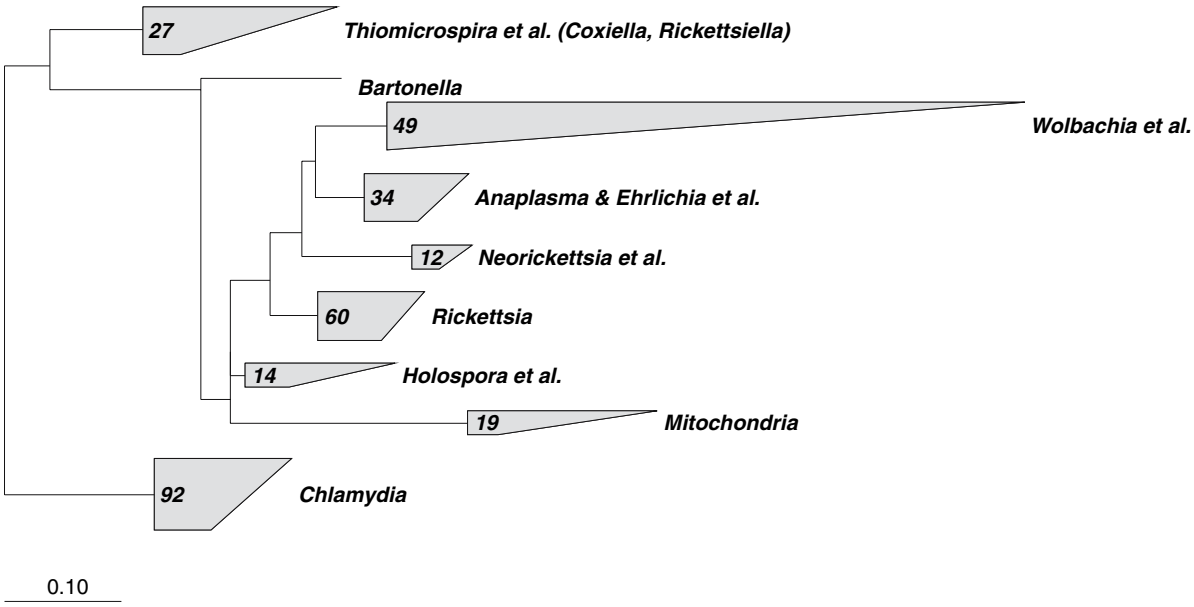

Fig. 1. Phylogentic tree containing the Rickettsiales and related intracellular bacteria as inferred from 16S rRNA sequence data. This tree shows the evolutionary relationships of the major bacterial groups to each other (http://www.arb-home.de, Technical University, Munich, Germany).

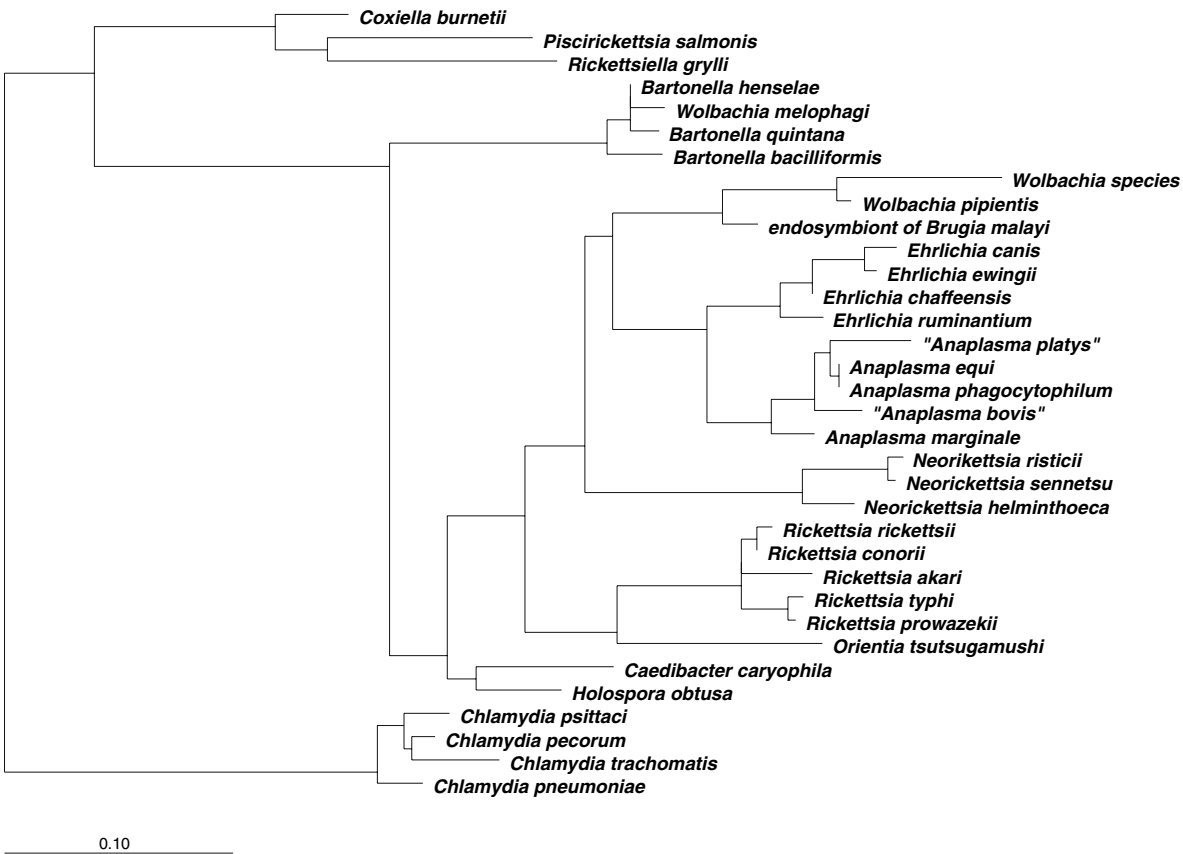

Fig. 2. A detailed phylogentic tree of selected intracellular bacteria as inferred from 16S rRNA sequence data. The bar represents 0.1 nucleotide substitutions per position and is a measure of evolutionary distance (http://www.arb-home.de, Technical University, Munich, Germany).

Table 1. The traditional classification scheme for the order Rickettsiales as described in *Bergey's Manual*.

Order	Family	Tribe	Genera
Rickettsiales	Rickettsiaceae	Rickettsieae	*Rickettsia*
			Rochalimaea
			Coxiella
		Ehrlicheae	*Ehrlichia*
			Cowdria
			Neorickettsia
		Wolbachieae	*Wolbachia*
			Rickettsiella
	Bartonellaceae		*Bartonella*
			Grahamella
	Anaplasmataceae		*Anaplasma*
			Aegyptianella
			Haemobartonella
			Eperythrozoon

organism to mammalian hosts. The rickettsiae have very small genomes of about 1.0–1.5 million bases. The true rickettsiae can be subdivided into three major groups (spotted fever, typhus and scrub typhus groups) based on clinical characteristics of disease and phylogenetic relationships (Weisburg et al., 1989; Tamura et al., 1991).

Spotted Fever Group *Rickettsia rickettsii* is the cause of Rocky Mountain spotted fever (RMSF) and the prototypical bacterium in the spotted fever group of rickettsiae (McDade and Newhouse, 1986). This bacterium is found in the Americas and is transmitted to humans through the bite of infected ticks. *Rickettsia rickettsii* infects human vascular endothelial cells, producing a vasculitis. The possibility of RMSF should be considered in patients from endemic areas presenting with fever, headache, and a rash, particularly during the summer and late spring. The rash tends to start on wrists and ankles and spreads centripetally (Walker and Raoult, 2000b).

Other spotted fever group rickettsiae that produce human rickettsioses include *R. conorii*, *R. mongolotimonae* and *R. slovaca* (bouteonneuse fever and similar illnesses), *R. akari* (rickettsial pox), *R. japonica* (Japanese spotted fever), *R. sibirica* (North Asian tick typhus), *R. africae* (African tick bite fever), *R. helvetica* (perimyocarditis), *R. australis* (Queensland tick typhus) and *R. honei* (Flinders Island spotted fever; Raoult et al., 1997; Fournier et al., 2000a; Fournier et al., 2000b; Nilsson et al., 1999). The spotted fever rickettsiae have been found on every continent except Antarctica. Other spotted fever group rickettsiae, including *R. montana*, *R. rhipicephali*, *R. parkeri*, *R. massiliae* and *R. aeschlimannii*, have been detected in ticks, but have not been clearly linked to human disease (Walker and Raoult, 2000b).

Typhus Group *Rickettsia prowazekii* is the cause of epidemic or louse-borne typhus and is the prototypical bacterium from the typhus group of rickettsiae (Saah, 2000c). Like other members of the rickettsiae, *R. prowazekii* infects human vascular endothelial cells, producing widespread vasculitis. In contrast to RMSF, louse-borne typhus tends to occur in the winter, with the rash starting in the axillary folds and spreading centrifugally. Infection usually is transmitted from person to person by the body louse and, therefore, tends to manifest under conditions of crowding and poor hygiene. The southern flying squirrel appears to be a reservoir in the United States, but the vector involved in transmission from the flying squirrel to humans is currently unknown. The disease has a worldwide distribution. Latent human infection may occur with subsequent reactivation disease (Brill-Zinsser disease).

Other rickettsiae in the typhus group include *R. typhi* and *R. felis*. Murine typhus is caused by transmission of *R. typhi* from rats, cats and opossums to humans via a flea vector (Dumler and Walker, 2000). Murine typhus is found worldwide and is endemic to areas of Texas and southern California in the United States. *Rickettsia typhi* infects the gut epithelium and sometimes the reproductive tissues of the flea vector. Murine typhus manifests as a nonspecific febrile illness; rash occurs in only about 50% of patients, usually as a late finding. Although *R. felis* is phylogenetically more closely related to the spotted fever group of rickettsiae than to the typhus group, it shares antigens with *R. typhi* and produces a murine typhus-like illness. *Rickettsia felis* has been detected in cat fleas and opossums (Higgins et al., 1996).

Scrub Typhus Group *Orientia tsutsugamushi* is the cause of scrub typhus. Originally called *Rick-*

ettsia tsutsugamushi, this organism was given its own genus designation because it is phylogenetically distinct from the other rickettsiae, though closely related (Tamura et al., 1995). This bacterium has a thicker outer leaflet to its cell wall and a minimal outer slime layer, in contrast to the situation with the other genogroups of rickettsia (Weiss and Moulder, 1984). *Orientia tsutsugamushi* is transmitted to humans by the bite of trombiculid mites (chiggers), which are the vector and host (Saah, 2000b). Scrub typhus occurs throughout much of Asia and Australia. The clinical presentation of scrub typhus is a nonspecific febrile illness with headache and myalgias. An inoculation eschar and/or lymphadenopathy are helpful clues in its diagnosis.

The Ehrlichia Group

The ehrlichia are small, Gram-negative, pleomorphic bacteria. They can be divided into several groups based on 16S rRNA phylogeny, ultrastructural features of infected cells, and pathological features of disease (Walker and Dumler, 2000a). The bacteria *Ehrlichia risticii* and *Ehrlichia sennetsu* have been designated as ehrlichiae, but it is clear from the phylogeny of these organisms that they form a distinct group with *Neorickettsia helminthoeca* and belong in a different but related genus (Pretzman et al., 1995; Popov et al., 1998). In this chapter, *Ehrlichia risticii* and *Ehrlichia sennetsu* are classified as neorickettsiae (next section) while awaiting further taxonomic designation. The ehrlichiae are obligate intracellular bacteria. Like chlamydiae, they exist in membrane-bound vacuoles within the cytoplasm of eukaryotic cells. Bacteria are only 0.5–1.0 μm in diameter, but can be visualized by light microscopy when they form groups of bacterial cells within vacuoles, called "morulae." The ultrastructure of morulae formed by each genogroup of ehrlichia is distinct (Popov et al., 1998).

THE *E. CHAFFEENSIS-E. CANIS* GROUP The true ehrlichiae form two distinct phylogenetic clusters or genogroups of organisms. One cluster includes the agent of human monocytic ehrlichiosis (HME), *E. chaffeensis*, as well as *E. canis*, *E. ewingii* and *E. muris*. Widely found in the United States, HME is a vector-borne zoonosis transmitted by ticks of the Amblyomma and Dermacentor genus. Dogs and deer appear to be natural reservoirs of infection. *Ehrlichia chaffeensis* parasitizes circulating and tissue-bound macrophages. Unlike rickettsiae, ehrlichiae do not infect human endothelial cells or produce a vasculitis. The clinical presentation of HME is similar to RMSF. However, HME differs from RMSF in that a rash is found in less than half of HME cases, leukopenia may be present, and intracytoplasmic morulae occasionally may be detected on peripheral blood smear. Patients with HME may have multiple abnormalities evident on blood chemistry and hematology tests (Walker and Dumler, 2000a).

Ehrlichia canis and *E. ewingii* are closely related to *E. Chaffeensis*. Both bacteria have been reported to cause disease in humans but are primarily canine pathogens (Buller et al., 1999). *Ehrlichia canis* infects macrophages in dogs, whereas *E. ewingii* infects granulocytes in dogs. *Ehrlichia muris* is another ehrlichia that clusters phylogenetically with this group, and it has been found in Japanese mice (Kawahara et al., 1993). Ticks transmit all of these bacteria.

Cowdria ruminantium is an obligate intracellular bacterium that is the cause of heartwater disease, an illness of African and Caribbean ruminants. This bacterium is found within vacuoles in the cytoplasm of host endothelial cells (Moulder, 1974). Ticks of the Amblyomma genus transmit *C. ruminantium*. Phylogenetically, *C. ruminantium* falls within the *E. Chaffeensis-E. Canis* group of ehrlichiae and probably should be renamed *Ehrlichia ruminantium* (Pretzman et al., 1995).

THE *E. PHAGOCYTOPHILA-E. EQUI* GROUP Another genogroup of the true ehrlichiae includes the agent of human granulocytic ehrlichiosis (HGE), which appears similar or identical to *E. equi* and *E. phagocytophila* (Walker and Dumler, 2000a). The agent of HGE is transmitted to humans by ticks of the Ixodes genus and has been described in the United States and Europe. Small mammals, such as the white-footed mouse, appear to be major reservoirs of this bacterium. *Ehrlichia equi* causes equine granulocytic ehrlichiosis in horses in the United States (Bullock et al., 2000). The clinical presentation of HGE is again similar to that of RMSF, except less than 10% of patients have a rash. As with HME, patients with HGE may have laboratory test abnormalities including thrombocytopenia, leukopenia and elevated serum levels of hepatic enzymes. Patients with HGE frequently have visible morulae in circulating granulocytes, whereas morulae are rarely detected in circulating monocytes of patients with HME.

The phylogenetic cluster related to *E. phagocytophila* includes *E. platys* and *E. bovis*. *Ehrlichia platys* infects dogs and targets platelets and macrophages (Woody and Hoskins, 1991). The vector is unknown. *Ehrlichia bovis* infects cattle and buffalo.

Anaplasma marginale is an obligate intracellular bacterium that infects cattle (Moulder, 1974). Anaplasmosis is found worldwide and is transmitted by ticks such as *Dermacentor andersoni*.

The bacterium occupies vacuoles within erythrocytes. *Anaplasma marginale* clusters phylogenetically with the *E. phagocytophila-E. equi* group of ehrlichiae, though it occupies a deep branch in this cluster (Drancourt and Raoult, 1994). Based on 16S rRNA phylogeny, *A. marginale* should be considered an ehrlichia.

The Neorickettsia Group

Although related to the ehrlichiae and the rickettsiae, the neorickettsiae form a distinct phylogenetic cluster within the Rickettsiales. *Ehrlichia sennetsu* and *E. risticii* are phylogenetically very closely related organisms that do not group with the true ehrlichiae (Pretzman et al., 1995). These bacteria are closely related to *Neorickettsia helminthoeca* (Rikihisa, 1991). Unlike the ehrlichiae that are transmitted by tick vectors, the neorickettsiae are associated with trematode worm vectors.

Neorickettsia helminthoeca is a small, pleomorphic, intracellular bacterium that causes salmon poisoning disease in canids such as coyotes and dogs (Foreyt et al., 1987). *Neorickettsia helminthoeca* infects cells of the canine reticuloendothelial system. A fluke vector (*Nonophyetus salmincola*) that infests salmonid fish transmits the disease. Dogs acquire the bacterium by eating fish containing infected flukes.

Ehrlichia risticii is the cause of Potomac horse fever (PHF), a febrile colitis of horses found in North America and Europe (Palmer, 1993). It has also been associated with equine abortions. Also, PHF has been transmitted to horses by inoculation with infected trematode worms harvested from freshwater snails (Barlough et al., 1998). The natural mode of acquisition is not known, but horses may become infected by drinking or standing in trematode-infested water or by ingesting aquatic insects containing infected metacercariae. *Ehrlichia risticii* has been propagated in mice. There is considerable 16S rRNA sequence diversity amongst the bacteria classified as *E. risticii*, suggesting that there are several different strains (Wen et al., 1995).

Ehrlichia sennestu is a human pathogen that has been reported to occur in Japan and Malaysia (Rapmund, 1984). It causes sennetsu fever, a mild mononucleosis-like illness marked by fever, lymphadenopathy and atypical lymphocytosis that usually resolves spontaneously. The vector has not been described, but an excellent candidate would be a trematode worm.

Another bacterium related to the neorickettsiae has been detected in California rainbow trout and the trematodes infesting these fish. It is not known whether this bacterium, which has not been named as yet, causes disease in mammals (Pusterla et al., 2000).

An illness that was first thought to be due to *E. sennetsu* was later linked to another member of the neorickettsiae. People in western Japan developed an illness (Hyuga fever) after eating raw gray mullet fish (Fukuda and Yamamoto, 1981). The SF agent was isolated from the trematode worm *Stellantchasmus falcatus*, which infests gray mullet (Wen et al., 1996). Phylogenetic analysis of the 16S rRNA gene suggests that SF is very closely related to *E. risticii*.

The Wolbachia Group

The Wolbachiae

The Wolbachiae are small, Gram-negative, intracellular bacterial symbionts of invertebrate hosts, particularly arthropods (Dobson et al., 1999). These bacteria can infect reproductive cells and thus have the potential to be vertically transmitted by cytoplasmic inheritance. Horizontal transmission of Wolbachiae also may occur. Wolbachiae are known to affect host reproduction by inducing cytoplasmic incompatibility, parthenogenesis, male killing, feminization of genetic males, and increases in fecundity (McGraw and O'Neill, 1999; Hadfield and Axton, 1999; Hurst et al., 2000). These reproductive changes induced in host arthropods appear to promote the spread of Wolbachia endosymbionts. *Wolbachia pipientis* and numerous unnamed Wolbachia species make up this group (McGraw and O'Neill, 1999). *Wolbachia pipientis* multiplies by binary fission in host cell vacuoles.

Wolbachia persica was isolated from the tick *Argas persicus*, now called *Argas arboreus* (Moulder, 1974). Phylogenetic analysis of the 16S rRNA gene shows that this bacterium does not cluster with the Wolbachiae, but rather belongs to the *Francisella* genus (Forsman et al., 1994). If this phylogeny proves correct, the name should probably be changed to *Francisella persica*.

Other bacterial endosymbionts of arthropods have been described that cluster phylogenetically with the Wolbachiae, but have been named by describing the infected host. These bacteria include symbionts of Trichogramma (Genbank L02886), Bangasternus (M85266), Muscifidurax (L02882), Brugia (AF051145), *Rhinocyllus* (M85267), and several incompatibility symbionts (M84692, M84689, ARB [short for "arbor," or "tree," in Latin] database, Technical University, Munich, Germany).

Wolbachia melophagi groups with the *Bartonellae* and is described in that section.

Other Bacteria in the Wolbachia Group

Bacteria that cluster with the Wolbachia based

on 16S rRNA phylogeny include bacterial symbionts associated with the algae *Cosmocladium saxonicum* (X79497) and *Pleurastrum paucicellulare* (Z47997) and a symbiont associated with the tick *Haemaphysalis longcornis* (AB001520). Some bacteria that are very closely related to the symbionts of *Cosmocladium saxonicum* have been called "Wolbachia species" (X64266) (http://www.arb-home.de, Technical University, Munich, Germany).

The Holospora Group

Another group of bacteria within the Rickettsiales includes *Holospora obtusa*, *Caedibacter caryophila*, and many unnamed bacteria detected in such diverse environments as marine and rumen ecosystems. *Holospora obtusa* and *C. caryophila* are lethal symbionts of the ciliate *Paramecium caudatum* (Springer et al., 1993; Fujishima and Fujita, 1985). Another bacterial endosymbiont from this group has been implicated in causing necrotizing hepatopancreatitis in shrimp (Loy et al., 1996).

Rickettsia-Like Prokaryotes

The Bartonellae

Members of the *Bartonella* genus are small (0.4 × 1.5 μm), Gram-negative, aerobic rods with typical cell walls (Weiss and Moulder, 1984). They may invade erythrocytes and endothelial cells or can occupy an epicellular location. Some *Bartonella* have flagella. In 1993, the bacteria previously classified in the Rochalimea genus were grouped and renamed with the *Bartonella* genus (Breitschwerdt and Kordick, 2000). In 1995, bacteria previously in the Grahamella genus were similarly absorbed into the *Bartonella* genus. Bartonellae are α-proteobacteria that are phylogenetically distinct from the Rickettsiae and are related to bacteria in the *Rhizobium* genus. An intracellular environment is not required for growth, as these bacteria can be propagated on complex axenic media in the laboratory (Spach and Koehler, 1998). Although many of these bacteria appear to grow intracellularly, the extent of intracellular versus extracellular replication in animal host tissues is not clear.

Bartonellae are important human pathogens, particularly in the immunocompromised host. A list of Bartonellae and the diseases they produce includes *B. bacilliformis* (Oroya fever and verruga peruana), *B. henselae* (cat scratch disease, bacillary angiomatosis, peliosis hepatis, bacteremia and endocarditis), *B. quintana* (trench fever, bacillary angiomatosis, endocarditis and bacteremia), *B. elizabethae* (endocarditis), *B. clarridgeiae* (cat scratch disease), *B. vinsonii arupensis* (bacteremia), *B. washoensis* (cardiac disease) and *B. grahamhii* (neuroretinitis).

Nonhuman mammals appear to be the primary hosts for most *Bartonella* species. For instance, cats are frequently infected with *B. henselae*, and they may have prolonged bacteremia (Heller et al., 1997; Chomel et al., 1995). Nonhuman reservoirs have not been identified for *B. bacilliformis* and *B. quintana*. Vectors for transmission of these agents are known: *B. bacilliformis* can be transmitted between humans via the sand fly, and *B. quintana* can be transmitted via the body louse.

Other members of the *Bartonella* genus not presently associated with human disease include *B. taylorii*, *B. doshiae*, *B. alsatica*, *B. tribocorum*, *B. weissii*, *B. koehlerae*, *B. vinsonii berkhoffii*, *B. vinsonii vinsonii*, *B. talpae* and *B. peromysci* (Breitschwerdt and Kordick, 2000). A diversity of mammals and insect vectors show high rates of infection with these bacteria.

Of note, the bacterium *Wolbachia melophagi* (Genbank X89110) does not group phylogenetically with the Wolbachiae genus, but rather groups with the Bartonellae genus. *Wolbachia melophagi* is closely related to *B. henselae* using 16S rRNA sequence phylogeny and should probably be renamed *Bartonella melophagi*.

The Thiomicrospira

The Thiomicrospira are γ-proteobacteria that include the human and animal pathogen *Coxiella burnetii*, the fish pathogen *Piscirickettsia salmonis*, and insect symbionts, such as *Rickettsiella grylli* and several unnamed tick symbionts. These bacteria live within cytoplasmic vacuoles of host cells and are phylogenetically distinct from the Rickettsiae and the Bartonellae that are α-proteobacteria. The Thiomicrospira are closely related to Legionella.

Coxiella burnetii is a small (0.2–0.4 × 0.4–1.0 μm), Gram-negative, obligate intracellular bacterium that passively enters cells where it replicates in the acidic environment of the phagolysosome (Maurin and Raoult, 1999). *Coxiella burnetii* can form an endospore that is resistant to degradation in the environment. Human infection is produced by inhalation of bacteria, though natural infection in animal populations probably involves a tick vector. In humans, *C. burnetii* is the cause of Q fever. A wide spectrum of organisms can harbor the bacterium, including insects, birds, fish and mammals.

A bacterial pathogen of salmonid fish, *Piscirickettsia salmonis*, causes epizootics of disease called "piscirickettsiosis" (Mauel et al., 1999; Fryer et al., 1992). The reservoir, vector and

mode of acquisition for this bacterium are not known.

Rickettsiella grylli is another member of the *Thiomicrospira* and is not related to the Rickettsiae despite the association implied by its name (Roux et al., 1997; Frutos et al., 1994). *Rickettsiella grylli* has a cricket host. The bacterium enters a host cell vacuole where it divides. Like chlamydia, these bacteria undergo a developmental cycle consisting of stages, including a large particle that replicates and a small dense particle that is infectious. The bacteria may form disk shapes and condense into crystalline bodies. Other members of this taxonomic group include symbionts of Rhipicephalus and Ornithodoros ticks.

The Chlamydiae

Chlamydiae are small bacteria (0.2–1.5 μm) that replicate within the phagosome of eukaryotic cells. Whereas rickettsiae exit the phagosome after phagocytosis and multiply in the cell cytoplasm, the chlamydiae (and ehrlichiae) remain in the phagosome and prevent fusion with lysosomes (Weiss and Moulder, 1984). Chlamydia do not metabolize glutamate, in contrast to the rickettsiae. Chlamydiae have some structural features of Gram-negative bacteria, but their cell wall lacks peptidoglycan. Chlamydiae have a biphasic life cycle. The infectious stage is more compact and called "the elementary body," whereas the replicative stage is larger and called "the reticulate body." The chlamydiae form their own distinct phylogenetic group within the eubacteria (Everett, 2000) and are peripherally related to the planctomycetes, a group of aquatic and terrestrial bacteria that also lack peptidoglycan in their cell walls (Weisburg et al., 1986).

The currently defined species of chlamydia include *C. psittaci*, *C. pecorum*, *C. trachomatis* and *C. pneumoniae*. *Chlamydia pecorum* is exclusively an animal pathogen, whereas the other three chlamydiae can cause human disease. *Chlamydia psittici* causes disease in birds and mammals. Humans acquire the bacterium via inhalation of aerosols generated from infected animals, with resulting pneumonia (Vanrompay et al., 1995). Closely related to *C. psittici*, *C. pecorum* causes disease in animals such as ruminants, swine and marsupials (Fukushi and Hirai, 1993). In humans, *C. trachomatis* causes ocular (trachoma), genital disease (lymphogranuloma venereum) and, less commonly, other syndromes (Stamm, 1999). *Chlamydia pneumoniae*, exclusively a human pathogen producing respiratory disease (Kuo et al., 1995), has also been linked to atherosclerosis in humans.

Prokaryotic Endosymbionts of Eukaryotic Cells

The relationships between prokaryotic and eukaryotic cells are discussed in detail (see section on Symbiotic Associations in this Chapter). This section will give examples of how some intracellular prokaryotes have evolved with their eukaryotic hosts.

Wolbachia pipientis, a member of the Rickettsiales, is a common cytoplasmic symbiont of insects. This bacterium is widely distributed in host tissues and can be transmitted vertically in the cytoplasm of infected host eggs, though horizontal transmission may also occur. The numerous effects of *W. pipientis* on insect reproduction increase the number of female offspring while suppressing the number of male offspring (McGraw and O'Neill, 1999; Stouthamer et al., 1999). Because female insects can vertically transmit *W. pipientis* to their offspring, this strategy benefits the endosymbiont. One mechanism for producing this result is crossing incompatibility. Infected females can mate with infected or uninfected males, but uninfected females cannot mate with infected males. Another mechanism promoting female representation is the direct killing of male embryos by *W. pipientis*.

Buchnera aphidicola is an obligate intracellular symbiont of aphids that is vertically transmitted and colonizes specialized host cells called "bacteriocytes" (Clark et al., 2000). This bacterium is not a member of the Rickettsiales, but rather is a member of the γ-proteobacteria. The complete genome of *Buchnera* has been sequenced (Shigenobu et al., 2000). *Buchnera* has genes for the biosynthesis of essential amino acids required by the host, but lacks those for nonessential amino acids produced by the host, demonstrating complete biochemical interdependence. Indeed, eradication of bacteria from bacteriocytes is lethal for aphids.

Mitochondria: The Ultimate Prokaryotic Endosymbiont

Mitochondria are specialized aerobic energy producing organelles of eukaryotic cells. Like chloroplasts, mitochondria have unique genomes that are distinct from the nuclear genomes of their associated eukaryotic cells (Gray, 1993). When mitochondrial DNA from diverse eukaryotes are compared, there are striking differences in the size, organization and gene content of these genomes (Gray et al., 1999). Yet, phylogenetic analyses suggest that all mitochondrial genomes descended from a common ancestor related to the α-proteobacteria (Lang et al.,

1999). The most complete mitochondrial genome has been found in the flagellated protozoan *Reclinomonas americana* (Lang et al., 1997). This genome contains 97 genes, including genes for bacterial rRNA and genes encoding a eubacteria-like multicomponent RNA polymerase. Of the bacterial genomes that have been sequenced, that of *Rickettsia prowazekii* is most closely related to the mitochondrial genome (Andersson et al., 1998).

These data suggest a bacterial origin for mitochondria. According to the endosymbiont hypothesis (Gray and Doolittle, 1982), about 1.5–2.0 billion years ago an α-proteobacterium entered a proto-eukaryotic cell where it established an endosymbiotic relationship. Progressive gene loss and specialization of function led to the development of mitochondria as known today. Mitochondria allowed eukaryotic cells to generate energy without using the plasma membrane. The host cell provided substrates for energy production and a protected niche for replication. Regulation of the mitochondrial genome eventually shifted to nuclear control.

The hydrogen hypothesis is an extension of the endosymbiont hypothesis for the origin of mitochondria (Rotte et al., 2000; Bui et al., 1996). This theory holds that a hydrogen-requiring archaebacterium engulfed a hydrogen-producing α-proteobacterium. Specialization of function by the endosymbiont led to the formation of hydrogenosomes for anaerobic energy production in eukaryotic cells. Hydrogenosomes occur in early amitochondric eukaryotic cells, such as Trichomonads. Although lacking in DNA, hydrogenosomes have proteins (e.g., heat shock proteins) that are phylogenetically related to mitochondrial proteins (Bui et al., 1996; Dyall et al., 2000). These data suggest a common origin for hydrogenosomes and mitochondria, though it is not clear if there was a single endosymbiotic event leading to two different organelles or multiple introductions with closely related prokaryotes.

Mitochondria appear to have originated from the introduction of a single α-proteobacterial endosymbiont into a eukaryotic or proto-eukaryotic cell (Gray et al., 1999). Although theoretically possible, a model in which different endosymbiont bacteria formed mitochondria is not supported by the data. Mitochondria are most closely related to the rickettsiae. However, one cannot conclude that the mitochondrial ancestor also belonged to this genus. It is possible that an α-proteobacterial ancestor gave rise to both the rickettsiae and the mitochondrial endosymbiont. The rickettsial and mitochondrial genomes are quite small. Both genomes are likely to have sustained gene loss over evolutionary time related to their common intracellular niche. Thus, the extensive loss of mitochondrial genes does not necessarily imply a rickettsial origin.

If evolutionary success is measured by the ability to pass a genome to succeeding generations, then the mitochondrial endosymbiont has been fantastically successful. Mitochondria in eukaryotic cells provide evidence that the strategy of intracellular prokaryotic existence is ancient and durable.

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Prokaryotes (2006) 5:467–492
DOI: 10.1007/0-387-30745-1_19

CHAPTER 3.1.18

The Genus *Bartonella*

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Phylogeny

Bartonella species share many general characteristics. Members of the genus are small (approximately 0.3 μm × 1 μm), Gram-negative, pleomorphic coccobacilli. The bacteria are facultative intracellular pathogens, many of which employ hemotrophy (infection of erythrocytes) as a parasitic strategy. All members of the genus are notoriously fastidious and grow slowly in vitro. *Bartonella* species have been shown to infect a variety of mammalian hosts, and at least three species (*B. bacilliformis*, *B. henselae* and *B. quintana*) are relatively common human pathogens. Vector-mediated transmission is another common theme within the genus. *Bartonella* spp. are typically transmitted between mammalian hosts by arthropods, with each bacterial species transmitted by a particular insect vector. Reservoirs for *Bartonella* spp. include a diverse array of mammals.

Based on 16S rRNA gene sequence comparisons, members of the genus *Bartonella* fall into the α-2 subgroup of the *Proteobacteria* (O'Connor et al., 1991). The genera closest to *Bartonella* include *Brucella*, *Rhizobium* and *Agrobacterium*. In general, the current genus *Bartonella* is relatively homogeneous with members exhibiting greater than 95% identity among aligned 16S rRNA gene sequences, resulting in a somewhat tightly branched phylogenetic tree (Fig. 1). Phylogenetic relationships determined using other loci, including *gltA* (the citrate synthase gene; Birtles and Raoult, 1996), *ftsZ* (a gene specifying a cell division [tubulin-like] protein; Kelly et al., 1998; Ehrenborg et al., 2000), and the 17-kDa antigen gene (Sweger et al., 2000), have resulted in dendrograms similar to that derived from the 16S rRNA gene sequences.

Taxonomy

The genus *Bartonella* has recently undergone a major taxonomic reorganization. Since the description of *Bartonella bacilliformis* in 1909, this species was the only representative from the genus described by A. L. Barton (Barton, 1909). However, bacteria once constituting the genera *Rochalimaea* and *Grahamella* were reclassified into the genus *Bartonella* (Brenner et al., 1993; Birtles et al., 1995), increasing the number of potential species to 15. Three *Bartonella* species are etiologic agents of major emerging infectious diseases in humans (Table 1). An additional four species (and two subspecies) of *Bartonella* also have been associated with human disease or described as human pathogens in isolated case reports (Table 2). Finally, ten species have not been associated with human disease but have been found in a variety of animal hosts (Table 3). The group's potential impact on human health and their ubiquitous presence in animal reservoirs have fueled an interest in understanding the basic biology of these bacteria as well as the epidemiology, clinical presentation and natural history of the resultant diseases.

The *Bartonellaceae* family has been removed from the order *Rickettsiales*; a group that originally included the *Anaplasmataceae*, *Rickettsiaceae* and *Bartonellaceae*. Members of the family *Bartonellaceae* share some rickettsia-like characteristics such as small size (e.g., the "virus-like particles" of *B. bacilliformis*), its transmission to humans through an arthropod vector (sandflies), and its ability to live within host cells (Moulder, 1974). However, they are not true obligate intracellular parasites like the rickettsiae, and several phenotypic properties differ between members of *Bartonellaceae* and the rickettsiae. For instance, *Bartonella*, *Grahamella* and *Rochalimaea* species and a few species of *Wolbachia* were the only *Rickettsiales* members that can be cultured in vitro, whereas true rickettsiae are strictly obligate intracellular parasites of eukaryotic cells. In addition, *B. bacilliformis* was the only flagellated member within the order (Weinman, 1974). Recently obtained DNA relatedness data, mainly 16S rRNA gene sequences, have clearly shown that *Rochalimaea* and *Bartonella* are more closely related to each other than to any rickettsia and most closely related to members of the α-2 subgroup of the *Proteobacteria*

Table 1. The three major human pathogens in the genus *Bartonella*.

Species	Common manifestation(s)	Vector(s)	Reservoir(s)
bacilliformis	Oroya fever, verruga peruana	Sandflies	Humans
henselae	Cat-scratch disease, endocarditis, bacillary angiomatosis, bacillary peliosis, bacteremic syndrome	Cats, fleas	Cats
quintana	Trench fever, endocarditis, bacillary angiomatosis, bacteremic syndrome	Body louse	Humans

Table 2. *Bartonella* species associated with human disease.[a]

Species	Clinical manifestation	Vector	Reservoir(s)	Reference
clarridgeiae	Cat-scratch disease?	Cats	Cats	Kordick et al., 1997
elizabethae	Endocarditis	Unknown	Unknown	Daly et al., 1993
grahamii	Retinitis	Unknown	Rodents	Kerkhoff et al., 1999
vinsonii sub sp. *aurepensis*	Bacteremia	Ticks?	Domestic dog, rodents	Welch et al., 1999
vinsonii sub sp. *berkhoffii*	Endocarditis	Unknown	Unknown	Roux et al., 2000

[a]Includes species associated with disease through serology or single case reports of disease associated with isolation or detection.

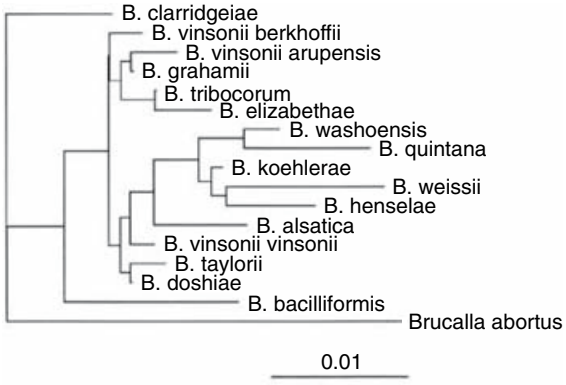

Fig. 1. Phylogenetic tree showing relationships of *Bartonella* spp. The 16S rRNA gene was aligned with over 1,272 bases. *Brucella abortus* is shown as an outgroup. Bar indicates a 1% nucleotide divergence. (From Breitschwerdt and Kordick, 2000, with permission.)

Table 3. *Bartonella* species that have not been demonstrated to be pathogenic for humans.

Species	Host(s)	Reference(s)
alsatica	Rabbits	Heller et al., 1999
doshiae	Rodents	Birtles et al., 1995
grahamii	Rodents, insectivores	Birtles et al., 1995
koehlerae	Domestic cat	Droz et al., 1999
peromysci	Rodents	Birtles et al., 1995
talpae	Moles	Birtles et al., 1995
taylorii	Rodents	Birtles et al., 1995
tribocorum	Rats	Heller et al., 1998
vinsonii sub sp. *vinsonii*	Voles	Weiss and Dasch, 1982
washoensis	Ground squirrels	Unpublished
weisii	Cats	Unpublished

class, especially the *Rhizobiaceae* (Birtles et al., 1991; O'Connor et al., 1991; Brenner et al., 1991; Relman et al., 1992). On the basis of these findings, *Rochalimaea* species were subsequently reclassified as *Bartonella*, and the *Bartonellaceae* family (containing both the *Grahamella* and *Bartonella* genera) was removed from the *Rickettsiales* order (Brenner et al., 1993). A subsequent study reclassified five *Grahamella* species as bartonellae based upon DNA relatedness data and phenotypic characteristics, thereby eliminating the *Grahamella* genus (Birtles et al., 1995).

The *Bartonella* genus presently contains the 18 species as listed in Tables 1–3. The phylogenetic relationship of *Bartonella* to other α-*Proteobacteria* indicates that the closest relatives to *Bartonella* include *Agrobacterium*, *Rhizobium* and *Brucella* species, the latter being the most closely related (Brenner et al., 1993). At first glance this grouping seems diverse, but its members do show similar natural histories. Specifically, all four genera have evolved towards a parasitic or mutualistic association with eukaryotic cells. *Bartonella* spp. and *Brucella* spp. are both intracellular parasites of mammalian cells, whereas *Agrobacterium* and *Rhizobium* species can parasitize or mutualistically associate with plant cells, respectively. None of these bacteria are obligate parasites, and all can be cultivated in vitro.

Interestingly, human diseases caused by *Brucella* and *Agrobacterium* spp. share superficial similarities with bartonelloses. Like with *Bartonella* spp., opportunistic infections with *Agrobacterium radiobacter* mainly occur in immunocompromised patients and can cause bacteremia and endocarditis (Edmond et al., 1993; Freney et al., 1985; Plotkin, 1980; Southern, 1996). In addition, some manifestations of brucellosis resemble the symptoms associated with

infection by *B. henselae* or *B. quintana*. For instance, brucellosis is a febrile illness characterized by granulomatous tissue in the lymph nodes, liver, spleen and bone and is accompanied by lymphadenopathy, bacteremia and occasionally endocarditis (Wilfert, 1992).

Habitat

Bartonella species have been isolated or detected in a wide range of animal species. Included in the list of potential animal reservoirs are cats, dogs, rodents, rabbits and cattle as well as a diverse group of wild animals including wildcats (bobcats, pumas and mountain lions), coyotes, deer, elk and foxes (for review, see Breitschwerdt, 2000). At least three species have been identified as major human pathogens (Table 1), and their disease syndromes are described below. An additional four species, including two subspecies, have been associated with human disease either indirectly or through isolated case reports (Table 2). However, their identification as major human pathogens awaits further reports associating them with human disease. Finally, currently eleven other species of *Bartonella* have only been isolated from animals (Table 3). It should be stressed that separation of individual species into human and animal agents is likely to change as specific diagnostic tests for all *Bartonella* species are developed and applied to testing human samples.

Transmission of *Bartonella* to humans occurs through an insect vector for most *Bartonella* species. The list of vectors associated with transmission includes flies, fleas, ticks, lice and mites (Tables 1 and 2). The possibility of direct mechanical transmission of *B. henselae* from cats to humans resulting in cat-scratch disease has been suggested. The role of the cat flea in this process seems likely and may involve the contamination of cat claws with infected flea feces. Transmission of *B. henselae* among cats appears to require the cat flea as a vector (Chomel et al., 1996). The epidemiological association of cat fleas with cases of human cat-scratch disease further supports the idea that contaminated flea feces is required for transmission (Zangwill et al., 1993).

Isolation

Isolation of *Bartonella* spp. from clinical specimens requires extended incubation times and specialized media. In addition, direct plating of specimens prepared by the lysis-centrifugation method (Wampole Laboratories, Cranbury, NJ) sometimes enhances isolation from blood (Slater et al., 1990). Alternatively, homogenized tissue can serve as the inoculum on blood plates or endothelial cell monolayers (Koehler et al., 1992). Blood or chocolate agar plates with a complex base medium (trypticase soy, heart infusion or Columbia agar) are used for culturing. The pH of the medium should be 7.0 to 7.5 for optimal growth. *Bartonella* spp. also require high humidity and, with the exception of *B. bacilliformis*, prefer 5% CO_2 within the growth chamber. The slow growth rate of these bacteria can compound primary isolation and often requires incubation times exceeding 3–4 weeks to visualize colonies. The combination of a slow growth rate and fastidious nature can make isolation and axenic culture of *Bartonella* spp. a challenging undertaking. In fact, endocarditis isolates of *B. henselae* were only recently obtained (Drancourt et al., 1996). Following isolation and pure culture of a strain, identification of the suspected *Bartonella* species can be accomplished using the techniques described below in "Identification."

Identification

In addition to a number of growth characteristics, structural, biochemical and genetic properties have been used to assist in the identification of *Bartonella* isolates. In general, polymerase chain reaction (PCR) amplification coupled with sequencing, restriction fragment-length polymorphism (RFLP) analysis or genetic probing recently has gained favor for rapid and definitive identification of isolates.

Colony Morphology

Bartonella colonies, typically small (1–3 mm diameter) and round, range from transluscent to opaque (white or cream) in color. Colonies may display a dry-to-mucoid phase variation with repeated passage. Colonies obtained from low-passage isolates typically adhere to and pit the medium, but this phenotype disappears following repeated passage in vitro. Colonies of *B. bacilliformis* freely interconvert between a small, transluscent round colony (T1) to a larger colony with an irregular edge (T2; Walker and Winkler, 1981). Colonies from primary isolates can take up to four weeks before becoming visible; however, growth is much more rapid (2–5 days) in subsequent passages. It has been suggested that this colony variation may correlate with antigenic phase variation seen with *B. henselae* and B. *quintana*, with fresh isolates being more highly reactive with human sera than isolates that have undergone extensive laboratory passaging (Regnery and Tappero, 1995).

Cell Morphology

Bartonella cells are Gram negative, non-acid fast, pleomorphic rods. *Bartonella* spp. stain poorly with safranin, but stain well with Giemsa or Gimenez stains. Cells are typically coccobacilli or slightly curved rods with a polar enlargement(s). Cells also can be coccoid, beaded, filamentous or in chains. Annular (ring forms) and autoaggregates also can occur. Cell size is uniformly less than 3 μm in the greatest dimension, with most cells measuring 0.5 μm × 1.0 μm. Figure 2 shows examples of the two basic cell morphologies observed in *Bartonella* spp. including a flagellated and nonflagellated cell.

Biochemical Tests

Bartonella spp. are nonfermentative aerobes with unremarkable physiology. For this reason biochemical tests are usually not conclusive for species identification. One potential problem with standard biochemical tests is that they do not include hemin for *Bartonella* growth, and therefore test results must be judged cautiously. If hemin is added to physiological test media, test results for acid production from carbohydrates (lactose, maltose and saccharose), hippurate hydrolysis, pyrazinamidase and Voges-Proskauer can be used to differentiate *B. henselae* from *B. quintana* (Drancourt and Raoult, 1993). RapID ANA panels (Innovative Diagnostic Systems, Inc., Norcross, GA; Daly et al., 1993; Clarridge et al., 1995), DNA hybridization (Regnery et al., 1991; Welch et al., 1992) and pre-formed peptidases (Welch et al., 1992) also have been used with varying success for the identification of *Bartonella* species.

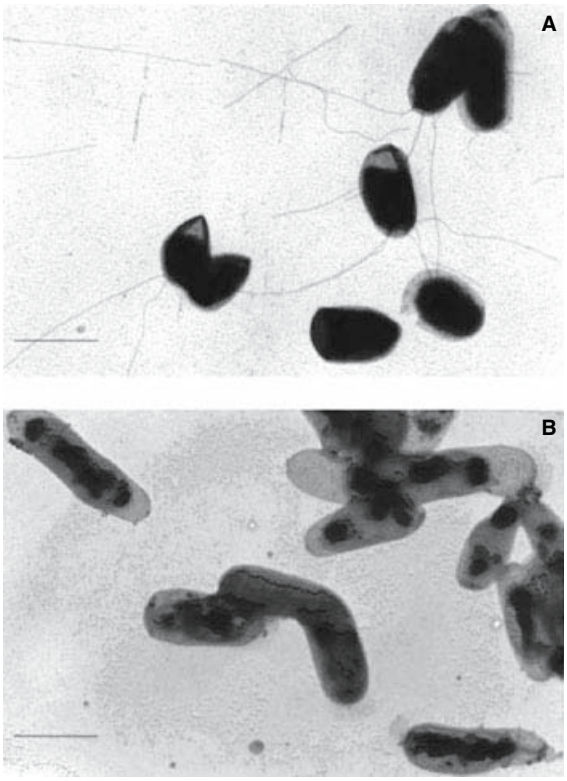

Fig. 2. Transmission electron micrographs showing the two basic cell morphologies observed in *Bartonella* species. Bacteria were grown by standard methods and stained with uranyl acetate. A) Flagellated cell morphology as exemplified by *B. clarridgeiae*, and B) a more typical, nonflagellated cell morphology as exemplified by *B. elizabethae* (Bars = 0.5 μm). (Courtesy of James A. Carroll.)

Polymerase Chain Reaction

The PCR is a sensitive and specific tool for identifying "non-culturable" *Bartonella* spp. in human samples or for confirming presumptive identification of isolates. Frequently, PCR has been used to amplify portions, or all, of the 16S rDNA gene from suspected *Bartonella* spp. The 16S rDNA PCR product is subsequently sequenced, and the data compared to known 16S rDNA sequences in GenBank to confirm presumptive identification (Relman et al., 1990; Regnery et al., 1992a; Koehler et al., 1992; Hadfield et al., 1993). To streamline identification, PCR strategies also have been devised using *Bartonella* genus-specific or species-specific targets. Targets have included the 16S rDNA gene (Dauga et al., 1996), a heat shock protein or stress endopeptidase *gene (htrA*; Anderson et al., 1993), the *gltA* gene (Regnery et al., 1991; Birtles and Raoult, 1996; Patel et al., 1999), the *ribC* gene (Bereswill et al., 1999), the *ftsZ* gene (Kelly et al., 1998), repetitive extragenic palindromic (REP) or enterobacterial repetitive intergenic consensus (ERIC) DNA sequences (Clarridge et al., 1995; Rodriguez-Barradas et al., 1995b) and the 16S-23S rDNA intergenic spacer (ITS; Minnick and Barbian, 1997b).

Restriction Fragment-Length Polymorphism

The RFLPs identifying *Bartonella* spp. employed rare-cutting restriction endonucleases together with genomic DNA (Slater et al., 1990; Maurin et al., 1994; Roux and Raoult, 1995), with PCR fragments of *gltA* (Norman et al., 1995), or with PCR products containing the 16S-23S rDNA intergenic spacer region (ITS; Matar et al., 1993; Roux and Raoult, 1995; Bergmans et al., 1996).

Cellular Fatty Acids

Cellular fatty acid (CFA) analysis also has been used to identify *Bartonella* spp. to the genus level. This technique is particularly useful, as *Bar-*

tonella spp. have an unusual fatty acid composition when compared to other bacteria. *cis*-11-Octadecanoate ($C_{18:1\ \omega7c}$; ~54%) and hexadecanoate ($C_{16:0}$; ~20%) are the predominant fatty acids of all *Bartonella* spp. *Bartonella elizabethae*, *B. henselae*, *B. clarridgeiae* and *B. quintana* contain considerable amounts of octadecanoate ($C_{18:0}$; ~23%), whereas *B. bacilliformis* contains very little (~2%). *Bartonella bacilliformis* also contains an unusually high quantity (~20%) of *cis*-11 hexadecanoate ($C_{16:1\ \omega7c}$) in contrast to most *Bartonella* species (<1%; Westfall et al., 1984; Slater et al., 1990; Daly et al., 1993; Clarridge et al., 1995; Kordick et al., 1995b).

Preservation

Bartonella spp. are highly resistant to freezing and freeze-thaw cycles (R. Regnery, personal communication). Strains can be stored indefinitely by adding glycerol to a final concentration of 12.5% (v/v) and storing at –70°C.

Physiology

Cell Structure

Bartonella cell walls are comparable to those of other Gram-negative bacteria (Kreier et al., 1991). Cellular fatty acids are unusual in composition, with greater than 50% being cis-11-octadecanoate ($C_{18:1\ w7c}$; see "Cellular Fatty Acids"). Motility has been observed in two pathogenic species (Table 3) and is conferred by unipolar lophotrichous flagella (*B. bacilliformis* and *B. clarridgeiae*; Scherer et al., 1993; Clarridge et al., 1995) or type IV pili (*B. henselae*; Batterman et al., 1995). There are no capsules or spore-like structures in any species. Spheroplasts of *Bartonella* can be made using lysozyme, and outer-membrane fractions can be prepared from these by cell lysis and collected by sucrose step-gradient centrifugation. The outer membrane of *B. bacilliformis* contains 14 outer-membrane proteins (OMPs) ranging from 11.2 to 75.3 kDa when analyzed by SDS-PAGE (Minnick, 1994). Further analysis of outer-membrane fractions by two-dimensional (2-D) SDS-PAGE suggests that the actual number of OMPs is closer to 50 (M. F. Minnick, unpublished observation). Nine surface proteins have been identified in *B. henselae* (Burgess and Anderson, 1998). The flagellin subunit (42 kDa) and a phage coat protein (31.5 kDa) are the major OMPs of *B. bacilliformis*. The outer membrane of *B. bacilliformis* contains a uniform lipopolysaccharide (LPS) molecule, migrating on SDS-PAGE at the equivalent of 5 kDa (Knobloch et al., 1988b; Minnick, 1994). A lack of multiple LPS bands suggests that only subtle differences exist in the O-side chains of *B. bacilliformis*. Early work on purified LPS from *B. quintana* showed that it contained 2-keto-3-deoxy-octonate and heptose and that it was reactive in chick embryo lethality, complement fixation and limulus amebocyte lysate tests (Hollingdale et al., 1980).

Growth and Metabolism

Excluding hemotrophy, the physiology of *Bartonella* spp. is not particularly exciting. Using standard tests, *Bartonella* spp. are strictly aerobic and do not utilize carbohydrates (no acid or gas) by preformed or de-novo enzymes. The oxidase test is usually negative for *Bartonella* spp., although variable and positive weak reactions have been reported for *B. quintana* and *B. vinsonii* (Daly et al., 1993; Kordick et al., 1996). Tests for catalase, indole production, nitrate reduction and urease activity are all negative (Clarridge et al., 1995; Birtles et al., 1995). In addition, tests for hippurate hydrolysis, alkaline phosphatase, tetrathionate reductase, pyrazinamidase, tributyrin, o-nitrophenyl-β-D-galactoside, esculin hydrolysis and arginine dihydrolase are all negative (Birtles et al., 1995). Voges-Proskauer tests are negative for all human pathogenic species, but positive for *Bartonella* spp. previously designated as *Grahamella* spp. (Birtles et al., 1995). Aminopeptidase hydrolysis of a variety of amino acids and peptides has been observed in the pathogenic *Bartonella* species, and hydrolysis of phenylalanine or trypsin might be useful for differentiation of some species (Clarridge et al., 1995; Kordick et al., 1995b; Birtles et al., 1995; Table 3). Utilization of succinate has been demonstrated for *B. quintana* (Weiss and Moulder, 1984).

Bartonellae are slow-growing bacteria with generation times of approximately 6 (i.e., 5.8–6.7) h under optimal conditions in vitro for *B. quintana* (Weiss and Dasch, 1982) and 6–8 h for *B. bacilliformis* (Benson et al., 1986). Because of long generation times, maximal colony size is only reached after several days of growth on plates. *Bartonella* spp. also can be cultivated in embryonated chicken eggs and tissue culture.

Extracellular Products

Only three extracellular products from *Bartonella* spp. have been described, including deformation factor (deformin), a defective bacteriophage, and hemolysin. A putative ABC transporter from *B. bacilliformis* has been cloned and sequenced (*txpA*; GenBank accession no. U68242) and shares homology with the ABC glucan exporter from *Agrobacterium tumefa-*

ciens. The TxpA protein may be involved in export from or import into *Bartonella* (for a review of ABC transporters see Fath and Kolter, 1993). Likewise, an operon encoding type IV secretion system genes with extensive similarity in sequence and gene arrangement to the *virB* operon of *A. tumefaciens* has been described for *B. henselae* (Schmiederer and Anderson, 2000; Padmalayam et al., 2000a). Notably, one gene (*virB5*) has been replaced by the gene encoding the 17-kDa antigen– previously described immunodominant protein of *B. henselae* (Anderson et al., 1995).

DEFORMATION FACTOR. *Bartonella bacilliformis* produces an extracellular protein termed "deformin" that can independently generate indentations and trenches in erythrocyte membranes. The pits and trenches produced with purified deformin are morphologically similar to those observed in infected cells (Benson et al., 1986; Fig. 3). The protein is actively secreted during growth of the bacterium and is a 130-kDa homodimer in its native state. Deformin is sensitive to heat (70–80°C) and proteases. Deformin activity is enhanced by pretreatment of the erythrocytes with trypsin or neuraminidase and abrogated if erythrocytes are pretreated with phospholipase D. Deformin-induced invaginations are reversible by vanadate, by dilauroylphosphatidyl choline (DLPC), or by increasing intracellular Ca^{2+} levels with ionophores (Mernaugh and Ihler, 1992; Xu et al., 1995). Whether other bartonellae produce deformin, or whether deformin is active against other cell types, is not known. The gene for deformin has not been characterized.

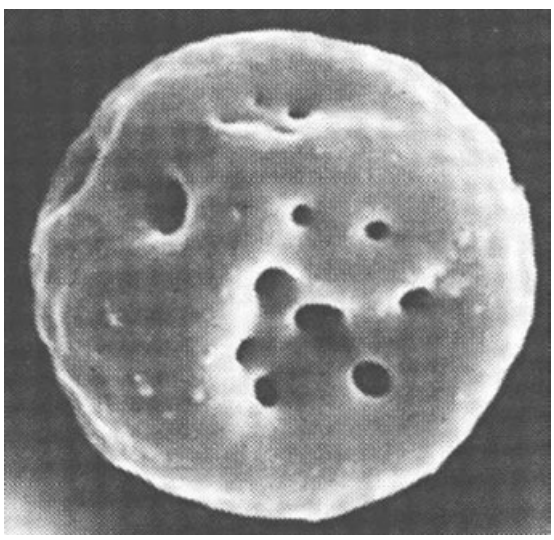

Fig. 3. Erythrocyte invaginations caused by *B. bacilliformis* deformation factor (deformin). Erythrocytes were treated with: trypsin, *B. bacilliformis* culture filtrate for 2 h, and purified deformin for 2 h. (From Xu et al., 1995, with permission.)

DEFECTIVE BACTERIOPHAGE. Uniform bacteriophage-like particles containing icosahedral heads (~40 nm in diameter) and a filamentous sheath-like tail structure (16 nm in length) were first observed in *B. bacilliformis* (Umemori et al., 1992). The authors suggested that the bacteriophage was temperate and that the *Bartonella* strains (KC583 and KC584) were lysogenic. In addition, observed decreases in *B. bacilliformis* yields with increased passage were thought to be due to bacteriophage infestation. Subsequent work showed that phage-like particles also can be obtained from *B. henselae*, but not *B. elizabethae* or *B. quintana*. Phages from *B. henselae* and *B. bacilliformis* consist of a heterogeneous, linear DNA of 14 kbp and at least three proteins (Anderson et al., 1994a). Two of the phage-associated proteins, Pap31 and PapA, have been characterized and are 31 and 36 kDa, respectively (Anderson et al., 1997b; Bowers et al., 1998). It is not currently known if this particle mediates transduction and genetic exchange among *Bartonella* spp, although packaging of a chromosomal marker has been demonstrated in the *B. bacilliformis* phage (Barbian and Minnick, 2000; Fig. 4).

HEMOLYSINS. These are produced by *B. bacilliformis* and *B. elizabethae*. Until recently, all literature on *B. bacilliformis* claimed that the bacterium was nonhemolytic. However, if *B. bacilliformis* cultures are plated on thin blood agar plates and cultured for at least four days, incomplete β-hemolysis can be observed. The hemolysin is extracellular and can pass through 0.2 μm filters into the underlying medium to produce a zone of hemolysis, which is limited to the outline of the colony (Minnick, 1997a). Likewise, an incomplete β-hemolysin with delayed appearance (four days growth) has been reported from *B. elizabethae* (Daly et al., 1993). The molecular nature of these hemolysins is not known.

Angiogenic Factor

The three major pathogenic *Bartonella* spp. produce a protein that stimulates angiogenesis and probably facilitates the generation of vascular lesions during infection. Fractions from *B. bacilliformis* containing the protein induce vascularization in vivo and can induce human umbilical vein endothelial cells (HUVECs) to proliferate in vitro (Garcia et al., 1990). Live *B. bacilliformis* (Garcia et al., 1992), *B. henselae* or *B. quintana* (Conley et al., 1994) stimulates endothelial cell proliferation when cocultured with HUVECs. The HUVECs also migrate towards bartonellae in cocultures (Conley et al., 1994). Mitogenicity

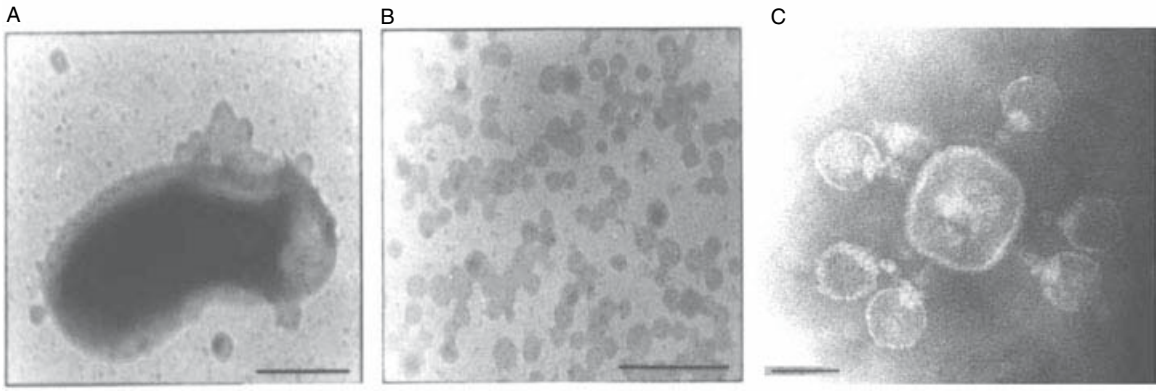

Fig. 4. Transmission electron micrographs showing: A) *B. bacilliformis* (strain JB585, a flagellin-minus mutant; Battisti and Minnick, 1999) infected with bacteriophage, B) purified bacteriophage from JB585 (Bars = 250 nm), and C) six bacteriophage with tails, infecting a bleb from *B. henselae*. (Panels A and B from Barbian and Minnick, 2000, with permission; Panel C courtesy of James A. Carroll.)

of the angiogenic protein is active on endothelial cells, but fibroblasts, smooth muscle or mesenchyme cells are unaffected (Garcia et al., 1990; Conley et al., 1994). In *B. bacilliformis*, the angiogenic factor has been shown to be a cytoplasmic protein (Garcia et al., 1990). The initial report for the angiogenic factor of *B. henselae* indicated that it was an insoluble membrane-associated protein (Conley et al., 1994); however, a more recent report indicated that it was a diffusible protein found in bacterial culture supernatants (Maeno et al., 1999). Thus, the bacterial localization of the angiogenic factor as well as the molecular nature of the protein and its mechanism of action remains unclear.

Enzymes

Few enzymes have been characterized and no exotoxins have been described from any *Bartonella* species. Seven putative *Bartonella* enzymes have been deduced from nucleotide sequences. These include several GltAs (citrate synthase species; Birtles and Raoult, 1996), HtrA (Anderson et al., 1996), FtsZ (Padmalayam et al., 1997), GroEL (Haake et al., 1997), alanyl tRNA synthetase (AlaS), leucyl tRNA synthetase (LeuS), and orotidine monophosphate decarboxylase (PyrF). Only four *Bartonella* enzymes have been characterized at both the nucleotide sequence and protein level: a carboxy-terminal protease that is autolytic (CtpA; Mitchell and Minnick, 1997a), the invasion-associated locus A protein (IalA), a nudix hydrolase (Cartwright et al., 1999), the gyrase B subunit (GyrB; Battisti et al., 1998), and inorganic pyrophosphatase (PPase; Mitchell and Minnick, 1997b). In keeping with Ppases from other prokaryotes, the *B. bacilliformis* enzyme displays maximal activity at a pH of 8.0 and demonstrates high thermostability in the presence of Mg^{2+} (highest activity at 55°C).

Pathogenesis

Hemotrophy is a striking aspect of the physiology of most *Bartonella* spp. Parasitism of erythrocytes is very unusual for bacteria and is undertaken by species from two other genera, *Anaplasma* and *Haemobartonella* (Kreier and Ristic, 1981). Hemotrophy undoubtedly fulfills the growth requirement for blood or hemin by all bartonellae. Heme uptake is employed by several pathogenic bacteria to acquire iron and porphyrin (Reidl and Mekalanos, 1996). Work done with *B. quintana* shows that a high concentration of heme (20–40 µg/ml), but not protoporphyrin, is essential for growth. In addition, serum is not a required growth factor (Myers et al., 1969). The gene encoding an outer-membrane, hemin-binding protein of *B. quintana* has been identified and characterized (Carroll et al., 2000). The protein appears to be a homolog of the phage-associated protein of *B. henselae*, Pap31 (Bowers et al., 1998).

Bartonella spp. are adapted to infect a variety of hosts (invertebrates and vertebrates) and several cell types (erythrocytes, epithelial and endothelial cells). Thus, these pathogens provide a splendid opportunity to investigate host-parasite interactions at the cellular and molecular level. Research on molecular pathogenesis primarily has been done using *B. bacilliformis* as a model system for the genus.

COLONIZATION OF THE HOST. *Bartonella* spp. are inoculated directly into the blood

by the bite or scratch of a contaminated arthropod or cat. Subsequent colonization of the host lymphatics and circulatory system is likely enhanced by blood flow and, if possible, bacterial motility. *Bartonella bacilliformis* and *B. clarridgeiae* (previously 94-F40), a novel *Bartonella* species recently isolated from cats (Clarridge et al., 1995; Kordick et al., 1997), are highly motile and employ peritrichous flagella. Twitching motility has been observed in low-passage isolates of *B. henselae* and is thought to be conferred by type IV bundle-forming pili (Batterman et al., 1995). Putative type IV pili also have been observed in *B. bacilliformis*, but probably play a subordinate role to flagella in motility (Minnick et al., 1996). *Bartonella bacilliformis* flagella have been biochemically characterized and consist of multiple 42-kDa flagellin subunits that are highly resistant to protease treatment (Scherer et al., 1993). The N-terminus for flagellin (Scherer et al., 1993) and the nucleotide sequence of the *fla* gene (GenBank accession no. L20677) have both been characterized. Recently, the gene encoding the flagellin subunit from *B. clarridgeiae* also was cloned and sequenced and displays extensive sequence similarity with the *fla* gene of *B. bacilliformis* (Sander et al., 2000).

ADHERENCE TO HOST CELLS. *Bartonella bacilliformis* and *B. henselae* show a correlation between colony morphology and adherence to host cells in in vitro assays. Adherence rates for *B. bacilliformis* obtained from colony type T2 (see "Colony Morphology") were nearly twice that of bacteria derived from colony type T1 (Walker and Winkler, 1981). Similarly, high-passage phase variants of *B. henselae* from mucoid colonies showed a decrease in adherence to Hep-2 cells relative to low-passage bacteria obtained from dry and embedded colonies (Batterman et al., 1995). Reduction in *B. henselae* adherence is believed to result from repeated-passage phase variation, causing the loss of expressed type IV bundle-forming pili (BFP) on the surface of the pathogen (Batterman et al., 1995). The recent discovery of a putative type-IV BFP on low-passage *B. bacilliformis* suggests that other *Bartonella* spp. employ these appendages as well (Minnick et al., 1996). Figure 5 shows a low-passage *B. henselae* cell expressing BFP on its surface.

Bartonella bacilliformis can associate with human umbilical vein endothelial cells (HUVECs) or epithelial cells (HEp-2) with equal binding efficiency, and the majority of adherence occurs within the first 60 min of a 3-h incubation period (McGinnis-Hill et al., 1992). This is in contrast to erythrocyte adherence, where maximal complexing occurs at approximately 6 h after incubation (Benson et al., 1986).

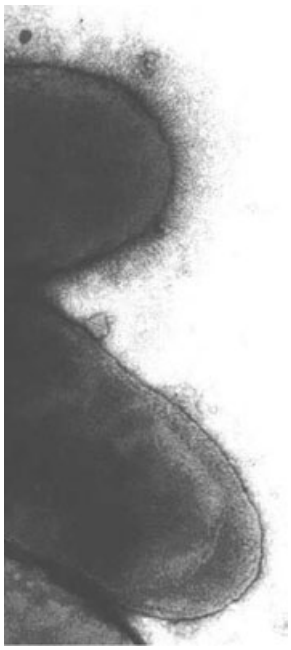

Fig. 5. Transmission electron micrograph showing piliated (upper) and non-piliated (lower) *B. henselae* cells from the same culture. (Courtesy of James A. Carroll.)

Disparate binding kinetics suggests that unique receptor-ligand interactions occur between each type of host cell and *Bartonella*. Adhesion to erythrocytes can be inhibited by reagents that inactivate proton-motive force (N-ethyl maleimide) or respiration (potassium cyanide; KCN), but is unaffected by pretreating the erythrocyte with inhibitors of glycolysis (sodium fluoride; NaF) or proton-motive force (N-ethyl maleimide). These observations suggest that adhesion is energy dependent (Walker and Winkler, 1981). However, it is believed that the red blood cell is passive during the process and cannot contribute to energy-dependent adhesion. The identity of the host cell receptor is not known. However, pretreating the erythrocyte with pronase or subtilisin enhances adhesion, whereas α- or β-glucosidase treatment decreases adhesion. Presumably, protease treatment of red cells exposes a glycolipid receptor that can be subsequently destroyed by glucosidase. It also is known that human erythrocytes are preferentially bound by *Bartonella* as compared to red blood cells from rabbits or sheep, suggesting that human red cells possess a more appropriate receptor or possess greater receptor density or accessibility than other types of erythrocytes possess (Walker and Winkler, 1981). Given the ability of *B. henselae* to infect cats and humans, it would be interesting to compare its binding efficiency with cat and human erythrocytes.

More recently, studies show that *B. bacilliformis* and *B. henselae* recognize five and six proteins, respectively, from human erythrocyte membranes (Iwaki-Egawa and Ihler, 1997).

Flagella also may serve as adhesins. A polar tuft of fibrous projections on *B. bacilliformis* was observed to make contact with the erythrocyte membrane during adhesion (Walker and Winkler, 1981). The fibrous tuft closely resembles the peritrichous flagella of the bacterium. It is also known that nonmotile bacteria bind poorly to erythrocytes, suggesting that an adhesin (flagella?) is missing in nonmotile bacteria or that nonmotile mutants have fewer bacteria-erythrocyte collisions (Benson et al., 1986). If *B. bacilliformis* is treated with rabbit anti-flagellin antiserum, there is a significant reduction (~41%) in bacterial association with red cells as compared to controls conducted with preimmune rabbit serum (Scherer et al., 1993). These data suggest that flagella may possess adhesive qualities and/or they increase the number of bacteria-host cell collisions.

The endothelial cell receptor for *Bartonella* spp. has not been characterized. The observation that *B. bacilliformis* binding to epithelial cells and endothelial cells displays a similar degree of efficiency suggests that the pathogen's apparent predilection for endothelial cells may actually be due to tissue site (e.g., circulatory system), rather than receptor-mediated constraints (McGinnis-Hill et al., 1992). In addition, binding data also suggest that both cell types contain a suitable, if not the same, receptor(s). The role of surface-exposed *Bartonella* polypeptides in adhesion is largely unknown. However, recent work with *B. henselae* has identified five biotinylated proteins (28–58 kDa) capable of binding to intact HUVECs. Of these, a 43-kDa polypeptide was identified as the major adhesin of the pathogen. It is significant to note that the 43-kDa protein also was recognized by reciprocal probing with biotinylated HUVEC surface proteins (Burgess and Anderson, 1998). The exact nature of the 43-kDa adhesin is currently under investigation.

INVASION OF HOST CELLS. Invasion of erythrocytes has been documented for *B. bacilliformis* (Benson et al., 1986), *B. henselae* (Kordick and Breitschwerdt, 1995a; Mehock et al., 1998), and *Bartonella* spp. previously classified as *Grahamella* species (Birtles et al., 1995; Table 2; Fig. 6). Host cell invasion by *B. quintana* and *B. elizabethae* has not been demonstrated, although both require blood or hemin for growth. In fact, *B. quintana* is believed to epicellularly associate with erythrocytes (Merrell et al., 1978). In addition to invasion of red blood cells, invasion of other host cell types (epithelial and endothelial cells) has been demonstrated for *B. henselae* (Batterman et al., 1995; Dehio et al., 1997b; Fig. 7) and *B. bacilliformis* (Garcia et al., 1992; McGinnis-Hill et al., 1992) and *B. quintana* (Brouqui and Raoult, 1996).

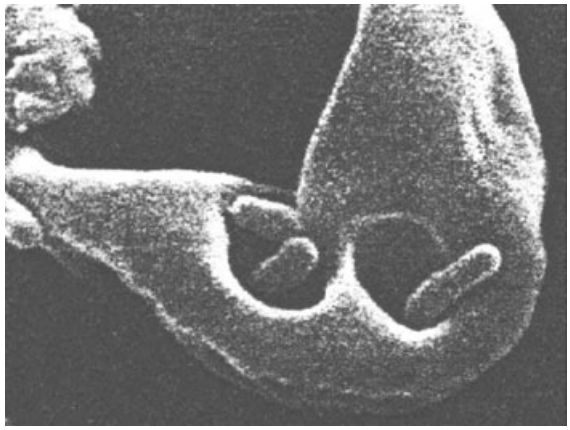

Fig. 6. Human erythrocyte being invaded by *B. bacilliformis*. (From Benson et al., 1986, with permission.)

Because *Bartonella* spp. can enter a variety of cells, molecular mechanisms for entry probably depend on the type of cell being invaded. Virulence studies with *B. bacilliformis*, together with cultured epithelial or endothelial monolayers, demonstrate that host cells can be induced by *Bartonella* to reconfigure the cytoskeleton, thereby enhancing bacterial uptake. Internalization is significantly reduced (~30% of controls) if actin filament formation is inhibited with cytochalasin D or if bacteria are pretreated with anti-*Bartonella* antiserum (McGinnis-Hill et al., 1992). These inhibition studies suggest that the bacterium is not passive during internalization and that the process involves a surface-borne molecule(s) that is accessible to antibody. Erythrocyte invasion by *Bartonella* spp. is very different because red cells are necessarily passive (non-endocytotic) and cannot contribute to bacterial uptake. Recent work by Dehio et al. (1997b) shows that *B. henselae* enters endothelial cells by a novel structure termed "an invasome" (Fig. 8). In in vitro models of infection, *B. henselae* cells are contacted and moved rearward to form an aggregate on the leading lamella of the endothelial cell. The "clumps" of bacteria are subsequently engulfed by membrane protrusions that are rich in cortical F-actin, intercellular adhesion molecule (ICAM-1), and phosphotyrosine. Based upon chemical inhibition studies, invasome activity is actin-dependent and microtubule-independent. Although most clinical strains of *B. henselae* were internalized via formation of the invasome, a natural mutant of *B. henselae* was internalized

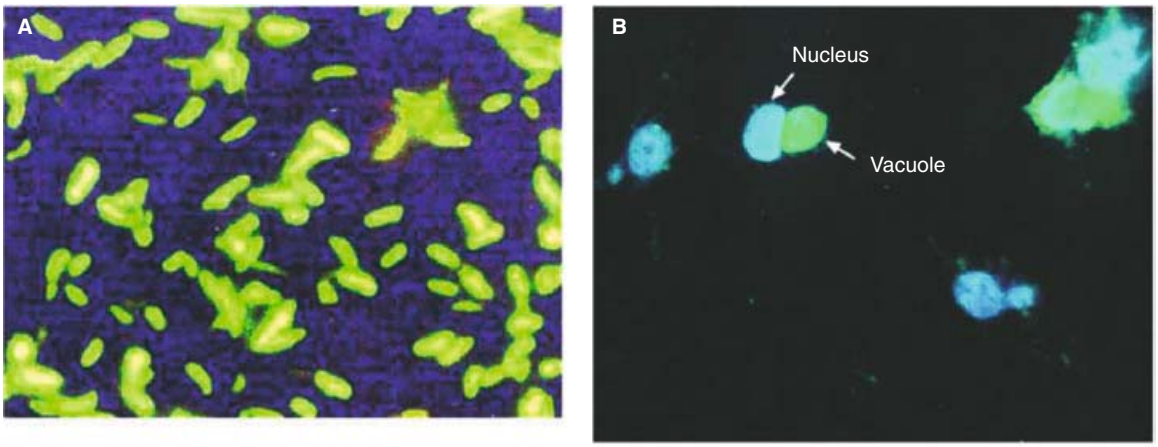

Fig. 7. Fluorescence microscopy showing a human microvascular endothelial cell with internalized *B. henselae*. A) *Bartonella henselae* expressing green fluorescent protein (GFP). B) A microvascular endothelial cell vacuole with numerous GFP-expressing *B. henselae* (green) in contrast to the nucleus (blue). (Magnification 400×; From Resto-Ruiz et al., 2000, with permission.)

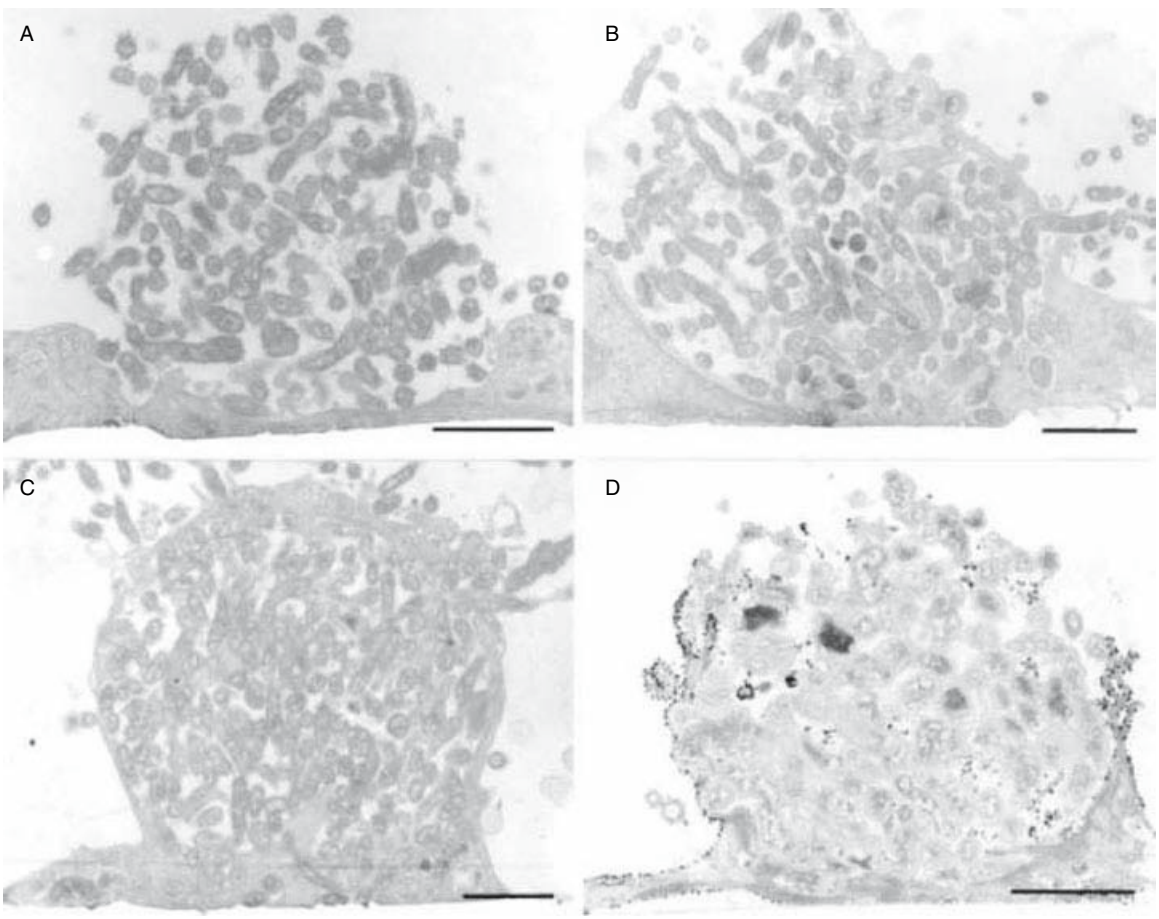

Fig. 8. Invasome structure observed when *B. henselae* is internalized by vascular endothelial cells. Transmission electron microscopy of: A) initial invasome structure, B) and D) engulfment of the bacterium, and C) internalization. Panel D shows immunogold labeling with mAbs to ICAM-1. Bars = 2 µm. (From Dehio et al., 1997b, with permission.)

via an alternate process and was ultimately located in a perinuclear phagosome. The authors hypothesized that invasome-mediated internalization may somehow interfere with the perinuclear phagosome formation. It is also possible that the mutant lacked the appropriate surface ligand for triggering formation of the structure.

Bartonella quintana was shown to invade epithelial cells in classical in vitro experiments using Hep-2 cells (Vinson and Fuller, 1961). More recent work has shown that the pathogen is also invasive for human endothelial cells in vitro and, more importantly, in cardiac valve tissue from endocarditis patients (Brouqui and Raoult, 1996). In vitro studies with *B. quintana* and human endothelial cells show that bacteria can become internalized within only one minute of coincubation. Concurrently, host cells exhibit ruffling and the bacterial cell wall becomes modified to produce surface appendages (20–40 nm wide by up to 500 nm in length). The appendages are apparently lost after adherence or internalization of the pathogen and are similar to those observed in *Salmonella typhimurium*. The authors hypothesize that the appendage is mediating or directing host cell adherence and endocytosis. Following host cell uptake, *B. quintana* multiplies in a vacuole, culminating in formation of morulae resembling those seen during infection by *Ehrlichia* spp. or *Chlamydia* spp. Older *B. quintana* and endothelial cell cocultures showed that morulae contain both bacteria and vesicle-like blebs presumably derived from the bacterial membrane. However, membrane blebs were not observed in cardiac tissue samples. It is interesting to note that *B. bacilliformis* (M. F. Minnick, unpublished data), *B. henselae* and *B. quintana* (Brouqui and Raoult, 1996) all produce blebs during in vitro growth, but their potential role in pathogenesis remains a mystery.

Three virulence determinants have been implicated in *Bartonella* invasiveness, including deformin, flagella, and proteins encoded by the invasion-associated locus of *B. bacilliformis*. Deformin-induced invaginations in the plasma membrane of erythrocytes (see "Extracellular Products") likely produce entry portals for colonizing the red cell. However, even with deformin, bacteria cannot enter the red cell cytosol unless they are motile (Mernaugh and Ihler, 1992). Perhaps *B. bacilliformis* swims into the trenches produced by deformin. If *B. bacilliformis* cells are treated with monospecific antibodies generated against the flagellin subunit, invasiveness of human erythrocytes is nearly abrogated in in vitro virulence assays (Scherer et al., 1993). Invasion of *B. bacilliformis* into Hep-2 cells is thought to require host cell tyrosine phosphorylation (Williams-Bouyer and Hill, 1999). In addition, invasion of human endothelial cells by *B. bacilliformis* requires host cell GTPase-Rho (Verma et al., 2000).

Recent work also shows that *B. bacilliformis* possesses an invasion-associated locus containing two genes, termed "*ialAB*" (Mitchell and Minnick, 1995; Fig. 9). The locus is approximately 1,500 bp and contains two ORFs (*ialA* and *ialB*) that confer an invasive phenotype on minimally invasive strains of *Escherichia coli* (strains HB101 and DH5α-) when combined with human red cells in vitro. Both genes are required to produce the invasiveness phenotype. The *ialA* gene codes for a (di)nucleoside polyphosphate hydrolase that may function to reduce stress-induced dinucleotide "alarmones" during host cell invasion and therefore enhance pathogen survival (Cartwright et al., 1999). The *ialB* gene codes for a protein with similar molecular mass and approximately 60% amino acid sequence similarity to the adhesion and invasion locus (Ail) protein of *Yersinia enterocolitica* (Miller et al., 1990) and the resistance to complement killing (Rck) protein of *Salmonella typhimurium* (Heffernan et al., 1994). Both Ail and Rck are implicated in host cell attachment, invasion and serum resistance. Whether the *Bartonella* invasion-associated locus mediates or facilitates the invasion of other cell types is unknown.

Characterization of genes that flank the *ialAB* locus suggests that *ialA* and *ialB* are components of a larger pathogenicity gene cluster. A gene encoding a carboxy-terminal protease, *ctpA*, lies upstream of *ialA* and *ialB* (Mitchell and Minnick, 1997a; Fig. 9). The encoded protease may be involved in *B. bacilliformis* virulence, espe-

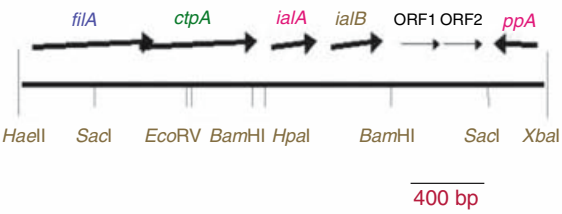

Fig. 9. The invasion-associated locus of *B. bacilliformis* plus flanking sequences characterized to date. Genes include *filA* (filament A gene), *ctpA* (carboxy-terminal processing protease gene), *ialA* (invasion-associated locus A gene), *ialB* (invasion-associated locus B gene), ORFs 1 and 2 (unknown function) and *ppA* (inorganic pyrophosphatase gene). The GenBank accession numbers are U73652, L37094, L25276 and L46591, respectively. Similar gene clusters have been found in *B. quintana* and *B. henselae*.

cially considering that the *prc* carboxy-terminal protease of S. *typhimurium* is involved in intracellular survival. The *prc* protease is thought to degrade abnormally folded stress-response proteins generated within the intracellular environment of the macrophage, thereby enhancing survival of S. *typhimurium* (Baumler et al., 1994). Though no evidence for CtpA involvement in invasion of human erythrocytes was found (Mitchell and Minnick, 1997a), the possible role that CtpA plays in *B. bacilliformis* intracellular survival is unknown.

Immediately upstream of the *ctpA* gene is a 1,200-bp ORF termed "*filA*" (M. F. Minnick and S. J. Mitchell, unpublished data). Characteristics of the predicted FilA polypeptide include: 1) a typical secretory signal sequence that follows the -3,-1 rule, 2) a 60% α-helical secondary structure, 3) a C-terminal hydrophobic domain that could anchor the protein in a membrane, 4) leucine-rich composition (12%) with numerous leucine repeats, a characteristic found in proteins that engage in protein-protein interactions (Kobe and Deisenhofer, 1995), and 5) amino acid similarity to a variety of filamentous proteins including smooth muscle myosin and the M1 protein of *Streptococcus pyogenes*, a virulence determinant involved in adhesion and invasion (Fischetti, 1989; Kehoe, 1994). The FilA polypeptide's similarity to M1 protein, the potential surface location and filamentous nature, plus the possibility that FilA provides for protein-protein interaction all suggest that the protein is a virulence determinant. The 3′ end of the virulence gene cluster is presumably demarcated by an inorganic pyrophosphatase (Ppase) gene (*ppa*) that is located 1,022 bp downstream of the locus (Mitchell and Minnick, 1997b). The Ppase is not involved in virulence because this enzyme plays an essential metabolic housekeeping role in many organisms.

DNA hybridization data suggest that *B. quintana* and *B. henselae* both possess homologues of *ialA*, *ialB* and *ctpA*, whereas *B. vinsonii*, an agent that does not infect humans, contains *ialA* and *ctpA* but may not contain an *ialB* homologue. *Bartonella elizabethae* showed very poor hybridization to any of the probes for the three genes (Mitchell and Minnick, 1997a). A homolog to the *ialAB* locus was recently cloned from *B. henselae* and was found to confer an invasive phenotype upon *E. coli* to approximately 100-fold over controls and possessed identical gene linkage with approximately 70–85% sequence identity to the *B. bacilliformis* locus (Murakawa, 1997). Likewise, invasiveness by *B. tribocorum* for rodent erythrocytes required the activity of IalB (Gille et al., 1999). Collectively, these data suggest that the invasion locus is conserved in most pathogenic *Bartonella* spp.

Genetics

Genome

The G+C content for *Bartonella* species is 40 + 1 mol%, with values ranging from 38.5 mol% for *B. quintana* (Tyeryar et al., 1973) to 41.1 mol% for *B. vinsonii* (Daly et al., 1993). In addition to a highly conserved G+C content, DNA hybridization results indicate a high degree of sequence homology (32–67% relatedness at 55°C) between the genomes of *Bartonella* species (Welch et al., 1992; Daly et al., 1993). The genome sizes of *B. bacilliformis* and *B. quintana* are approximately 1.6 and 1.5 Mbp, respectively (Krueger et al., 1995; Myers et al., 1979). Thus, the typical *Bartonella* genome is less than half the size of that from *E. coli*. Sequencing of the *B. henselae* genome is nearing completion and updated information from this project may be obtained. Genome sequencing projects for other *Bartonella* species are not currently in progress. An extrachromosomal, linear DNA band of 14 kbp is frequently observed in genomic DNA preparations from *B. henselae* and *B. bacilliformis*. The DNA is heterogeneous in nature and derived from a putative defective bacteriophage for bartonellae (Anderson et al., 1994a; Barbian and Minnick, 2000). Plasmids have not been described from any *Bartonella* spp.

Gene Structure

Because of their modest G+C content, genes in *Bartonella* spp. are biased for A or T in the second and third positions of their codons. For example, codon usage analysis of the 2,275-bp *gyrB* gene (gyrase B subunit) of *B. bacilliformis* (Battisti et al., 1998) or the 1,512-bp *htrA* gene (heat-shock antigen) of *B. henselae* (Anderson et al., 1996) shows an A or T 60% of the time in the second position and 77% of the time in the third position of their respective codons. However, a clear bias is not observed in the first position of the codons. Although promoters have never been characterized fully from a *Bartonella* species, all genes characterized are preceded by putative -35 and -10 hexamers with homology to the *E. coli* consensus promoter (McClure, 1985). Likewise, these genes are immediately preceded by a ribosomal binding site similar to that of *E. coli* (Gold et al., 1981). Indeed, the recent complementation of *E. coli* mutants with a cloned *ppA* gene (inorganic pyrophosphatase) and *gyrB* (gyrase B) from *B. bacilliformis* suggests that there is conservation of promoter sequence and function between the two bacteria (Mitchell and Minnick, 1997b; Battisti et al., 1998). The majority of the characterized *Bartonella* ORFs end

with a TAA stop codon, and putative transcriptional terminators have been observed downstream of the *ialA*, *ialB*, *ctpA* and *ppA* genes from *B. bacilliformis* (Mitchell and Minnick, 1995; Mitchell and Minnick, 1997a; Mitchell and Minnick, 1997b).

Genetic Systems

No naturally occurring systems for genetic exchange have been demonstrated for any *Bartonella* species. The presence of the bacteriophage particle in *B. henselae* and *B. bacilliformis* and the packaging and exportation of a diverse collection of chromosomal DNA fragments have raised the question about a possible role of this particle in transduction (Anderson et al., 1994a; Barbian and Minnick, 2000). The introduction of exogenous DNA into *B. bacilliformis* by electroporation (Batttisti and Minnick, 1999) and into *B. henselae* by transconjugation (Dehio and Meyer, 1997a; Lee and Falkow, 1998) and electroporation (Resto-Ruiz et al., 2000) has recently been reported.

Furthermore, the construction of specific mutants by the use of suicide plasmids and site-specific recombination recently has been demonstrated (Battisti and Minnick, 1999) and will undoubtedly prove valuable for a number of different types of studies.

Epidemiology and Control

Bartonellosis has been reported in rodents, insectivores, dogs, cats and humans (Breitschwerdt and Kordick, 2000). The pathogen usually is transmitted to mammals by arthropods. If chronically infected, the host may serve as a reservoir. Transmission also may be enhanced by persistent infection of the vector. For example, *Bartonella* (previously *Grahamella*) species (endemic in rodent populations) also infect the flea, thereby conferring the status of reservoir to the insect (Kreier and Ristic, 1981). Bartonelloses are usually sporadic and epidemic in nature, suggesting that infection of the vector or reservoir is cyclical, rather than continuous, in nature.

Bartonella bacilliformis The only known risk factor for *B. bacilliformis* infection is exposure to bites from phlebotamine sandflies (*Lutzomyia verrucarum*) in South America. The bacterium was once thought to be endemic to the high altitude regions of the Andes because its vector was restricted to that habitat. However, a recent review reports that related sandflies may serve as vectors for the agent and that numerous cases of Oroya fever have occurred in areas of much lower altitude (Alexander, 1995). Because of this, *B. bacilliformis* may be an emerging infectious agent in South America. The high carrier rate and seropositivity of individuals in endemic areas strongly suggest that these individuals serve as a reservoir for the pathogen. In addition, the sandfly vector is possibly infected by the bacterium (Hertig, 1942; Kreier and Ristic, 1981). The best control measure is insecticide application to eradicate sandflies. The use of antibiotics has produced a dramatic decline in the number of deaths caused by Oroya fever. No vaccine is currently available.

Bartonella henselae Data suggest that exposure to cats is the most significant risk factor for contracting bartonellosis from *B. henselae* (Zangwill et al., 1993; Koehler et al., 1994). A very high incidence (89%) of prolonged bacteremia with this pathogen has been observed in cats belonging to CSD patients (Kordick et al., 1995b). In addition, a high incidence of this agent (9–41%) has been seen in control cats of all ages from the United States and Japan (Koehler et al., 1994; Kordick et al., 1995b; Maruyama et al., 1996). Transmission routes include cat scratches and bites and possibly by infected fleas (Zangwill et al., 1993; Koehler et al., 1994). Epidemiologic evidence suggests that fleas also might serve as a vector (Welch et al., 1992; Lucey et al., 1992). *Bartonella henselae* is currently the only *Bartonella* species known to be transmitted to humans by non-arthropod means. Control of the disease includes decreased exposure to cats during immunocompromised states, antibiotic therapy of infected cats and humans, vaccination of the cat reservoir and flea control. A vaccine is not yet available, but is being developed for use in cats.

Bartonella quintana Trench fever is transmitted to humans by exposure to infected human body lice (*Pediculus humanus*). Infected lice perennially shed *B. quintana* in their feces, and humans are subsequently exposed through openings in the skin (e.g., bite site or scratches). The insects can transmit the pathogen for long periods of time (Vinson and Fuller, 1961). Contact with the vector is increased by conditions of overcrowding and poor hygiene. However, lice have not been clearly implicated in the re-emergence of *B. quintana* in the inner city. Overt risk factors for "urban trench fever" include homelessness, non-Caucasian descent and alcoholism (Spach et al., 1995; Jackson and Spach, 1996). Although the arthropod vector of urban trench fever is still unclear, it probably is a blood-sucking insect such as a louse, mite or flea. Spread of trench fever has historically been controlled by de-lousing and hygienic measures. There are no vaccines currently available.

Bartonella elizabethae The epidemiology of *B. elizabethae* is a mystery. The endocarditis patient

from whom the species was isolated had no known exposure to small mammals, nor did he have predisposing factors (cardiac valvular abnormalities, immunosuppression, HIV infection, etc.) that could have contributed to infection (Daly et al., 1993).

Bartonella clarridgeiae Associated with cats, *B. clarridgeiae* may be transmitted to humans by cat bite or (Kordick et al., 1997) by other forms of cat trauma and ectoparasites such as fleas. The coincidental infection of house cats with *B. henselae* and *B. clarridgeiae* suggests the potential for cotransmission of these bacteria to humans (Gurfield et al., 1997).

Disease

Clinical Presentation

CAT SCRATCH DISEASE. This disease (CSD) typically manifests as granulomatous skin lesions (papules or pustules) that develop within one week following a scratch or bite from an infected cat (Fig. 10A). Skin lesions contain areas of necrosis bordered by histiocytes, lymphocytes and giant cells (Johnson and Helwig, 1969). Lymphadenitis is usually unilateral and characteristic, with the proximal draining lymph node displaying adenopathy at 2–3 weeks following infection. Like the skin papules, lymph node lesions are granulomatous microabscesses that contain infiltrated lymphocytes and giant cells, together with follicular hyperplasia (Carithers, 1985). In addition to skin and lymph node involvement, patients with CSD can present with mild fever, malaise and gastrointestinal distress. Complications involving the central nervous system, bone, lung, liver, spleen and eyes also have been reported (Carithers, 1985; Milam et al., 1990; McCrary, 1994; Doyle et al., 1994; Caniza et al., 1995). Within 8–12 weeks, CSD lymphadenopathy typically self-resolves.

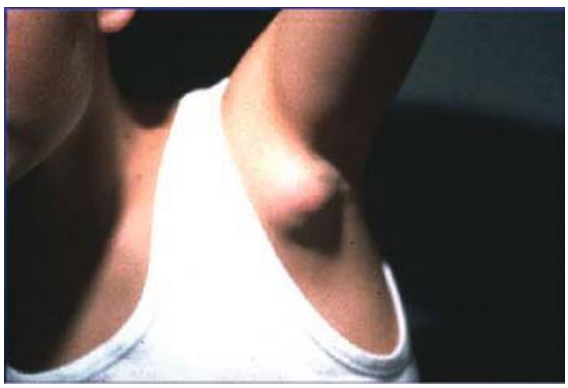

Fig. 10A. The varied clinical manifestations of bartonellosis including: A) cat-scratch disease (CSD), B) bacillary angiomatosis (BA), C) bacillary peliosis (BP) of the liver (hepatis peliosis) and D) aortic valve from a homeless patient with *Bartonella quintana* endocarditis. Note the small vegetation on the valve surface and infiltration with mononuclear cells. The valvular stroma presents with an extensive fibrosis (hematoxylin-phloxine-saffron, original magnification 100×). (CSD photo from Sander, A, 1998; BA photo from Koehler et al., 1992; hepatis peliosis photo from Perkocha et al., 1990; and endocarditis photo courtesy of Hubert Lepidi and Pierre-Edouard Fournier; all with permission.)

The etiologic agent of CSD was the source of speculation for many years. The putative agent was isolated in 1988 from the lymph node of a patient with lymphadenopathy (English et al., 1988) and later named "*Afipia felis*" (Brenner et al., 1991). However, a further link between *A. felis* and CSD weakened when other CSD patients were not found to be seropositive for *A. felis*, and new isolates of the bacterium from other cases were not obtained (Regnery and Tappero, 1995). Later, antibodies to *B. henselae* were found in the sera of patients with CSD, providing a serologic link (Regnery et al., 1992b). Subsequently, isolation and detection of *B. henselae* in CSD patients as well as epidemiological links removed any doubt that *B. henselae* was a cause of CSD (Welch et al., 1992; Regnery et al., 1992a; Zangwill et al., 1993; Dolan et al., 1993; Perkins et al., 1992; Anderson et al., 1993; Bergmans et al., 1995; McGinnis-Hill et al., 1992). However, *B. henselae* does not appear to cause (based on the inability to detect *B. henselae* in clinical specimens or antibodies to *Bartonella* in patient sera) a small but significant percentage of CSD cases. Thus, the possibility exists for an additional etiologic agent of CSD.

The identity of a second etiologic agent (*B. clarridgeiae*) of CSD was recently proposed when high titers of specific anti-*B. clarridgeiae* antibodies were found in patients with CSD (Kordick et al., 1997; Margileth and Baehren, 1998). In addition, *B. clarridgeiae* has been isolated from cats residing with patients diagnosed with CSD (Clarridge et al., 1995; Kordick et al., 1997). More recently, specific antibodies to the flagellin protein of *B. clarridgeiae* were found in 3.9% of sera from patients diagnosed with CSD (Sander et al., 2000). Although it is clear *B. clarridgeiae* is found in cats, the detection or isolation of *B. clarridgeiae* in clinical specimens from patients with CSD is necessary to confirm the etiologic role of this agent and has not been reported as yet.

BACILLARY ANGIOMATOSIS. Also known as "BA," this illness results from infection with either *B. henselae* or *B. quintana* (Relman et al., 1990; Relman et al., 1992; Koehler et al., 1992; Welch et al., 1992). The syndrome was first observed in AIDS patients in the 1980s (Stoler

et al., 1983; Cockerell et al., 1987; Berger et al., 1989) and, although it usually affects immunodeficient individuals (Stoler et al., 1983; Koehler and Tappero, 1993), it has been reported in immunocompetent patients (Cockerell et al., 1990; Lucey et al., 1992; Tappero et al., 1993a). The course of disease can be subacute and insidious in immunodeficient patients, whereas it is sudden in immunocompetent individuals (Schwartzman, 1992). Like CSD, risk factors for BA include exposure to infected cats or cat fleas (Koehler et al., 1994). The disease is characterized by pseudoneoplastic cutaneous or subcutaneous vascular lesions (Fig. 10B), and unlike those of CSD, BA lesions lack granulomatous tissue. The papule or nodule-like lesions of BA contain extensive vascular channels bordered by cuboidal, protuberant endothelium and a multicellular inflammatory infiltrate with polymorphonuclear leukocytes that display leukocytoclastic characteristics (LeBoit et al., 1989; Cockerell et al., 1990). In addition, the lesions usually contain aggregates of bartonellae when stained by Warthin-Starry silver stain (LeBoit et al., 1988; Angritt et al., 1988). Cutaneous lesions of BA are similar to verruga peruana and superficially resemble vascular neoplasms such as Kaposi's sarcoma (LeBoit et al., 1988; Webster et al., 1992) or pyogenic granulomas (Koehler and Tappero, 1993). The lesions can last for several months (Koehler et al., 1992). Subcutaneous or visceral BA can involve a host of organ systems including the brain, bone, lymph nodes and eyes (Koehler et al., 1992; Spach et al., 1992; Koehler and Tappero, 1993; Tappero et al., 1993b; Kemper et al., 1990; Waldvogel et al., 1994; Slater et al., 1994; Golnik et al., 1994; Tappero et al., 1993b).

BACILLARY PELIOSIS. *Bartonella henselae* also is the agent of bacillary peliosis (BP; Marullo et al., 1992; Slater et al., 1992; Welch et al., 1992; Tappero et al., 1993b). The BP lesions are characterized by cystic blood filled cavities (Perkocha et al., 1990; Garcia-Tsao et al., 1992; Fig. 10C). Gastrointestinal distress, fever and chills may accompany peliosis, and the syndrome may occur alone or in combination with BA or bacteremic syndrome. The disease can involve single or multiple organs such as the liver, spleen and lymph nodes, and the lesions contain bartonellae when stained by Warthin-Starry silver stain (Perkocha et al., 1990). In the liver, BP can produce hepatomegaly, and patients typically show elevated serum levels of liver enzymes (e.g., γ-glutamyl transferase and alkaline phosphatase; Perkocha et al., 1990). Death following peliosis-induced liver failure also has been reported (Perkocha et al., 1990).

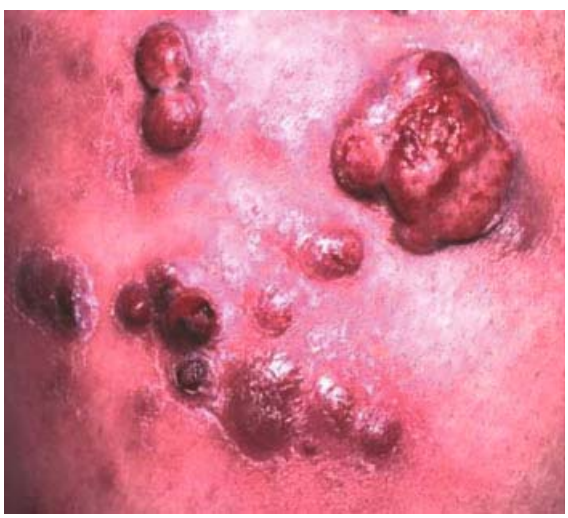

Fig. 10B. The varied clinical manifestations of bartonellosis including: A) cat-scratch disease (CSD), B) bacillary angiomatosis (BA), C) bacillary peliosis (BP) of the liver (hepatis peliosis) and D) aortic valve from a homeless patient with *Bartonella quintana* endocarditis. Note the small vegetation on the valve surface and infiltration with mononuclear cells. The valvular stroma presents with an extensive fibrosis (hematoxylin-phloxine-saffron, original magnification 100×). (CSD photo from Sander, A, 1998; BA photo from Koehler et al., 1992; hepatis peliosis photo from Perkocha et al., 1990; and endocarditis photo courtesy of Hubert Lepidi and Pierre-Edouard Fournier; all with permission.)

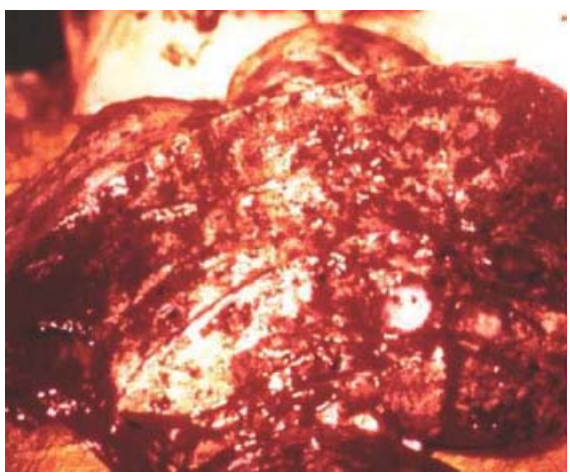

Fig. 10C. The varied clinical manifestations of bartonellosis including: A) cat-scratch disease (CSD), B) bacillary angiomatosis (BA), C) bacillary peliosis (BP) of the liver (hepatis peliosis) and D) aortic valve from a homeless patient with *Bartonella quintana* endocarditis. Note the small vegetation on the valve surface and infiltration with mononuclear cells. The valvular stroma presents with an extensive fibrosis (hematoxylin-phloxine-saffron, original magnification 100×). (CSD photo from Sander, A, 1998; BA photo from Koehler et al., 1992; hepatis peliosis photo from Perkocha et al., 1990; and endocarditis photo courtesy of Hubert Lepidi and Pierre-Edouard Fournier; all with permission.)

OROYA FEVER. Also referred to as "Carrion's disease" or "verruga peruana," this disease is endemic to South America (Alexander, 1995), particularly in Peru, Colombia and Ecuador (Kreier and Ristic, 1981), and has afflicted travelers visiting this region (Matteelli et al., 1994). The course of disease is very unusual and transpires sequentially in two disparate stages. The primary (hematic) phase is characterized by an acute bacteremia that occurs within four weeks of being bitten by a contaminated phlebotamine sandfly. From the inoculation site, *B. bacilliformis* efficiently colonizes the entire circulatory system. Nearly every erythrocyte becomes parasitized and approximately 80% are lysed (Hurtado et al., 1938), presumably by splenic culling (Reynafarje and Ramos, 1961). Case fatalities can reach 40–88% without antibiotic therapy (Weinman, 1965; Gray et al., 1990). The acute phase can be accompanied by anorexia, headache and coma in fatal cases (Roberts, 1995). Secondary infectious diseases such as salmonellosis, shigellosis, or recurrence of toxoplasmosis or tuberculosis are not uncommon during the hematic phase and can complicate patient prognosis (Urteaga and Payne, 1955; Cuadra, 1956; Gray et al., 1990). Patients present with secondary (tissue) phase symptoms, approximately four weeks following resolution of the primary phase. Tissue involvement results from bacterial invasion of the endothelial cells lining the capillary beds and generates bacteria-filled vacuoles (termed "rocha lima inclusions") and localized cellular proliferation leading to the formation of nodule or papule lesions termed "verruga peruana" (Arias-Stella et al., 1986). Verruga eruptions are usually cutaneous but may involve mucous membranes and viscera (Ricketts, 1949). Cutaneous lesions are found on the skin of the head and extremities and can persist for several weeks to months. Though the secondary phase is rarely fatal, verrugas can bleed and scar the patient (Weinman, 1965). Anemia is no longer present during the tissue stage (Ricketts, 1949), but viable bacteria can be isolated from the blood, marrow and hemangioma tissue of patients with verruga peruana.

TRENCH FEVER. A louse-borne disease caused by *B. quintana*, it is distributed throughout the world. The illness was first reported during World War I and was responsible for several epidemics among troops (McNee and Renshaw, 1916). Only influenza caused a greater morbidity and loss of man-hours during World War I (Strong, 1918). After a brief period of quiescence, trench fever reappeared during World War II (Kostrzewski, 1950) and has occurred sporadically thereafter. Currently, infection with *B. quintana*, the so-called "urban trench fever," is re-emerging in homeless, inner-city populations of the United States (Jackson and Spach, 1996). Cases of urban trench fever also have been reported in France (Raoult et al., 1996). Trench fever occurs approximately nine days following inoculation by the bite of an infected louse (Byam, 1919). Although symptoms of trench fever vary significantly between patients, the disease usually presents with mild to moderately severe fever, chills, malaise, myalgia and bone pain that is especially prominent in the tibia (hence "shinbone fever"; Varela et al., 1969). Occasionally, patients develop splenomegaly and a maculopapular rash resembling the rose spots of typhoid fever (Strong, 1918). The illness usually lasts about one week, but febrile episodes may be recurrent and the bacteremia protracted. Recent data also have implicated *B. quintana* as an emerging etiologic agent of bacillary angiomatosis and endocarditis.

ENDOCARDITIS. Although many microorganisms can cause endocarditis, data suggest that blood culture-negative cases often involve *Bartonella* species (Raoult et al., 1996; Fig. 10D). Endocarditis has been linked to infection with *B. henselae* (Hadfield et al., 1993; Drancourt et al., 1996; Raoult et al., 1996), *B. quintana* (Spach et al., 1993; Drancourt et al., 1995; Jalava et al., 1995; Raoult et al., 1996) and *B. elizabethae* (Daly et al., 1993). Endocarditis caused by bartonellae has been reported for both immunocompetent and immunosuppressed individuals.

BACTEREMIC SYNDROME AND FEVER OF UNKNOWN ORIGIN. Cases of relapsing

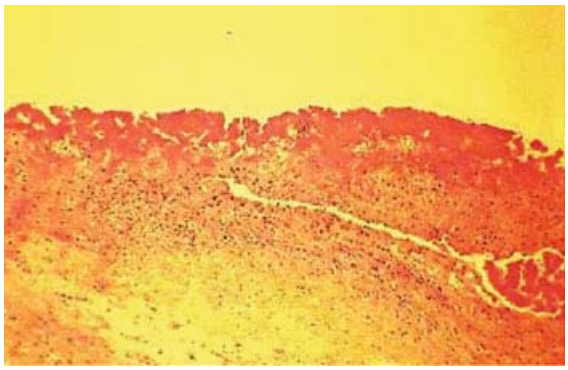

Fig. 10D. The varied clinical manifestations of bartonellosis including: A) cat-scratch disease (CSD), B) bacillary angiomatosis (BA), C) bacillary peliosis (BP) of the liver (hepatis peliosis) and D) aortic valve from a homeless patient with *Bartonella quintana* endocarditis. Note the small vegetation on the valve surface and infiltration with mononuclear cells. The valvular stroma presents with an extensive fibrosis (hematoxylin-phloxine-saffron, original magnification 100×). (CSD photo from Sander, A, 1998; BA photo from Koehler et al., 1992; hepatis peliosis photo from Perkocha et al., 1990; and endocarditis photo courtesy of Hubert Lepidi and Pierre-Edouard Fournier; all with permission.)

fever with persistent bacteremia have been reported in patients infected with *B. henselae* (Slater et al., 1990; Welch et al., 1992; Welch et al., 1993; Regnery et al., 1992a; Lucey et al., 1992) and *B. quintana* (Koehler et al., 1992; Maurin et al., 1994). The possibility that *B. henselae* is a relatively common cause of fever of unknown origin (FUO) in pediatric patients was suggested by a study that found 7 of 146 patients diagnosed with FUO had high antibody titers to *Bartonella* (Jacobs and Schutze, 1998). In a separate study 34% of patients with prolonged fever or FUO were found to have anti-*Bartonella* antibodies (Tsukahara et al., 2000). These later two studies suggest that *Bartonella* spp. may be a relatively common cause of FUO in immuncompetent individuals.

Diagnosis

Because *Bartonella* spp. are difficult to culture from clinical isolates, indirect identification of the pathogen is usually based upon individual patient history and symptomology (see "Clinical Presentation"). If culture of the agent is possible, identification of the bacterium can be made using the techniques described in "Identification" above.

CAT-SCRATCH DISEASE. Patients are generally immunocompetent children or young adults. There is a history of cat bite or scratch resulting in a papule or pustule-like lesion. Diagnostic characteristics include a positive CSD-skin test (more recently been replaced by serologic testing), an unexplainable lymphadenopathy, and characteristic lesion histopathology (granuloma and abscess formation and bacilli present when sectioned and stained by Warthin-Starry silver stain). Diagnosis is based on tests for seroconversion (Regnery et al., 1992a; Zangwill et al., 1993; Patnaik et al., 1992) and a more sensitive ELISA system (Barka et al., 1993). A titer of >64 by immunofluorescence assay is considered positive for *B. henselae* by the United States Centers for Disease Control and Prevention (CDC; Regnery et al., 1992b). When available, PCR on lymph node aspirates or biopsies is often successful (Anderson et al., 1994b).

BACILLARY ANGIOMATOSIS. Patients usually are immunocompromised. Cutaneous and/or subcutaneous nongranulomatous vascular lesions affect a variety of organs. Bacilli in sections of affected tissue can be visualized using Warthin-Starry silver stain. Diagnostic testing includes an anti-*B. henselae* IgG indirect fluorescent antibody assay (Regnery et al., 1992a; Zangwill et al., 1993; Patnaik et al., 1992) and an ELISA system (Barka et al., 1993).

BACILLARY PELIOSIS. Patients usually are immunocompromised. Diagnosis based on symptoms is difficult and may require biopsy. Cystic blood-filled liver lesions that are similar to those of BA, together with bacilli in sections of affected tissue, are diagnostic. Hepatomegaly and elevated serum levels of liver enzymes are characteristic. Bacillary peliosis should be suspected if patient has BA or bacteremic syndrome. Detection reagents used for BA diagnosis also can be used for bacillary peliosis.

OROYA FEVER. Patients have a history of travel or residence in South America and may have history of sandfly bite. Diagnostic symptoms of the primary stage include a dramatically low erythrocyte count (~500,000/mm^3; Hurtado et al., 1938; Reynafarje and Ramos, 1961; Kreier and Ristic, 1981) and numerous infected erythrocytes when blood smears are stained with eosin and thiazine (Knobloch et al., 1985). Secondary (tissue) phase presents with cutaneous angiomatous lesions (verruga peruana) that contain bacteria when sectioned and stained with Warthin-Starry silver stain.

TRENCH FEVER. Patients likely have a history of infestation with body lice (*Pediculus humanus*). Chronic alcohol abuse, homelessness and non-Caucasian descent are risk factors (Jackson and Spach, 1996). Diagnostic characteristics include pain in the bones (especially the tibia), fever and persistent bacteremia for several weeks to months following resolution of symptoms (Vinson et al., 1969). The presence of seroconversion to *B. quintana* can be tested; however, the antibody response usually is not specific to only *B. quintana* owing to crossreactivity within the genus (Regnery et al., 1992a).

ENDOCARDITIS. *Bartonella* infection should be considered in blood culture-negative cases of endocarditis. Patients usually have predisposing heart conditions or are at high risk for contracting infection with *B. henselae* or *B. quintana* (see *B. henselae* or *B. quintana* under "Epidemiology and Control"). Vegetative lesions are common in cases of *Bartonella endocarditis* (Daly et al., 1993; Raoult et al., 1996). The difficulty of culture from patient isolates and serological crossreactivity between *Chlamydia* spp., *Bartonella* spp. and *Coxiella burnetii* can complicate diagnosis (Drancourt et al., 1995; Knobloch et al., 1988b; La-Scola and Raoult, 1996). Seropositivity for *Bartonella* infection can be tested with the detection reagents described for diagnosis of BA. Molecular techniques involving PCR also should be used, particularly if species-level identification is desired (see "Identification").

BACTEREMIC SYNDROME. Patients are usually immunocompromised. Persistent bacteremia and recurring fevers, together with chronic fatigue and malaise, are diagnostic. Diagnosis can be made by blood cultures with subsequent identification protocols (see "Identification") or

by testing for seropositivity to *B. henselae* or *B. quintana* as described for BA.

Antimicrobial Therapy

A variety of antibiotics have been used successfully to treat bartonelloses. Antimicrobial therapy varies depending upon *Bartonella* species and in some cases is syndrome-dependent. CSD and trench fever generally are self-limiting illnesses that do not require therapy unless the infection is systemic. In contrast, BA can be life threatening and requires prompt antibiotic therapy. The immune state of the patient is also an important consideration for prescribing the type and duration of antimicrobial therapy for bartonellosis. For example, BA therapy for healthy patients is approximately three weeks, whereas immunocompromised patients may require several weeks to months, or possibly a lifetime, of therapy to resolve the infection (Adal et al., 1994). Relapse of bartonellosis is common even with antimicrobial therapy. Bartonellae are typically sensitive to most antibiotics in vitro (Maurin and Raoult, 1996), but are frequently resistant to them during human therapy. This observation may reflect the inability of the chemotherapeutic agent to access the pathogen located within host cells. Aminoglycosides are particularly bactericidal for bartonellae in vivo (Musso et al., 1995) and are recommended for therapy. Although optimal antibiotic regimens are still unclear, a list of antimicrobials that have successfully been used in treating various manifestations of bartonellosis is given in Table 4.

Immunity

The general course of bartonellosis begins with transient or persistent bacteremia that may involve erythrocyte parasitism, depending upon bacterial species. The pathogen subsequently infects the vascular bed within a variety of tissues, where vascular lesions are produced. Tissue involvement can be chronic, and presumably the pathogen is shed from the affected tissues into the bloodstream. Lymphadenopathy is a frequent complication in bartonellosis. The immune status of the host is a risk factor for infection. Typically, immunocompromised patients are at higher risk of opportunistic infection by bartonellosis than healthy individuals are. As a rule of thumb, trench fever, CSD, endocarditis and Oroya fever take place regardless of immune status of the patient, whereas BA, bacillary peliosis and bacteremic syndrome occur mainly in immuocompromised individuals. Bartonellosis symptoms are generally more pronounced in immunodeficient patients. In cases of BA, many patients display a marked CD4+ lymphocyte leukocytopenia, with counts of less than 100 (LeBoit et al., 1989; Koehler and Tappero, 1993). At best, the human immune response against *Bartonella* is poorly characterized, and for some species (*B. elizabethae* and *B. clarridgeiae*) it has never been studied. Because *Bartonella* spp. are found in a variety of body fluids and cells, involvement of both humoral and cellular arms of the immune system needs to be invoked for resolution of infection.

BARTONELLA BACILLIFORMIS. Lifelong humoral immunity supposedly results from an

Table 4. Antimicrobial agents used to treat bartonelloses.

Syndrome	Antimicrobial Agent(s)	Reference(s)
BA	Erythromycin or doxycycline	Bartlett, 1996
	Also chloramphenicol, sulfa-trimethoprim, aminoglycosides, azithromycin	Milde et al., 1995
	Ciprofloxacin	
	Clarithromicin	
Bacteremic syndrome	Erythromycin or doxycycline	Adal et al., 1994
BP	Erythromycin[a]	Perkocha et al., 1990
		Bartlett, 1996
CSD[b]	Ciprofloxacin, sulfa-trimethoprim or gentamicin	Bartlett, 1996
	Erythromycin or doxycycline	Smith, 1997
	Azithromycin	Bass, 1998
Endocarditis	Nafcillin + gentamicin	Daly et al., 1993
Oroya fever	β-Lactams, tetracycline + streptomycin, aminoglycosides, chloramphenicol if coninfected by enterics	Weinman, 1965
Pulmonary nodules	Doxycycline	Caniza et al., 1995
Retinal vasculitis and vitreitis	Ciprofloxacin	Soheilian et al., 1996
Trench fever[a]	Tetracycline or chloramphenicol	Moulder, 1974

Abbreviations: BA, bacillary angiomatosis; BP, bacillary peliosis; and CSD, cat-scratch disease.
[a]Alternatives as in BA.
[b]Usually self-limiting (does not require antibiotic therapy unless systemic).

active infection with *B. bacilliformis* (Ricketts, 1949; Weinman, 1965). Seropositivity (mainly IgM isotype) in endemic areas of Peru can reach 60% of the population, and many of these individuals are healthy (Knobloch et al., 1985). However, in spite of circulating antibody, many individuals who are asymptomatic or posteruptive for verruga peruana are often blood culture-positive for *B. bacilliformis* (Howe, 1943), and carrier rates can reach 15% in some areas of Peru (Herrer, 1953).

Immunosuppression is characteristic and probably results from the acute anemia and leukocytosis. Although a variety of proteins from *B. bacilliformis* are immunogenic (Knobloch, 1988a), the most prominent antigens include a GroEL homologue (BB65) (Knobloch and Schreiber, 1990), the flagellin protein (42 kDa), a phage coat protein (31.5 kDa) and an uncharacterized protein of 45 kDa (Minnick, 1994). The gene encoding a 43-kDa lipoprotein of *B. bacilliformis* reactive with human sera from bartonellosis cases was recently cloned, sequenced and expressed (Padmalayam et al., 2000b).

BARTONELLA QUINTANA. Although symptoms during *B. quintana* infections are highly variable, patients generally produce low-titer, complement-fixing antibody during the early phase of convalescence (Varela et al., 1969). However, in spite of antibodies, patients frequently display persistent and protracted bacteremia (Varela et al., 1969; Koehler et al., 1992; Maurin et al., 1994). In addition, *B. quintana* is markedly resistant to killing by complement fixed by classical or alternative pathways (Myers and Wisseman, 1978). Infection frequently is accompanied by leukocytosis (Vinson et al., 1969), possibly enhancing bacteremia.

BARTONELLA HENSELAE. Patients infected with *B. henselae* are generally seropositive when assayed by indirect fluorescence antibody tests (Regnery et al., 1992a). Antibodies apparently opsonize the pathogen and enhance production of oxygen radicals following phagocytosis, but do not enhance complement activation by the classical pathway. In fact, complement fixation mainly proceeds via the alternative pathway (Rodriguez-Barradas et al., 1995a). Persistent bacteremia with *B. henselae* is common in both humans and cats (Koehler et al., 1994), and respective antibody titers do not necessarily correlate with protection against the pathogen. A 17-kDa antigen has been shown to be highly reactive with human sera from patients with CSD (Anderson et al., 1995). The gene encoding this antigen is conserved among most *Bartonella* spp. but shows extensive amino acid sequence divergence (Sweger et al., 2000). A second protein of 83-kDa has been reported to react with human sera from patients with CSD (McGill et al., 1998). In addition to antibody reactivity, the cellular immune response in humans with CSD has been well recognized (Gerber et al., 1986). The aggravating cellular immune response to *B. henselae* both contributes to the pathogenesis of CSD and forms the basis of diagnostic skin test. This delayed-type hypersensitivity and T helper 1-type cytokine response has been recently reproduced in mouse models (Arvand et al., 1998; Karem et al., 1999).

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Prokaryotes (2006) 5:493–528
DOI: 10.1007/0-387-30745-1_20

CHAPTER 3.1.19

The Order Rickettsiales

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Introduction

Rickettsiae are genetically related α-Proteobacteria with fascinating obligately intracellular lifestyles usually involving alternation between vertebrate and invertebrate hosts. These feats are accomplished with small, evolutionarily selected genomes. The agents classified as *Rickettsia*, *Orientia*, *Ehrlichia*, *Anaplasma*, *Wolbachia* and *Neorickettsia* cause diseases (such as epidemic louse-borne typhus fever, Rocky Mountain spotted fever, scrub typhus, human ehrlichioses, and bovine anaplasmosis) that have repeatedly altered the course of history (Zinsser, 1935), unexpectedly kill previously healthy tick-exposed persons, occur as highly prevalent endemic febrile illness, and are veterinary pathogens of major economic importance.

Although mostly known as pathogens transmitted by hematophagous ticks and insects, some of the Rickettsiales are insect endosymbionts (*Wolbachia*) that have evolved dramatic abilities to manipulate their host populations, and others (*Neorickettsia*) have a life cycle within a trematode that involves a series of aquatic hosts including snails, fish and insects.

The biology of the rickettsiae is difficult to define and investigate separately from the biology of their hosts. As a result of obligately intracellular growth and propagation difficulties in antibiotic-free cell culture or even in embryonated eggs, rickettsial physiology and metabolism are challenges to elucidate. Consequently, the study of these microorganisms and their diseases has been relatively neglected and there is the mistaken impression that they must be unimportant and uninteresting. Nothing could be further from the truth. The state of Rickettsiales knowledge presented in this chapter is to suggest to microbiologists opportunities for increasing scientific and medical understanding in this field.

On the basis of phylogenetic analysis of the 16S rRNA gene sequence (Fig. 1), the order Rickettsiales is a monophyletic group (Roux and Raoult, 1999) that currently includes the families Rickettsiaceae and Anaplasmataceae (Stothard and Fuerst, 1995; Dumler et al., 2001). All bacteria in the order Rickettsiales are Gram-negative α-Proteobacteria. The arrangement of the rRNA genes in Rickettsiales is unusual. While the 16S, 23S, and 5S rRNA genes are linked together in other bacteria, in rickettsial organisms, the 16S rRNA gene is separated from the 23S and 5S rRNA gene cluster, and the 23S rRNA gene is preceded by a gene that codes for methionyl-tRNA formyltransferase (Andersson et al., 1999; Massung et al., 2001). The genomic rearrangement of the rRNA genes preceded the divergence of the two families.

On the basis of phylogenetic analysis of 16S rRNA gene sequences, many species classified traditionally as Rickettsiales do not cluster with and have been removed from the Rickettsiaceae and Anaplasmataceae families. These include *Rickettsiella grylli*, *Coxiella burnetii*, *Wolbachia persica*, *Bartonella*, *Grahamella*, *Eperythrozoon ovis*, *Hemobartonella felis* and *H. muris* (Roux and Raoult, 1999).

The family Anaplasmataceae includes four genera, *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia*. Members of the genera *Anaplasma*, *Ehrlichia*, and *Neorickettsia* in the family Anaplasmataceae are small obligately intracellular, Gram-negative, pleomorphic, coccoid to ellipsoidal organisms that reside in membrane-bound cytoplasmic vacuoles and form characteristic microcolonies resembling mulberries, termed morulae (Latin *morum* = mulberry; Fig. 2). Electron microscopy reveals two distinct morphological forms: a larger reticulate and a smaller dense-core cell. Both forms divide by binary fission, which is strong evidence that they are not stages in a developmental cycle (Popov et al., 1998; Popov et al., 2000). All known members of these genera are pathogenic for mammals, and some are pathogenic for human beings. Most members of the Anaplasmataceae target hematopoietic cells. *Anaplasma marginale* infects the erythrocytes of cattle and wild ruminants, and *A. bovis* infects the mononuclear cells of cattle. *Anaplasma phagocytophila* and

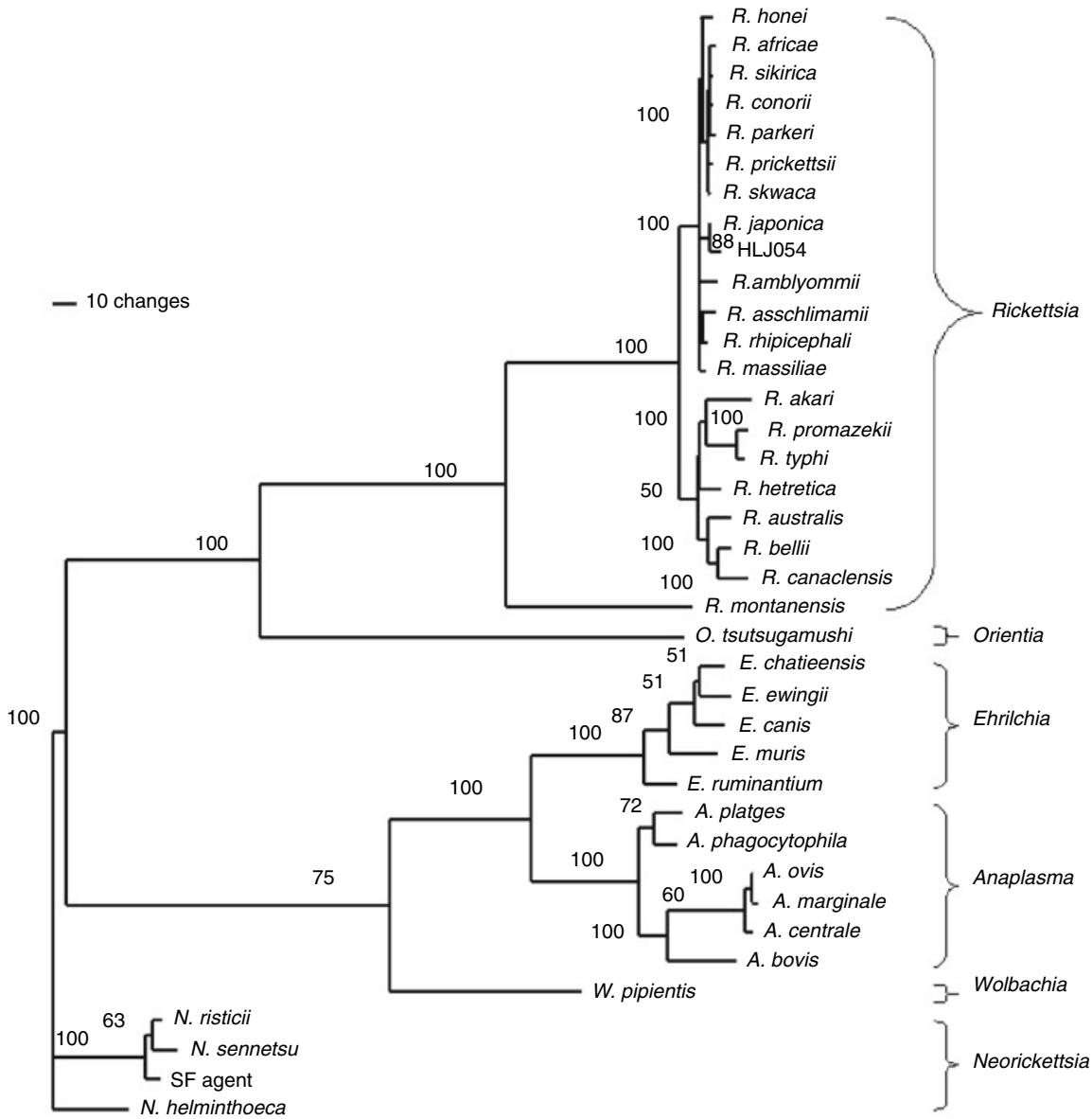

Fig. 1. Phylogenetic relationships of the organisms in the order Rickettsiales based on the DNA sequences of the 16S rRNA genes (GenBank accession numbers: *R. honei*, AF060705; *R. africae*, RIRRGDA; *R. sibirica*, RIRRS16SRG; *R. conorii*, RIRRGDH; *R. parkeri*, RIRRRDA; *R. rickettsii*, RIRRGDP; *R. slovaca*, RIRRGDX; *R. japonica*, RIRRGDL; Strain HLJ054, AF178037; *R. amblyommii*, RAU11012; *R. aeschlimannii*, RAU74757; *R. rhipicephali*, RIRRGDO; *R. massiliae*, RIRRGDI; *R. akari*, RAU12458; *R. prowazekii*, RIRGGSA; *R. typhi*, RIRRGDU; *R. helvetica*, RIRRGDK; *R. australis*, RAU17644; *R. bellii*, RBU11014; *R. canadensis*, RCU15162; *R. montanensis*, RIRRGDN; *O. tsutsugamushi*, RIRRTKP16B; *E. chaffeensis*, AF147752; *E. ewingii*, EEU96436; *E. canis*, AF162860; *E. muris*, EMU15527; *E. ruminantium*, CRDNA; *A. platys*, AF156784; *A. phagocytophila*, AY055469.1; *A. ovis*, AF309865; *A. marginale*, APMRR16SA; *A. centrale*, AF318944; *A. bovis*, EBU03775; *W. pipientis*, U23709; *N. risticii*, EHRRGBSA; *N. sennetsu*, EHRRRNAI; SF agent, EHRSF; and *N. helminthoeca*, NHU12457). The length of each pair of branches represents the distance between sequence pairs. The numbers on the branch indicate the bootstrap values.

A. platys infect mammalian neutrophils and canine platelets, respectively. All members of the genus *Ehrlichia* except *E. ruminantium* and *E. ewingii* infect monocytes. *Ehrlichia ruminantium* infects endothelium, and *E. ewingii* infects granulocytes.

Wolbachia pipientis and the genus *Anaplasma* form sister taxons by 16S rRNA gene and *groEL* gene sequence phylogeny (Wen et al., 1995; Yu et al., 2001). Thus, *W. pipientis* was reclassified into the family Anaplasmataceae (Dumler et al., 2001).

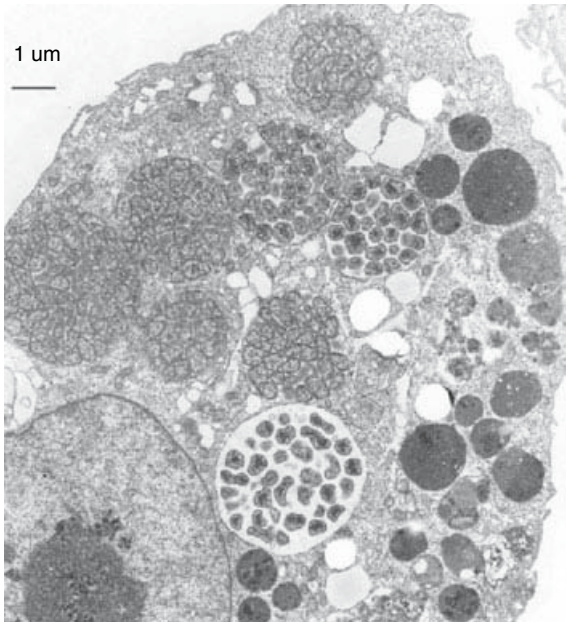

Fig. 2. Electron photomicrograph of a canine macrophage-like cell line (DH82) infected with *Ehrlichia canis* contains eight vacuoles (morulae) filled with ehrlichiae. Five morulae contain reticulate cell forms; three contain dense-core forms (particularly the morula in the lower center of the figure). See the Microbe Library website (http://www.microbelibrary.org/) for more electron micrographs of *Ehrlichia*.

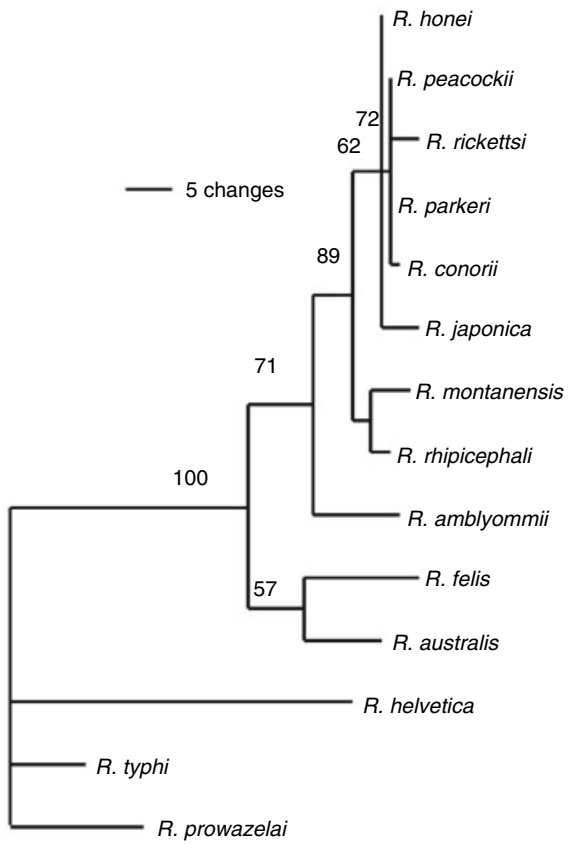

Fig. 3. Phylogenetic relationships of the organisms in the genus *Rickettsia* based on the DNA sequences of the 17-kDa protein genes (GenBank accession numbers for *R. honei*, A060704; *R. peacockii*, AF260571; *R. rickettsii*, RIRANTRR; *R. parkeri*, RPU17008; *R. conorii*, RIRANT17KA; *R. japonica*, RIR17KGCA; *R. montanensis*, RMU11017; *R. rhipicephali*, RPU11020; *R. amblyommii*, RAU11013; *R. felis* AF195118; *R. australis*, RIRTRAPRO; and *R. helvetica*, AF181036). The length of each pair of branches represents the distance between sequence pairs. The numbers on the branch indicate the bootstrap values.

Genus Rickettsia

Phylogeny

The evolution of the genus *Rickettsia* is demonstrated by phylogenetic trees developed from DNA sequence data that are based on the 16S rRNA, *groEL*, citrate synthase (*glt*), *rompA*, *rompB* and other genes (Roux and Raoult, 1995; Stothard and Fuerst, 1995; Roux et al., 1997; Fournier et al., 1998; Roux and Raoult, 2000; Fig. 3). The most ancestral species of the genus appear to be *R. bellii*, *R. canadensis*, and the AB male killing bacterium (Stothard et al., 1994). *Rickettsia* and mitochondria evolved from a common ancestor (Andersson et al., 1998). The phylogenetic tree may be overpopulated by species' names of spotted fever group rickettsiae, many of which are closely related and have been given separate names. The discovery of organisms very closely related to *R. bellii* in herbivorous pea aphids and associated with plant pathology (e.g., papaya bunchy top disease) suggests that knowledge and diversity of *Rickettsia* may have been determined more by medical investigations and medical entomology than by consideration of the complete picture (Chen et al., 1996; Davis et al., 1998).

Taxonomy

The family Rickettsiaceae consists of two genera: *Rickettsia* and *Orientia*. *Rickettsia* is further divided into two groups: the spotted fever group (SFG) and typhus group (TG), based on the difference in lipopolysaccharide (LPS) antigens. The TG rickettsiae include *R. prowazekii* and *R. typhi*. The SFG rickettsiae include *R. akari*, *R. australis*, *R. africae*, *R. conorii*, *R. honei*, *R. japonica*, *R. sibirica R. helvetica*, *R. slovaca*, *R. massiliae*, *R. rhipicephali*, *R. aeschlimannii*, *R. montanensis*, *R. parkeri*, and most likely *R. felis* (Roux and Raoult, 2000; Zhang et al., 2000; Bouyer et al., 2001). The numbers of named SFG rickettsiae have increased rapidly in recent years

owing to improved isolation approaches and molecular methods for identification; however, there is no consensus about the criteria used to define a species of SFG rickettsiae. Many designated species of SFG rickettsiae actually may be clones or strains of a single species, according to the standards used for classifying other bacteria. Other *Rickettsia* (such as *R. bellii*, *R. canadensis* and the AB male killing bacterium) do not fit into either the SFG or TG.

Habitat

Rickettsia are small (0.3–0.5 × 0.8–2.0 μm), Gram-negative, aerobic coccobacilli. They are obligately intracellular and reside free in the cytoplasm of the eukaryotic host cell where they divide by binary fission. SFG rickettsiae may also reside in the nucleus of the eukaryotic host cells. *Rickettsia* are dependent on arthropods (ticks, mites, fleas and lice) for their persistence in nature. For some, the arthropod host is both reservoir and vector. Transovarian transmission of the agent from the infected female to the next generation through the ova is the essential mechanism for many species (Burgdorfer, 1988).

Isolation

Isolation of rickettsiae from blood, buffy coat, or plasma historically employed inoculation of adult male guinea pigs or embryonated chicken eggs for *Rickettsia* and mice for *Orientia* (Walker, 1996a). More often at present Vero, L-929, HEL, and MRC5 cells in antibiotic-free media are inoculated with heparin-anticoagulated plasma or buffy coat ideally collected before antirickettsial treatment. Isolation of rickettsiae is enhanced by centrifugation of the inoculum onto monolayers in shell vials (LaScola and Raoult, 1996). Rickettsiae are detected by examining monolayers stained by Giemsa, Gimenez, or immunofluorescence methods, with 82% of positive samples identified after 48 hours incubation. Appropriate precautions should be taken for handling these P3 level pathogens.

Identification

Members of the genus *Rickettsia* have a typical Gram-negative bacterial cell wall that contains LPS, peptidoglycan, and outer membrane proteins. Isolated organisms can be identified as *Rickettsia* by the obligate intracellular parasitism, ultrastructural identification of cell wall morphology and of location free in the cytosol, reactivity with group-, species-, or strain-specific monoclonal or polyclonal antibodies, and restriction fragment length polymorphism of particular genes. Currently DNA sequence analysis of the genes of the 17-kDa lipoprotein, outer membrane proteins A and B, citrate synthase, and 16S rRNA is utilized most often to identify rickettsial isolates (Walker, 1996a).

Preservation

Stocks of *Rickettsia* are usually preserved frozen at –70 to –80°C or in the vapor phase of liquid nitrogen. They may also be preserved in a lyophilized state.

Physiology

Adherence to the Host Cell

Rickettsiae are inoculated into the dermis of the skin by a tick bite or through damaged skin from the feces of lice or fleas. Rickettsiae spread through the bloodstream and infect the endothelium. Adherence to the host cell is the first step of rickettsial pathogenesis. The adhesins must be outer membrane proteins. The outer membrane protein OmpA has been implicated as an adhesin of *R. rickettsii* because monoclonal antibodies to OmpA block *R. rickettsii* attachment (Li and Walker, 1998). The rickettsial adhesin must have evolved prior to the divergence of the SFG and TG rickettsiae because both groups are obligately intracellular. Lack of OmpA in TG rickettsiae indicates that there must be a more conserved or even more important adhesin in rickettsiae than OmpA for mammalian cells. There is evidence that both OmpA and OmpB are adhesins of *R. japonica* (Uchiyama, 1999). The receptor for *Rickettsia* has yet to be identified. Although the main target cells of *Rickettsia* in vivo are endothelial cells, rickettsiae can infect virtually every cell line in vitro. Thus, either the receptor for *Rickettsia* is ubiquitous among cells, or rickettsiae can bind to different receptors.

Invasion of the Host Cell

Upon attaching to the host cell membrane, rickettsiae are phagocytosed by the host cell. *Rickettsia* and *Orientia* are believed to induce host cell phagocytosis because they can enter cells that normally do not phagocytose particles (Walker, 1984a). Once phagocytosed by the host cell, rickettsiae and orientiae are observed to enter quickly the cytoplasm outside of the phagosome. Thus, they are said to escape the phagosome. Neither the molecules nor the mechanisms involved in rickettsial escape from the phagosome have been determined. Phospholipase A_2 (PLA_2) has been suggested to be involved in the lysis of the phagosomal membrane by *Rickettsia* (Winkler and Miller, 1982; Walker et al., 1984b; Winkler

and Daugherty, 1989; Silverman et al., 1992; Manor et al., 1994; Winkler et al., 1994; Ojcius et al., 1995); however, genome sequencing did not reveal a gene encoding a PLA_2. To search for the rickettsial PLA_2 gene by comparing rickettsial genes with PLA_2 genes of bees or snakes might not be warranted since the rickettsial PLA_2 involved in the lysis of host cell membrane may be very different. We have found a rickettsial protein (Accession no. RPXX03) with a calcium-independent PLA_2 motif (Yu et al., 2000c). The rickettsial protein is homologous to the exoenzyme (ExoU) of *Pseudomonas aeruginosa*. ExoU expression in *P. aeruginosa* is linked to acute cytotoxicity (Vallis et al., 1999). The enzymatic activity of the rickettsial protein has yet to be characterized.

Movement within and Release from the Host Cell Observations in cell culture systems suggest that the mechanisms of intracellular movement and destruction of the host cell differ among the spotted fever and typhus group rickettsiae (Silverman and Santucci, 1988; Silverman, 1997). TG rickettsiae are released from host cells by lysis of the cells. After infection with *R. prowazekii* or *R. typhi*, the rickettsiae continue to multiply until the cell is packed with organisms and then bursts. Cell death possibly results from apoptosis or membranolytic activity that previously has been hypothesized to be due to phospholipase A_2 (Winkler and Miller, 1982; Walker et al., 1984b; Winkler and Daugherty, 1989; Silverman et al., 1992; Manor et al., 1994; Winkler et al., 1994; Ojcius et al., 1995). Before lysis, TG rickettsia-infected host cells have a normal ultrastructural appearance.

SFG rickettsiae seldom accumulate in large numbers and do not burst the host cells. They escape from the cell by stimulating polymerization of host cell-derived F-actin tails (Fig. 4), which propel them through the cytoplasm and into tips of filopodia, from which they emerge (Schaechter et al., 1957; Teysseire et al., 1992; Heinzen et al., 1993; Heinzen et al., 1999). Infected cells exhibit signs of membrane damage associated with an influx of water, which is sequestered within cisternae of dilated, rough endoplasmic recticulum (Walker and Cain, 1980a). The means by which rickettsiae damage host cell membranes is uncertain, but there is experimental evidence to suggest a role for free radicals of oxygen, a phospholipase, or a protease (Walker et al., 1983b; Walker et al., 1984b; Walker et al., 2002; Silverman and Santucci, 1988; Silverman and Santucci, 1990; Eremeeva and Silverman, 1998; Eremeeva et al., 2001). The protein responsible for the actin-based movement in SFG rickettsiae has yet to be identified. The genome sequence of *R. conorii* did not reveal a rickettsial protein homologous to ActA or Ics, the proteins responsible for actin polymerization by *Listeria monocytogenes* and *Shigella flexneri*, respectively; however, a hypothetical protein of 520 residues (RC0909) exhibits an overall organization similar to that of ActA. Both proteins are highly charged at the N-terminus and have a central proline-rich region. RC0909 has a weak similarity to the Wiskott-Aldrich.

Syndrome protein (WASp) homology domain 2 regulates the formation of the actin filaments (Ogata et al., 2001).

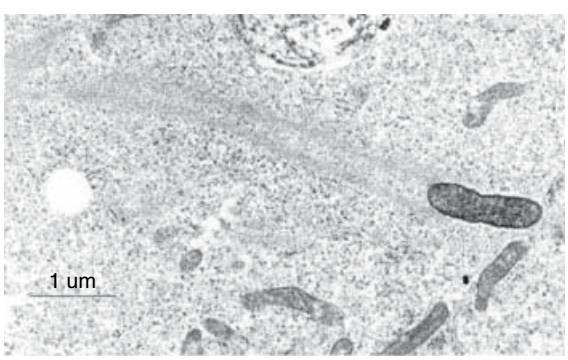

Fig. 4. Electron photomicrograph of an organism of *Rickettsia conorii* (right) with a long actin tail extending from its left pole to the left of the figure. Photomicrograph provided by Vesevolod Popov.

Reactivation of Rickettsia *Rickettsia rickettsii* loses its pathogenicity and virulence for guinea pigs in starved ticks (Spencer and Parker, 1923). Injection of triturated, starved, infected ticks into guinea pigs does not cause disease, but causes asymptomatic seroconversion; however, incubation of the tick vector at 37°C for 24–48 h or feeding the ticks on an animal for 10 h or longer before trituration results in clinically manifest disease in the inoculated guinea pigs. Spencer and Parker (1923) postulated that virulence of *R. rickettsii* in the tick vector is linked directly to the physiological state of the tick and defined this phenomenon as "reactivation." The mechanism of rickettsial reactivation is not understood; however, the reactivation may result from growth of rickettsiae or differential expression of rickettsial virulence factors at the elevated temperature or after stimulation by components of the blood meal. *Rickettsia rickettsii* increases 100-fold in the hemolymph of partially engorged ticks compared to unfed infected ticks (Wike and Burgdorfer, 1972). Differential expression of rickettsial proteins and ultrastructural changes has been confirmed by immunoblot and electron microscopy. Electron microscopy reveals

that reactivated *R. rickettsii* in ticks incubated at 37°C or in ticks fed on animals has a discrete microcapsular layer and a discrete electron-lucent slime layer outside the microcapsular layer. In starved ticks, the microcapsular layer and the slime layer of rickettsiae are inconspicuous or ragged (Hayes and Burgdorfer, 1982). Immunoblot analysis indicates that rickettsial proteins of 42, 43, 48, 75 and 100 kDa are induced in a tick cell line when shifted from 28 to 34°C (Policastro et al., 1997).

Genetics

The genomes of *R. prowazekii* and *R. conorii* have been completely sequenced (Andersson et al., 1998; Ogata et al., 2001). The *R. conorii* genome (1,268,755 bp) is slightly larger than that of *R. prowazekii* (1,111,523 bp). There are 804 common open reading frames (ORFs) between *R. conorii*, which has 1374 ORFs, and *R. prowazekii*, which has 834 ORFs; 552 ORFs were found only in *R. conorii* and 229 of them have a homologous noncoding remnant in *R. prowazekii*. The G+C content is 32.4 mol% for *R. conorii* and 29.0 mol% for *R. prowazekii*. The *R. conorii* genome contains more repetitive DNA than that of *R. prowazekii*. The higher G+C content of *R. conorii* is accounted for by the repetitive DNA, which is G+C rich (40%; Ogata et al., 2001).

Rickettsia genomes are very small, only about a quarter of the genome size of *Escherichia coli* strain K12 (4,639,221 bp, accession no. NC000913), a free-living Gram-negative bacterium. The small genomes of *Rickettsia* are the result of genome reduction, which shapes the relationship of intracellular bacteria and their hosts. Many genes involved in the biosynthesis and regulation of biosynthesis of amino acids and nucleosides were deleted inasmuch as host genes fulfill these functions. *Rickettsia* not only contain fewer genes but also contain more non-coding sequences (19% in *R. conorii* and 24% in *R. prowazekii*) than their free-living bacterial cousins contain (Andersson et al., 1998; Ogata et al., 2001). A large portion of the non-coding DNA in rickettsiae is hypothesized to be degraded remnants of unnecessary genes that have not yet been removed from the genome (Andersson et al., 1998).

Evolutionarily, rickettsiae were derived from a free-living bacterial ancestor. Once a free-living aerobic bacterium entered and established an intracellular parasitic relationship with the pro-eukaryote cell, many chemical substrates could be readily obtained from the host cells and used without further metabolic modification by the bacteria. The genes of the bacterium involved in metabolic pathways such as glycolysis, fermentation and biosynthesis of small molecules were functionally similar to their host cell's genes. These redundant genes then could mutate and become genes with new functions or pseudogenes. Pseudogenes eventually are deleted from the genome to increase the energy use efficiency of the bacterium. Owing to loss of genes essential to a free-living mode, the bacterium became further dependent on the host cell and eventually lost its ability to live outside the host cell.

All the enzyme genes for the tricarboxylic acid cycle are present in the rickettsial genome. *Rickettsia* retains genes for ATP synthesis complexes, despite the acquisition of an ATP/ADP translocator gene (Andersson et al., 1998). The ATP/ADP translocases are unique to *Rickettsia* and *Chlamydia* among bacteria and might have originated from plants (Wolf et al., 1999). This enzyme allows the importation of ATP from the host cell cytoplasm. The structural differences between the ATP/ADP translocases of mitochondria and those of *R. prowazekii* indicate their evolutionary origins are different, despite the evidence that mitochondria and rickettsiae evolved from a common ancestor (Andersson et al., 1998).

Genetic Manipulation of the Rickettsial Genome No resident plasmid or bacteriophage has been found in rickettsiae (Andersson et al., 1998; Ogata et al., 2001). Lack of genetic tools to manipulate the rickettsial genome has hampered our current understanding of the biology of rickettsiae. Recent advances in transforming rickettsiae may facilitate study of their molecular biology (Troyer et al., 1999; Rachek et al., 2000).

Autotransporter Proteins In Gram-negative bacteria, proteins are transported across the cytoplasmic or inner membrane and the outer membrane by several pathways including types I, II, III, IV and autotransporter secretion. Types I, II, and III secretion systems require the assistance of a variety of accessory proteins that function in combination with the general secretory pathway (Salmond and Reeves, 1993; Galan and Collmer, 1999). In contrast, autotransporters do not need accessory proteins for assistance in self-translocation. An autotransporter protein consists of three functional domains: the amino-terminal leader sequence, the secreted mature (α) protein and a carboxy-terminal (β) domain. The signal peptide inserts itself into the inner membrane and directs the export of the precursor molecule via the general secretion pathway into the periplasm. The signal peptidase cleaves the precursor, releasing the mature polypeptide into the periplasm. Once in the periplasm, the β-

domain of the protein is inserted into the outer membrane to form a β-barrel pore, and the passenger domain is translocated to the cell surface through the pore. The β-domain of autotransporters is conserved among even distantly related bacteria, whereas passenger domains are diverse, suggesting that autotransporters were either derived originally from a single gene or from recombination of genes for unrelated proteins with the β-domain gene (Henderson et al., 1998).

A striking feature of rickettsiae is that all the identified rickettsial outer membrane proteins belong to the autotransporter family. These proteins include OmpA, OmpB, and four ORFs encoding the hypothetical proteins Sca1 (accession no. PR018), Sca2 (RP081), Sca3 (RP451), and Sca5 (RP704; Andersson et al., 1998; Bouyer et al., 2001). Sca1 and Sca2 consist of only the β-domain without the passenger peptide. Thus, Sca1 and Sca2 may be involved in the transport of other rickettsial proteins. The precursor of OmpB is a 168-kDa protein that is post-translationally processed to the 135-kDa mature OmpB by cleavage of a 32-kDa β-peptide (Hackstadt et al., 1992). The amino acid sequences of the β-peptides of all five rickettsial autotransporters are highly homologous and also share homology with autotransporters from distantly related bacteria (Henderson et al., 1998). Most of the autotransporter proteins are adhesins or proteases of bacteria, indicating that OmpA, OmpB, Sca3 and Sca5 are possibly important rickettsial virulence factors.

OmpA, a 160–190-kDa protein, has been found in all tick-borne SFG but not TG rickettsiae. The flea-borne SFG rickettsia, *R. felis*, has an OmpA that is truncated by the presence of premature stop codons. Over 40% of the amino acid sequence of OmpA is devoted to a hydrophilic region of tandem repeat units (Anderson et al., 1990b). The repeat units are not identical and thus are divided into two types (I with 75 amino acids and II with 72 amino acids). The type II repeats are less conserved and further divided into two subtypes (IIa and IIb; Anderson et al., 1990b). The *rompA* sequences upstream and downstream of the repeat region are conserved among SFG rickettsial species. Although conserved among *R. rickettsii*, *R. conorii* and *R. akari*, the repeat sequences vary in number and order of repeat unit arrangement among SFG rickettsiae (Gilmore, 1993); however, the *R. australis* repeat unit differs greatly from other SFG rickettsial repeat units. *Rickettsia australis* has only one type of repeat unit with only 21% identity to the type I unit of *R. rickettsii* (Stenos and Walker, 2000). The antigenic diversity of SFG rickettsiae is determined in large part by the number, order, and type of repeat units. Recombinant OmpA stimulates immune protection in guinea pigs against rickettsial challenge (Sumner et al., 1995). OmpB, found in all rickettsiae, is the most abundant rickettsial surface (S-layer) protein (Ching et al., 1990) and contains species-, group- and genus-specific epitopes (Anacker et al., 1987). OmpB stimulates immune protection in mice and guinea pigs against challenge with *R. typhi* (Dasch et al., 1999).

Type IV Secretion System Genome sequencing revealed that *R. prowazekii* contains the genes *virB4*, *virB8*, *virB9*, *virB10*, *virB11* and *virD4* (Andersson et al., 1998), which are homologues of the type IV secretion systems founded in *Agrobacterium tumefaciens* (*virB* operon; Stachel and Nester, 1986; Kuldau et al., 1990), *Brucella abortus* (*virB*; Sieira et al., 2000), *E. coli* (*tra* genes; Winans and Walker, 1985; Pohlman et al., 1994), *Bordetella pertussis* (*ptl* genes; Weiss et al., 1993; Kotob et al., 1995), *Legionella pneumophila* (*dot-icm* genes and *lvh* genes; Brand et al., 1994; Segal and Shuman, 1997; Segal et al., 1999), *Helicobacter pylori* (*cag* genes; Covacci et al., 1999), *E. chaffeensis*, *A. phagocytophilum* (Ohashi et al., 2002), and *Wolbachia* (Brand et al., 1994; Masui et al., 2000; Sieira et al., 2000). The type IV secretion systems inject either bacterial DNA or proteins directly into the cytosol of eukaryotic host cells (Winans and Walker, 1985; Weiss et al., 1993; Christie, 1997; Covacci et al., 1999). In the facultative intracellular bacteria *L. pneumophila* and *B. abortus*, the type IV secretion systems are essential for the intracellular multiplication of the two organisms (Brand et al., 1994; Sieira et al., 2000). *Legionella pneumophila icm* mutant is deficient in its ability to cause lysis of the host cell. The dot/icm protein secretion apparatus of *L. pneumophila* secretes RalF, a protein required for recruiting ADP ribosylation factor (ARF) to the phagosome containing *L. pneumophila* and for vacuole biogenesis (Nagai et al., 2002). ARF is a highly conserved small GTP-binding protein that acts as a key regulator of vesicle traffic from the ER and Golgi apparatus. An *R. prowazekii* gene (G71694) similar to the *ralf* gene of *L. pneumophila* has been found by BLASTN search (Nagai et al., 2002). Because they are homologous to the eukaryotic ARF-specific guanine nucleotide exchange factors and not found in other bacteria, RalF and its rickettsial analogue might have been acquired from a eukaryotic gene by horizontal gene transfer (Nagai et al., 2002). On the basis of similarity with the RalF of *L. pneumophila*, the rickettsial protein encoded by gene G71694 may be the component that is transported by the rickettsial type IV secretion system; however, unlike *L. pneumophila*, *Rickettsia* do not live in vacuoles.

Thus, the function of rickettsial gene G71694 is still not clear. The role of the type IV secretion system in the pathogenesis of other bacterial infections suggests that the rickettsial *virB* genes may be associated with rickettsial intracellular survival.

Rickettsial Lipoprotein A 17-kDa protein has been identified in all *Rickettsia* species (Anderson, 1990a). The corresponding gene sequence is highly conserved among rickettsial species, indicating the importance of the protein to the survival of the rickettsiae. The protein, predicted to be a lipoprotein and known to be partly surface exposed, has been speculated to play a scaffolding and protective role in the rickettsiae (Anderson, 1990a).

Ecology

The importance of transovarial maintenance for the ecology of rickettsiae varies from essential (in the nonpathogenic *R. peacockii* where it is the only mechanism ensuring persistence in the environment) to necessary, but not sufficient (in the highly pathogenic *R. rickettsii*; McDade and Newhouse, 1986; Niebylski et al., 1996; Niebylski et al., 1997). The nonpathogenic organisms appear to coexist with their arthropod host, neither harming them nor possessing the virulence traits for horizontal transmission. In contrast, *R. rickettsii* under some circumstances harms its tick host resulting in its death or reduced production of offspring (Niebylski et al., 1999); however, the virulence mechanisms that harm the tick also permit horizontal transmission to small rodents and sufficient levels of rickettsemia to establish new lines of transmission to feeding ticks (Gage et al., 1990). In the United States, generally fewer than 5% of vector ticks are infected with rickettsiae, most of which are nonpathogenic *R. montanenisis* or *R. bellii* (Philip and Casper, 1981a; Philip et al., 1981b). Fewer than 1 in 1000 vector ticks carry virulent *R. rickettsii*, presumably a result of their deleterious interaction. Higher rates of rickettsial carriage by ticks are observed with less virulent pathogens. Application of specific diagnostic methods might reveal that some organisms previously of undetermined pathogenicity, such as *R. amblyommii*, *R. rhipicephali* and *R. helvetica*, are actually at least mildly pathogenic. This situation has occurred for *R. slovaca* and strain Thai tick-118 (now established as *R. honei*; Stenos et al., 1998).

Our view of *Rickettsia* ecology is highly anthropocentric and hematophagous arthropod- and medically oriented. Identification of strains closely related to *R. bellii* in herbivorous insects and even in association with a plant disease, papaya bunchy top disease, suggests that our knowledge of the diversity and evolutionary origin of *Rickettsia* might be dreadfully skewed (Chen et al., 1996; Davis et al., 1998).

Epidemiology

Twelve species of *Rickettsia* have been associated with human disease: *R. rickettsii* (Rocky Mountain spotted fever), *R. conorii* (boutonneuse fever, Mediterranean spotted fever, Kenyan tick typhus, Israeli spotted fever, Indian tick typhus, and Astrakhan spotted fever), *R. africae* (African tick-bite fever), *R. sibirica* (North Asian tick typhus), *R. japonica* (Japanese spotted fever), *R. australis* (Queensland tick typhus), *R. honei* (Flinders Island spotted fever), *R. akari* (rickettsialpox), *R. felis* (cat flea typhus), *R. slovaca* (tick-borne lymphadenopathy), *R. prowazekii* (epidemic louse-borne typhus, recrudescent typhus fever, and sylvatic typhus), and *R. typhi* (murine typhus). Each rickettsiosis has a particular geographic distribution determined by its natural history involving an arthropod host and in some cases a zoonotic vertebrate host (Table 1). Often during a particular season of the year, rickettsial diseases occur when humans encounter infected ticks, fleas, or mites that are seeking a blood meal. Louse-borne typhus fever occurs particularly in cold climates among impoverished persons who are infested with lice (Wolbach et al., 1922; Perine et al., 1992). Epidemics have been associated with war and natural disasters where there is crowding, prevalence of human body lice, and lack of opportunity to bathe and wash clothes (Patterson, 1993; Raoult et al., 1998).

Disease

General Considerations Rickettsioses vary in clinical severity according to the virulence of the *Rickettsia* and host factors, such as older age, male gender, and alcoholism, and other underlying diseases (McDade, 1990; Walker, 1990). The most virulent rickettsiae are *R. rickettsii* and *R. prowazekii*, which kill a significant portion of previously healthy infected persons, unless they are treated sufficiently early in the course of illness with an effective antirickettsial agent, usually doxycycline. *Rickettsia conorii* and *R. typhi* also possess the virulence to kill 1–2% of infected persons (Ruiz-Beltran et al., 1985; Raoult et al., 1986; Dumler et al., 1991). The other spotted fever rickettsioses can cause disseminated infection with multisystem involvement and illness of two weeks or more duration, but fatalities are rare and (for some entities) have never been reported.

Table 1. Geographic distribution, maintenance in nature, and human transmission.

Agent	Geographic distribution	Maintenance in nature	Human transmission
R. rickettsii	North, Central and South America	Transovarial maintenance in *Dermacentor*, *Rhipicephalus*, and *Amblyomma* ticks: less extensive horizontal transmission from tick to mammal to tick	Tick bite
R. conorii	Mediterranean basin, Africa, Asia	Transovarial maintenance in *Rhipicephalus* ticks	Tick bite
R. africae	Sub-Saharan Africa, Caribbean	Transovarial maintenance in *Amblyomma* ticks	Tick bite
R. sibirica	Russia, China, Mongolia, Pakistan, Kazakhstan, Kirgiziya, Tadzhikistan	Transovarial maintenance in *Dermacentor*, *Haemaphysalis*, and *Hyalomma* ticks; horizontal transmission from tick to mammal to tick	Tick bite
R. japonica	Japan, China	Presumably a transovarial tick host	Tick bite
R. australis	Eastern Australia	Transovarial transmission in *Ixodes* ticks	Tick bite
R. honei	Southern Australian islands, Thailand	Transovarial transmission in *Aponomma* ticks	Tick bite
R. akari	United States, Ukraine, Croatia, possibly worldwide	Transovarial transmission in *Liponyssoides sanguineus* mites; horizontal transmission from mite to mouse to mite	Mite bite
R. felis	North and South America and Europe	Transovarial transmission in *Ctenocephalides felis* fleas	Unknown
R. prowazekii	South and Central America, Africa, Asia, Mexico	Man to *Pediculus humanus corporis* louse to man	Louse feces scratched into skin
R. prowazekii	United States	Flying squirrel to louse and flea ectoparasites to flying squirrel	Presumably fleas of flying squirrels to man
R. prowazekii	Worldwide	Reactivation of latent human infection years after acute illness	None
R. typhi	Worldwide, predominantly tropical and subtropical	Rat to *Xenopsylla cheopis* flea to rat; opossum to cat flea C. *felis* to opossum	Flea feces scratched into skin; rubbed into conjunctiva or inhaled

All infections with organisms of the genus *Rickettsia* usually begin with introduction of rickettsiae into the skin, either through a tick bite or, in the case of contaminated flea or louse feces, through cutaneous abrasions. Rickettsiae enter dermal cells including endothelium and proliferate locally intracellularly with endothelial cell-to-cell spread for most SFG rickettsioses resulting in an eschar or tache noire, a zone of dermal and epidermal necrosis approximately 1 cm in diameter with a surrounding zone of erythema (Walker et al., 1988b). Eschars do not occur in epidemic and murine typhus and are rarely observed in Rocky Mountain spotted fever and Israeli spotted fever (Walker et al., 1981; Sarov et al., 1990). SFG rickettsioses often manifest regional lymphadenopathy in the drainage of the eschar, suggesting that rickettsiae may spread via lymphatic vessels from the tick bite inoculation site early in the infection. Rickettsiae spread throughout the body and infect mainly endothelial cells, establishing many foci of contiguous infected blood vessel-lining cells. Injury in these local sites causes vascular damage manifesting as rash, interstitial pneumonia, encephalitis, interstitial nephritis, and interstitial myocarditis, as well as lesions in the liver, gastrointestinal wall, pancreas, or indeed nearly any vascularized tissue of the body (Walker, 1988a; Walker, 1996b).

The most important pathophysiologic effect is increased vascular permeability with consequent edema, loss of blood volume, hypoalbuminemia, decreased osmotic pressure, and hypotension (Harrell and Aikawa, 1949). These effects can be life threatening in the lungs as non-cardiogenic pulmonary edema and adult respiratory distress syndrome (Donohue, 1978; Lankford and Glauser, 1980; Walker et al., 1980b). Hypovolemia and hypotension may result in prerenal azotemia or, if shock occurs, in acute tubular necrosis (Walker and Mattern, 1979). In the most severe cases of Rocky Mountain spotted fever, louse-borne typhus, boutonneuse fever, and murine typhus, central nervous system involvement is a critical life-threatening factor (Helmick

et al., 1984). The precise relative contributions of the altered blood-brain barrier, hypoperfusion, hypoxemia, cerebral edema, and other pathophysiologic effects have not been determined, but progressively severe illness involves confusion, delirium, stupor, ataxia, coma, focal neurologic signs, and seizures (Miller and Price, 1972a; Miller and Price, 1972b; Horney and Walker, 1988). Cerebrospinal fluid abnormalities may include pleocytosis and increased protein concentration. Hyponatremia occurs frequently with secretion of antidiuretic hormone as a manifestation of the appropriate response of the hypothalamus and posterior pituitary to hypovolemia (Kaplowitz and Robertson, 1983a). Despite interstitial inflammation in the myocardium, left ventricular function is maintained, and in a small proportion of cases, the principal pathophysiologic effect on the heart is arrhythmia, presumably owing to vascular lesions involving the cardiac conduction system (Schmaier et al., 2001).

Despite disseminated infection of the vascular endothelium, frequent occurrence of thrombocytopenia, and the stimulation of a procoagulant state, neither disseminated intravascular coagulation nor life-threatening hemorrhage occurs in rickettsial diseases except on rare occasions, as a remarkable manifestation of the homeostatic mechanisms of the coagulation system (Elghetany and Walker, 1999).

The mechanisms of immune clearance of disseminated intraendothelial rickettsiae have been determined for SFG and TG rickettsiae (Valbuena et al., 2002). Rickettsial infection stimulates an early innate immune response with activation of natural killer cells and production of gamma interferon (IFN-γ), which act in concert to dampen rickettsial growth. Adaptive immunity develops with clonal expansion of CD4 and CD8 T lymphocytes and antibody-producing B cells. Clearance of intraendothelial rickettsiae is achieved by rickettsicidal effects due to cytokine activation of the infected endothelial cells themselves. In mice, IFN-γ and tumor necrosis factor-α stimulate nitric oxide synthesis and nitric oxide-dependent rickettsial killing. In different human target cells activated by IFN-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-Iβ, and RANTES (regulated on activation, normal T-cell expressed and secreted), rickettsicidal mechanisms include various combinations of nitric oxide, reactive oxygen species, and tryptophan degradation. Ultimate clearance of rickettsiae is mediated by major histocompatibility class (MHC) I-matched, cytotoxic CD8 T-lymphocyte activity that is largely perforin-dependent. The mechanisms by which the cytokine-secreting and cytotoxic cells are directed to the foci surrounding infected endothelium are important and undetermined. The production of particular chemokines in these foci suggests that the endothelium plays a key role. Remarkably, antibodies (including those directed at epitopes of OmpA and OmpB) also play a role in protective immunity.

Rocky Mountain Spotted Fever Rocky Mountain spotted fever is the most severe rickettsiosis. In the pre-antibiotic era, 20–25% of previously healthy, infected persons died of the illness (Parker, 1941). Today, even with particular antimicrobial agents that are highly effective, 3–5% of persons die owing mainly to late or missed diagnosis and delayed or ineffective antimicrobial treatment (Dalton et al., 1995). The onset of disease follows an infective bite by a week (range 2–14 days), beginning with fever, severe headache, and myalgia. When patients present to a physician during the first three days of illness, only 3% have the classic triad of fever, rash, and history of tick bite (Helmick et al., 1984). The reasons are that up to 40% of patients are unaware of a tick bite, which is painless and may go unnoticed or be forgotten, and that the rash does not usually appear until day 3–5 of illness. To further confound the difficulty of diagnosis, symptoms such as nausea, vomiting, diarrhea, abdominal pain, and cough may suggest other diagnoses including enterocolitis, acute surgical abdomen, or pneumonia (Table 2). The rash typically appears on the ankles and wrists as faint pink 1–5 mm macules that represent a focus of vascular infection and surrounding vasodilation that blanches on pressure. These lesions may progress to become maculopapular, owing to the leakage of edema fluid from the affected blood vessels, with the development of a hemorrhage (petechia) in the center of the lesions in approximately half of cases. The rash will spread to involve the entire extremities, trunk and, in about half of the patients, the palms and soles. Skin necrosis or gangrene occurs as a result of rickettsial damage to the microcirculation in only 4% of patients and in some cases necessitates amputation (Kaplowitz et al., 1981; Kirkland et al., 1993; Hove and Walker, 1995).

Just as the spread and growth of rickettsiae and damage to a greater portion of the vascular system occur in other organs, the involvement of the microcirculation of vital organs such as the lungs and brain causes life-threatening adult respiratory distress syndrome and encephalitis. In most fatal cases, death occurs 8–15 days after onset. If treatment is initiated during the first five days of symptoms, death is averted (Hattwick et al., 1978); however, in another clinical form, fulminant Rocky Mountain spotted fever, death

Table 2. Symptoms, signs, and laboratory data.

Feature	RMSF	BF	% ATBF	ET	MT
Fever	99–100	100	88	100	100
Headache	79–91	56	NA	89–100	42–91
Rash	88–90	97	46	100	20–80
Tache noire	<1	72	95	0	0
Multiple eschars	0	0	54	0	0
Myalgia	72–83	36	63	54	46–71
Nausea and/or vomiting	56–60	NA	NA	32	31–59
Abdominal pain	34–52	NA	NA	30	11–31
Petechial rash	45–49	10	4	34	NA
Conjunctivitis	30	9	NA	53–87	45
Lymphadenopathy	27	NA	43	NA	NA
Stupor	21–26	10	NA	NA	4
Diarrhea	19–20	NA	NA	7	5–26
Edema	18–20	NA	NA	NA	NA
Ataxia	5–18	NA	NA	NA	1
Meningismus	18	11	NA	NA	NA
Splenomegaly	14–16	6	NA	3	5
Hepatomegaly	12–15	13	NA	13	16–24
Jaundice	8–9	2	NA	17	3–11
Pneumonitis	12–17	NA	NA	74	NA
Cough	33	10	NA	38	35–47
Dyspnea	NA	21	NA	NA	NA
Coma	9–10	NA	NA	6	NA
Seizures	8	NA	NA	NA	2–4
Shock and/or hypotension	7–17	NA	NA	NA	NA
Decreased hearing	7	NA	NA	NA	NA
Arrhythmia	7–16	NA	NA	NA	NA
Myocarditis	5–26	11	NA	NA	NA
Death	4–8	2.5	0	0–10	1–2
Increased AST level	36–62	39	NA	63	90
Thrombocytopenia	32–52	35	NA	43	48
Anemia	5–24	NA	NA	48	NA
Hyponatremia	19–56	25	NA	NA	60
Azotemia	12–14	6	NA	31	27

Abbreviations: RMSF, Rocky Mountain spotted fever; BF, boutonneuse fever; ATBF, African tick bite fever; ET, epidemic typhus; MT, murine typhus; AST, aspartate aminotransferase; and NA, not applicable.

itself occurs within five days of onset (Parker, 1938). This course is so rapid that a rash appears only terminally, if at all. Glucose-6-phosphate dehydrogenase deficient males are particularly prone to fulminant Rocky Mountain spotted fever, apparently as a result of an effect of hemolysis, presumably on rickettsial growth or oxidative injury (Walker et al., 1983a).

Boutonneuse Fever and African Tick-Bite Fever Boutonneuse fever and its agent were first described in North Africa in 1910, and variants of *R. conorii* have been identified in South Africa, Kenya, Somalia, Israel, Morocco, Ethiopia, Russia, India and Pakistan (Conor and Bruch, 1910; Walker et al., 1995). In parts of Africa, tick-transmitted diseases caused by *R. conorii* and *R. africae* overlap geographically (Kelly et al., 1996; Table 1). Although their clinical manifestations also overlap, the differences were sufficient for a South African physician, Pijper, to suggest in 1936 that there were two different disease agents. Generally milder than boutonneuse fever, African tick bite fever has a lower incidence of rash, which is more often vesicular and sparse, a higher incidence of eschars that are frequently multiple, and more prominent regional lymphadenopathy (Raoult et al., 2001a). Each of these diseases has been diagnosed in the United States after patients return from vacation abroad, particularly from African safaris.

Rickettsialpox Recognized mainly in the urban United States as an agent maintained in a mite-mouse cycle with humans as an accidental host, *R. akari* may have a broader host range and geographic distribution (Kass et al., 1994). A papule appears at the site of mite feeding in the skin during the incubation period, and over 2–7 days,

it evolves into an eschar. Later fever, chills, malaise, headache, and myalgia develop, followed after 2–6 days by a macular rash that becomes maculopapular and then vesicular before crusting and healing. Fatalities have not been reported.

Cat Flea Typhus Despite the widespread geographic distribution and prevalence of *R. felis* in cat fleas, on very few occasions have clinical investigations of this potential diagnosis been undertaken.

Among eight reported cases of human infection with *R. felis* (five diagnosed by polymerase chain reaction [PCR] and three by differential antibody titers), all had fever and constitutional symptoms (Schriefer et al., 1994; Zavala-Velazquez et al., 2000; Raoult et al., 2001b). The majority manifested rash, headache, and central nervous system (CNS) involvement, and variable proportions suffered nausea, vomiting, diarrhea, abdominal pain, myalgia and conjunctivitis. The actual spectrum of illness of this infection requires further careful clinical studies.

Typhus Fever *Rickettsia prowazekii* infections occur in three situations: louse-transmitted epidemics, subsequent recrudescence of a long-standing latent infection, and zoonotic infection transmitted from flying squirrels by their ectoparasites (Sonenshine et al., 1978; McDade et al., 1980; Duma et al., 1981; Walker, 2001). Wolbach's study of typhus in Poland after World War I detailed a prodrome in 88%, followed by onset of fever, chills, headache, and myalgia. Macules of 2–6 mm usually appeared first on the trunk on day 5 and later spread to the extremities (Wolbach et al., 1922). Rales, conjunctival injection, and delirium were frequent manifestations.

Recrudescent typhus is a milder version of the same signs and symptoms (Brill, 1910; Zinsser and Castaneda, 1933). Flying squirrel-associated typhus too has been described as less severe; whether this is due to antimicrobial treatment or less virulent strains of rickettsiae is unclear.

Murine Typhus Flea-borne *R. typhi* infections cause extreme discomfort but are seldom fatal in previously healthy young persons (Silpapojakul et al., 1991a; Whiteford et al., 2001). The difficulty in detecting a rash in darkly pigmented skin was evident in a study finding only 20% of experimentally infected African-American volunteers had rashes, compared to 80% of Caucasian volunteers (Woodward, 1988). The infection can follow a mild course in children with as many as half suffering only fever at night, but necessitates intensive care unit support in 10% of hospitalized adult patients (Dumler et al., 1991; Silpapojakul et al., 1991a). Pneumonitis or meningoencephalitis can be the major manifestation in some patients (Silpapojakul et al., 1991b).

Diagnosis

The physician's suspicion, based on the patient's potential exposure to infected vectors and on clinical manifestations, is needed for early diagnosis of *Rickettsia* and *Orientia* infections, when effective and specific antimicrobial treatment (most antibiotics are ineffective) can be administered. Making an early diagnosis (i.e., within the first three days of infection) is difficult because key diagnostic features (history of tick bite, fever, and rash) at medical presentation are seen in only a small minority of patients, e.g., in 3% of patients with Rocky Mountain spotted fever (Helmick et al., 1984). For rickettsialpox, boutonneuse fever, African tick bite fever, North Asian tick typhus, Queensland tick typhus, Japanese spotted fever, Flinders Island spotted fever, and scrub typhus, careful inspection for and recognition of an eschar is a useful diagnostic clue. For scrub typhus, hearing loss and generalized lymphadenopathy are helpful when present (Watt and Walker, 2001).

In most clinical situations, both in developed and developing countries, local clinical microbiology laboratories offer little assistance for diagnosis during the acute stage of illness (Walker, 1989a). Methods that offer a timely laboratory diagnosis include immunohistochemical detection of SFG and TG rickettsiae and *Orientia* in biopsies of a rash or eschar, immunocytochemical detection of SFG rickettsiae in circulating endothelial cells captured on immunomagnetic beads coated with a monoclonal antibody against an endothelial cell surface antigen, and PCR detection of circulating organisms (Walker et al., 1980b; Walker et al., 1989b; Walker et al., 1997; Walker et al., 1999b; Kaplowitz et al., 1983b; Montenegro et al., 1983; Tzianabos et al., 1989; Furuya et al., 1991; Furuya et al., 1995; Sugita et al., 1993; Sexton et al., 1994; Williams et al., 1994; LaScola and Raoult, 1996; LaScola and Raoult, 1997; Zavala-Velazquez et al., 2000; Moron et al., 2001). These techniques are available only in a few research laboratories. Few patients have diagnostic antibody titers early in the course of illness, and few laboratories routinely perform the most sensitive and specific assays for antibodies to *Rickettsia* and *Orientia*.

Immunocytochemical examination of circulating endothelial cells has a sensitivity and specificity of 50 and 94%, respectively, in *R. conorii* infections. Immunohistochemical detection of

Rickettsia has been achieved in Rocky Mountain spotted fever, boutonneuse fever, African tick bite fever, rickettsialpox, murine typhus, and louse-borne typhus; *R. australis*, *R. sibirica* and *R. japonica* have been identified by immunohistochemistry in the tissues of experimentally infected animals, demonstrating further clinical feasibility. For Rocky Mountain spotted fever, the sensitivity and specificity of immunohistochemical diagnosis are 70 and 100%, respectively. PCR has been applied diagnostically to the detection of *R. conorii*, *R. africae*, *R. rickettsii*, *R. japonica*, *R. felis*, *R. slovaca*, *R. helvetica*, *R. typhi*, *R. prowazekii* and *O. tsutsugamushi*. For *Rickettsia*, the 17-kDa lipoprotein gene is the usual target, although DNA of the genes for citrate synthase, 16S rRNA, and OmpA have also been amplified diagnostically. The 56-kDa protein gene of *Orientia* is the usual target of PCR amplification for diagnosis of scrub typhus. For Rocky Mountain spotted fever, the diagnostic sensitivity of PCR has been disappointing, presumably owing to the presence of only a low concentration of these obligately intracellular organisms in the circulating blood. PCR amplification of DNA of *R. prowazekii* from lice removed from patients has been utilized effectively to diagnose typhus even in samples sent through the mail, providing an excellent opportunity to identify outbreaks before they progress to epidemic proportions (Roux and Raoult, 1999).

Currently used serologic assays for the diagnosis of rickettsioses include indirect immunofluorescence assay (IFA), indirect immunoperoxidase assay, latex agglutination, enzyme immunoassays based on antigens of whole organisms and recombinant antigens, line blot, Western immunoblot, and *Proteus vulgaris* OX-19 and OX-2 and *Proteus mirabilis* OX-K agglutination. IFA detects antibodies at a diagnostic titer of ≥64 usually in the second week of illness in patients with Rocky Mountain spotted fever. The diagnostic titer cut off may vary not only in individual laboratories but also in endemic areas for particular diseases. At an IFA cutoff titer for antibodies to *O. tsutsugamushi* of ≥400, the sensitivity was only 48% and the specificity was 96% in a scrub typhus endemic region (Brown et al., 1983). At a cutoff titer of ≥100, the sensitivity improves to 84% but the specificity is only 78%. IFA reagents are commercially available for SFG and TG rickettsiae and *Orientia*. Dot-enzyme immunoassay kits are available commercially for *R. rickettsii*, *R. conorii*, *R. typhi* and *O. tsutsugamushi*. The sensitivities and specificities appear to be satisfactory for murine typhus (88 and 91%, respectively, when compared with an IFA titer of ≥64), and this kit detects crossreactive antibodies in patients infected with *R. prowazekii* (Kelly et al., 1995); however, the specificities for diagnosing scrub typhus are only 77 and 66% when compared with IFA titers of ≥64 and ≥128, respectively (Weddle et al., 1995). Newer 56-kDa protein recombinant enzyme immunoassays are more encouraging, with sensitivities, specificities, and early detection of antibodies that appear superior to those of the well-established IFA (Kim et al., 1993; Ching et al., 2002). This assay is rapid, detects IgM and IgG separately, and is appropriate for point-of-care clinical use; however, in many situations the only laboratory method available is the Weil-Felix test. Although discredited owing to poor sensitivity and specificity, the Weil-Felix test is still useful in developing countries where the choice is between a *Proteus* agglutination assay and nothing at all for the detection of an epidemic of louse-borne typhus or for recognition of the increasing incidence of cases that might represent murine typhus, scrub typhus, or an SFG rickettsiosis. Indeed, antibodies detected by *Proteus* agglutination led to the identification of two newly emerging human pathogens, *R. japonica* and *R. honei*.

The precise etiologic agent of an SFG rickettsiosis is difficult to determine because immunodominant antigens (lipopolysaccharide, OmpA and OmpB) are shared among these organisms (Vishwanath, 1991). The situation for epitopes of lipopolysaccharides and Omp B shared between *R. typhi* and *R. prowazekii* is similar. The criterion of a four-fold or greater IFA titer distinguishes infections caused by *R. conorii* and *R. africae* in only 26% of cases and infections by *R. typhi* and *R. prowazekii* in only 34% of cases. The use of detection of species-specific antibodies against OmpA and OmpB by Western immunoblotting only identifies the agent of half of these cases. Cross-absorption of sera before IFA or Western immunoblotting is more effective but is too expensive and cumbersome for practical use. There is a serious need in clinics for more useful methods not only to diagnose life threatening, readily treatable diseases such as Rocky Mountain spotted fever but also for potential identification of bioterrorist-disseminated *R. prowazekii* and other rickettsiae.

Treatment

Doxycycline is the drug of choice for the treatment of infections caused by *Rickettsia* except in cases of pregnancy and tetracycline hypersensitivity (Raoult and Drancourt, 1991; Watt et al., 1996; Watt et al., 1999; Watt et al., 2002; Walker and Sexton 1999a). Recent studies have shown that doxycycline is superior to chloramphenicol for the treatment of Rocky Mountain spotted fever as it is associated with a lower

case fatality rate and a lower hospitalization rate (Holman et al., 2002). Because of the teratogenic risk of doxycycline, chloramphenicol has been used to treat pregnant patients with Rocky Mountain spotted fever in whom monitoring of serum chloramphenicol levels is recommended to avoid toxicity to the fetus. Several fluoroquinolones, josamycin, azithromycin, and clarithromycin, have been used successfully to treat boutonneuse fever but are not recommended for more pathogenic rickettsioses (Bella et al., 1990; Cascio et al., 2001). It should be emphasized that rickettsiae are highly resistant to most antibiotics. Most fatal cases of Rocky Mountain spotted fever have received substantial courses of antimicrobial treatment, including beta lactams, aminoglycosides, and erythromycin. Sulfonamide antimicrobials actually appear to exacerbate the severity of rickettsial infections.

Genus Orientia

Phylogeny

The genus *Orientia* consists of a single species *Orientia* (formerly *Rickettsia*) *tsutsugamushi* (Tamura et al., 1995). Its closest relatives are organisms in the genus *Rickettsia* (Fig. 1). Unlike members of the genus *Rickettsia*, *Orientia* does not have LPS and peptidoglycan in its cell wall (Tamura et al., 1995). *Orientia tsutsugamushi* has evolved in mites to form a distinct set of divergent clones in each geographic location of its vast geographic distribution (Dasch et al., 1996).

Habitat

Orientia tsutsugamushi is maintained by transovarial transmission in its various trombiculid mite hosts and requires no other host than the mite in its life cycle. *Orientia* resides free in the cytoplasm of its host cell. Although wild rats become infected and chiggers acquire rickettsiae when feeding on rats, the chiggers do not transmit the acquired organisms to the next generation (Traub and Wisseman, 1974).

Isolation

Orientia tsutsugamushi is isolated by inoculation of mice, cell culture, or embryonated chicken eggs.

Identification

Orientia tsutsugamushi may be identified by PCR amplification of the DNA of a species-specific gene, DNA sequencing of genes available in GenBank (ncbi.nim.nih.gov4/genbank), and reactivity with species-specific antibodies.

Preservation

Stocks of *O. tsutsugamushi* are usually preserved frozen at –70 to –80°C or in the vapor phase of liquid nitrogen. They may also be preserved in a lyophilized state.

Physiology

Orientia are obligately intracellular bacteria that reside free of the cytosol of the host cell. They possess an unusual Gram-negative cell wall that lacks lipopolysaccharide and peptidoglycan. They enter the host cell by attachment and induced phagocytosis. The adhesin has not been identified, but host cell surface heparan sulfate glucosaminoglycan plays a role as a receptor (Ihn et al., 2000). To escape the phagosome, *Orientia* has to be metabolically active (Rikihisa and Ito, 1982). Movement of *Orientia* occurs within the cytoplasm to the perinuclear microtubule organizing center, where it replicates, and is mediated by microtubules through rickettsial interaction with dynein, the minus end-directed microtubule-associated motor protein, rather than via F-actin tails (Kim et al., 2002b). *Orientia* is released from infected cells by budding. In mouse peritoneal mesothelial cells, *O. tsutsugamushi* multiplies in the cytoplasm, moves to the cell periphery and, surrounded by a host cell membrane, separates from the cell surface. *Orientia* enveloped by the host membrane enters other mesothelial cells, apparently by a phagocytic mechanism. Organisms escape from the phagocytic vacuole as the vacuole membrane and host cell membrane coat are disintegrated (Ewing et al., 1978). Scanning electron microscopy and ruthenium red staining clearly show that the budding rickettsiae are surrounded with a host cell membrane (Tsuruhara et al., 1982). Cells heavily infected with *Orientia* undergo apoptotic cell death in association with decreased content of focal adhesion kinase and paxillin, decreased actin stress fiber polymerization, and decreased expression of anti-apoptotic bcl-2 (Kee et al., 2002).

Genetics: Variable Immunodominant Antigens

Although *Orientia* consists of a single species, it contains many antigenic variants. Antigenic variability among geographic isolates of *Orientia* is determined by a type-specific protein (TSA), a 56-kDa immunodominant surface protein. The

TSAs of six *Orientia* antigenic variants range from 55,308 to 56,745-daltons with 521–532 amino acids. TSA has alternating hydrophobic and hydrophilic regions, which is a feature of transmembrane proteins. Comparison of the TSAs of *Orientia* variants revealed four variable domains with spans of 16–40 amino acids. The variable domains are located in the hydrophilic regions of the molecule and show different amino acid sequences among the strains (Ohashi et al., 1992). The antigenic variability of *Orientia* poses problems for vaccine design. Immunization with *Orientia* confers relatively strong protection lasting only 1–3 years against challenge by the homologous strain, whereas protection is very weak and short-lived against heterologous strains (Seong et al., 2001a).

Epidemiology

Scrub typhus cases occur when larval mites (chiggers) encounter humans as a source of a tissue fluid meal taken from the skin. Each mite host species has its own geographic distribution and seasonal activity pattern that determines the occurrence of scrub typhus (Audy, 1968). Often the geographic location of infected chiggers is highly focal. Of the 18,000 cases of scrub typhus that occurred among Allied troops in the Pacific and Asian theaters of operations during World War II, some were intensely focal, affecting persons in one location but not others very nearby (Audy, 1968).

Disease

Orientia tsutsugamushi is inoculated into the skin during feeding by the larva (chiggers) of trombiculid mites. A small, painless papule forms, enlarges, and undergoes central necrosis and crusting to form an eschar with associated regional lymphadenopathy during the incubation period, which averages 10 days (Brown, 1988). Bacteremia precedes onset of symptoms by 1–3 days. Onset is characterized by abrupt fever, headache, and often myalgia. About one third of patients will also experience hearing loss (Watt and Walker, 2001). Rash is observed at the end of the first week in half of primary infections, being recognized in a smaller proportion of those reinfected and with dark skin. A recent investigation of the 22.7% of scrub typhus patients with gastrointestinal symptoms showed severity of lesions assessed by gastric endoscopy was associated with cutaneous manifestations (Kim et al., 2000). The most serious organ involvement, pulmonary or neurological, may manifest clinically as cough, tachypnea, radiographic pulmonary infiltrates progressing to dyspnea and, in the most severe cases, as adult respiratory distress syndrome or CNS-related confusion and apathy, with seizures and coma occurring only rarely (Kim et al., 2002a; Tsay and Chang, 2002). These manifestations and peripheral vascular collapse lead to an age-dependent risk of death that was 10–30% in the pre-antibiotic era.

T-lymphocytes are critically important in immunity to *Orientia*, and antibodies to particular strain-specific epitopes of the major 56-kDa protein also play a role in inhibiting establishment of infection (Shirai et al., 1976; Jerrells and Geng, 1994; Seong et al., 2001b). Immune cytokines (including IFN-γ and TNF-α) play an anti-*Orientia* role in model systems and, along with cytotoxic T-lymphocytes, are likely to be important effectors of immunity to *Orientia* (Rollwagen et al., 1986; Hanson, 1991; Geng and Jerrells, 1994). *Orientia* infection of a murine macrophage cell line stimulated NF-κB-dependent expression of mRNA of macrophage inflammatory proteins 1 α/β and 2 and macrophage chemoattractant proteins, providing clues to the mechanism of development of perivascular lymphohistiocytic infiltrates in the multiple foci of vascular endothelial infection, presumably as a manifestation of the host defenses (Cho et al., 2001; Moron et al., 2001).

Treatment

Doxycycline is the drug of choice for the treatment of infections caused by *Orientia* except in pregnant and tetracycline-hypersensitive patients and in patients in northern Thailand where some strains of *O. tsutsugamushi* are resistant to tetracyclines and chloramphenicol (Walker and Sexton, 1999a). In northern Thailand where the case fatality rate of scrub typhus is 15% and doxycycline-resistant *O. tsutsugamushi* is prevalent, rifampicin has been shown to be more effective than doxycycline for mild scrub typhus infections (Watt et al., 2002). Azithromycin has been shown effective in vitro against doxycycline-susceptible strains of *Orientia* (Strickman et al., 1995). Azithromycin has been used successfully to treat two pregnant patients with scrub typhus, and three Japanese patients were treated effectively with clarithromycin (Miura et al., 2002).

Genus Anaplasma

Phylogeny

The evolution of the genus *Anaplasma* is demonstrated by phylogenetic trees developed from

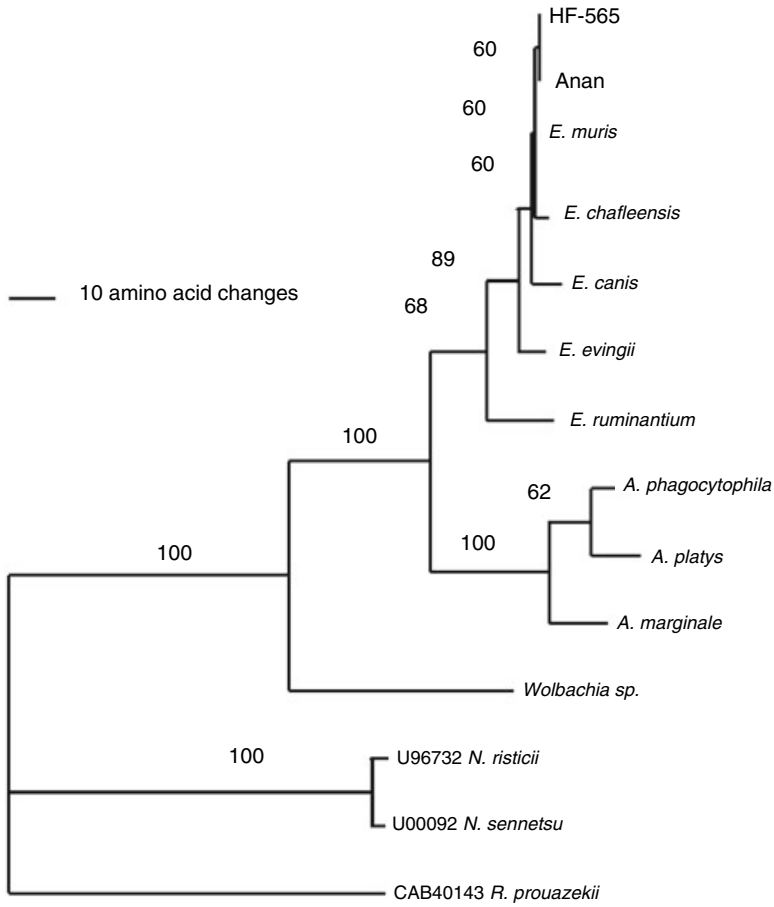

Fig. 5. Phylogenetic relationships of the organisms in the *Anaplasmataceae* family based on the amino acid sequences of the GroEL (GenBank accession numbers for strain HF-565, AB03271; strain Anan, AB032711; *E. muris*, AF210459; *E. chaffeensis*, L10917; *E. canis*, ECU96731; *E. ewingii*, AF195273; *E. ruminantium*, CRU13638; *A. phagocytophila*, U96729; *A. platys*, AY008300; *A. marginale*, AF165812; *Wolbachia* sp., AB002286; *N. risticii*, U96732; *N. sennetsu*, U88092; and *R. prowazekii*, CAB40143.) The length of each pair of branches represents the distance between sequence pairs. The numbers on the branch indicate the bootstrap values.

DNA sequence data based on the 16S rRNA gene, *groEL*, and other genes (Fig. 5).

Taxonomy

The genus *Anaplasma* includes *A. marginale*, *A. ovis*, *A. bovis*, *A. phagocytophilum* and *A. platys* (Dumler et al., 2001). The latter three species were formerly classified in the genus *Ehrlichia*. *Ehrlichia equi*, *E. phagocytophila*, and the human granulocytic ehrlichiosis agent have been consolidated to a single species, namely *A. phagocytophilum*.

Habitat

Anaplasma are obligately intracellular bacteria, residing in cytoplasmic vacuoles of their tick host's cells or in the vertebrate host's erythrocytes (*A. marginale*), neutrophils (*A. phagocytophilum*), platelets (*A. platys*), or monocytes/macrophages (*A. bovis*). Leukocytotropic *Anaplasma* have evolved mechanisms to avoid intracellular host defenses, including the effects of reactive oxygen species and fusion of lysosomes with their modified endosomal vacuole.

Isolation

Anaplasma have been isolated from their hosts in antibiotic-free cell cultures employing continuous lines of tick cells, such as IDE8 or leukocytes, e.g., HL-60 cells (Goodman et al., 1996; Munderloh et al., 1996).

Identification

Anaplasma have been identified historically by their host (mammals and ticks) and target cells on a morphologic basis. Contemporary criteria

for identification usually rely upon DNA sequence data (e.g., 16S rRNA, *groEL*, and *msp2* sequences).

Preservation

Anaplasma are fragile, obligately intracellular organisms that are best preserved as a stabilate of infected cells. *Anaplasma* stabilate is often frozen in a solution containing dimethylsulfoxide, so that thawing results in a portion of the infected cells being viable and containing infectious organisms.

Physiology

Anaplasma enter the skin through a tick bite. The adhesin of *A. marginale* is Msp-1, which is a heterodimer composed of two structurally unrelated polypeptides: Msp-1a and Msp-1b. Msp-1b adheres only to bovine erythrocytes and not to tick cells (de la Fuente et al., 2001). Thus, Msp-1a and Msp-1b are differential adhesins for vertebrate and invertebrate hosts. P-selectin on the neutrophil has been demonstrated as the receptor for *Anaplasma phagocytophilum* (Herron et al., 2000). The host cell receptors for other organisms of *Anaplasma* have yet to be identified. Upon attachment to the host cell, the organisms of Anaplasmataceae are phagocytosed by the host cell. *Anaplasma* induce their own entry into the host cell, e.g., *A. marginale* can invade nonprofessional phagocytic cells in cell culture, and the target cell of *A. marginale* is the erythrocyte. Inhibition of lysosomal fusion by *Anaplasma* may be critical for intracellular survival and replication of these organisms since *A. phagocytophilum* inhibits phagosome-lysosome fusion (Wells and Rikihisa, 1988; Barnewall et al., 1997; Mott et al., 1999). The *A. phagocytophilum* inclusion is neither an early nor a late endosome, and it does not fuse with lysosomes or Golgi-derived vesicles (Barnewall et al., 1997; Mott et al., 1999).

Genetics

Outer Membrane Protein Multigene Family and Persistent Infection with Anaplasma

Most of our knowledge of the molecular microbiology of *Anaplasma* comes from the study of *A. marginale*. The genome of *Anaplasma* is 1.2 Mb with a G+C content of 56 mol% (Alleman et al., 1993). Despite its small genome, *Anaplasma* contains a large amount of repetitive DNA sequences that encode outer membrane proteins. These repetitive genes are considered to be multigene families. Two multigene families (*msp-2* and *msp-3*) encode outer membrane proteins in *A. marginale* (Alleman et al., 1997; Palmer et al., 1994). The *msp-2* multigene family has been identified in all species of *Anaplasma* species except *A. platys*, which has yet to be investigated (Palmer et al., 1994; Palmer et al., 1998; Alleman et al., 1997; Ijdo et al., 1998; Murphy et al., 1998b). In *A. phagocytophilum* the *msp-2* genes have also been referred to as *p44* genes (Ijdo et al., 1998). The *msp-3* gene family has also been found in *A. ovis*. The actual total numbers of *msp-2* and *msp-3* genes have yet to be determined. The *msp-2* and *msp-3* genes have common features. All genes of the *msp-2* and *msp-3* families have a central hypervariable region flanked by 5′ and 3′ highly conserved sequences. Genes of the *msp-2* and *msp-3* gene families share conserved sequences, indicating a common evolutionary origin.

The multigene families of *Anaplasma* are involved in antigenic variation (Brayton et al., 2001). The evasion mechanism of persistent *A. marginale* infection is antigenic variation of surface proteins. Although *Anaplasma* contain multiple copies of the *msp-2* genes, only one *msp-2* gene is expressed in an individual *A. marginale* organism. The remaining *msp-2* genes are pseudogenes. The expressed *msp-2* gene is linked to a polycistronic expression site, which contains a promoter and three additional genes upstream of the *msp-2* gene (Barbet et al., 2000). The entire expression site is transcribed as a single polycistronic mRNA unit. Recombination between the *msp-2* gene in the expression site and a pseudogene of the *msp-2* family results in a new phenotypic Msp-2 and causes antigenic switching to a new serotype. Thus, segmental gene conversion of the expression site to link hypervariable *msp-2* sequences to the promoter and polycistron causes antigenic variation (Barbet et al., 2000). Although the *msp-2* and *msp-3* genes have different sequences, they are flanked by conserved sequences. Thus, recombination between the *msp-2* gene and *msp-3* gene through the conserved flanking sequences also causes gene switching and results in antigenic variation. Gene recombination occurs not only between the expression site and pseudogenes but also between *msp-2* pseudogenes. Gene recombination between *msp-2* genes and between *msp-2* and *msp-3* genes results in unlimited antigenic variation of *Anaplasma*.

One report indicates that Msp-2 antigens are predominantly expressed in human but not tick cells (Jauron et al., 2001). Thus, Msp-2 may be involved in regulatory changes that mediate survival of *A. phagocytophilum* by immune evasion after tick transmission. Msp-1a is encoded by a single gene, *msp-1α*, and Msp-1b is encoded by two genes, *msp-1β1* and *msp-1β2*

(Barbet et al., 1987; Viseshakul et al., 2000). Msp-1a is variable in molecular mass among geographic isolates because of a variable number of tandem 28 or 29 amino acid repeats (Allred et al., 1990).

Potential Glycoproteins of Anaplasma The 130- and 100-kDa proteins (p130 and p100) of *A. phagocytophilum* contain repeat units, and the repeats' motifs have limited homology with the *E. chaffeensis* 120-kDa glycoprotein repeats (Storey et al., 1998). Of particular interest regarding the p130, p100 and gp120 is that they all have larger molecular sizes than predicted on the basis of amino acid sequences. The aberrant size of these proteins is most likely caused by the glycosylation of the amino acids.

Ecology

The life cycle of *Anaplasma* includes two hosts: a tick and a vertebrate host. The organisms multiply in the tick midgut and salivary glands. Ticks transmit these bacteria in the course of feeding on mammals. The bacteria are transmitted in ticks from stage to stage (transstadially) when the tick molts or are passed from infected to noninfected ticks via vertebrate hosts that develop ehrlichemia. All species of *Anaplasma* cause persistent infection in their natural mammalian hosts. Thus, mammalian hosts serve as a reservoir.

Anaplasma marginale can also be transmitted mechanically by blood-contaminated instruments and biting flies as well as biologically by tick bite (Kocan et al., 1992; Kocan et al., 2000b). *Anaplasma marginale* has been experimentally transmitted by *Boophilus decoloratus*, *Rhipicephalus evertsi evertsi*, *R. simus*, *Hyalomma marginatum rufipes* (De Waal, 2000), *B. microplus* (Ribeiro and Lima, 1996), *Dermacentor andersoni* Stiles, *D. variabilis* Say (Stiller et al., 1989), and *B. annulatus* (Samish et al., 1993). *Rhipicephalus simus* experimentally transmits both *A. marginale* and *A. centrale* (Potgieter and van Rensburg, 1982; Potgieter and van Rensburg, 1987). Intrastadial transmission of *A. marginale* by male ticks is believed to be an important mechanism of transmission of anaplasmosis because the male tick seeks mates by feeding on multiple hosts (Kocan et al., 2000b). Tick infection rates are unaffected by bovine antibodies ingested during tick feeding on *A. marginale*-immune cattle (Kocan et al., 1996). *Anaplasma* is not maintained through transovarial transmission in the tick (Stich et al., 1989). Instead of inefficient maintenance mainly in the arthropod vectors, efficient maintenance of *Anaplasma* is achieved in mammalian hosts, whose prolonged infection facilitates spread to new vectors. Thus, *Anaplasma* has evolved a mechanism of persistent infection in their natural animal hosts. Cattle infected with *Anaplasma marginale* are carriers and maintain a low level of rickettsemia, which cannot be detected microscopically (Zaugg et al., 1986). Despite a strong immune response, animals surviving *A. marginale* infection become persistently infected with cyclic rickettsemia, with each rickettsemic cycle occurring every 6–8 weeks (French et al., 1998).

Ixodes ticks are the vectors of *A. phagocytophilum*. In the northeastern United States, the vector of *A. phagocytophilum* is *Ixodes scapularis* Say (Schauber et al., 1998), whereas on the Pacific coast of the United States, it is *I. pacificus* (Barlough et al., 1997). *Ixodes ricinus* and *I. persulcatus* are the vectors of *A. phagocytophilum* in Europe and Asia, respectively (Schouls et al., 1999; Cao et al., 2000).

Anaplasma phagocytophilum causes persistent infection in sheep (Kocan et al., 1986) and in *Peromyscus leucopus* (Telford et al., 1996). Human beings are an accidental host of *A. phagocytophilum* and play no role in the natural cycles of these organisms; however, human cases of persistent infection with *A. phagocytophilum* have been reported (Dumler and Bakken, 1996; Horowitz et al., 1998a). Other important mammalian hosts of *A. phagocytophilum* include white-tailed deer, roe deer, red deer, and wood rats.

Epidemiology

Bovine anaplasmosis is a worldwide tick-transmitted disease with substantial prevalence in the tropics and subtropics, and significant occurrences have affected beef production in the United States.

The first recognized case of human *A. phagocytophilum* infection occurred in 1990 (Bakken et al., 1994). By 1997, more than 449 human cases had been diagnosed in the United States, and human cases had been diagnosed in Europe (Lotric-Furlan et al., 1998; McQuiston et al., 1999). Most cases have occurred in areas endemic for Lyme borreliosis, which shares the same tick vectors in the northeastern and upper midwestern states, California, and Europe. Infections occur in suburban as well as rural areas. The incidence in New York and Connecticut is 3–16 cases per 100,000 population, and active surveillance in Connecticut and northwestern Wisconsin revealed an incidence greater than 50 cases per 100,000 population (Bakken et al., 1996; McQuiston et al., 1999). Tick bites or exposures are recalled in 75–85% of cases. Men and older patients (median age 40–60 years) are disproportionately affected. Most cases occur during sum-

mer with peak occurrence in June and July transmitted by nymphal ticks and a smaller peak in November coinciding with the emergence of adult ticks.

Disease

ANAPLASMOSIS IN CATTLE AND RUMINANTS *Anaplasma marginale* and *A. centrale* cause bovine anaplasmosis in cattle and wild ruminants. *Anaplasma marginale* identified by Theiler in South Africa in 1909 was first described as the "marginal points" in bovine erythrocytes with "marginale" (referring to the peripheral location of the organism in the host erythrocyte). Later, Theiler identified the less virulent *A. centrale* that was named after its central location within erythrocytes (Kumar et al., 1982). *Anaplasma marginale* infects only erythrocytes in cattle. The major clinical sign is anemia without hemoglobinemia and hemoglobinuria. Anemia is caused by extravascular destruction of *A. marginale*-infected erythrocytes by phagocytosis. Cattle that survive acute infection become persistently infected carriers with cyclic low-level rickettsemia.

ANAPLASMOSIS IN HUMANS Human granulocytotropic ehrlichiosis (HGE), in actuality human anaplasmosis, results from infection of neutrophils with *A. phagocytophilum*. The disease varies from a self-limited undifferentiated febrile illness to severe multisystem disease, and seroprevalence studies suggest the significant occurrence of subclinical infections (Aguero-Rosenfeld et al., 1996; Horowitz et al., 1998a; Horowitz et al., 1998b; Bjoersdorff et al., 1999; Bakken and Dumler, 2000). Presenting with chills and fever frequently accompanied by headache, myalgia, and mylaise, fewer than half of cases develop nausea, vomiting, diarrhea, abdominal pain, cough and confusion. Rash occurs only rarely. Most patients have thrombocytopenia with or without leukopenia and mild-to-moderate increases in serum concentrations of hepatic transaminases. The case fatality rate is 0.5–1% with a sepsis-like picture, adult respiratory distress syndrome, and opportunistic fungal and viral infections occurring in some patients as life threatening events. Host factors including older age and underlying diseases play a role in severity of illness. Although persistent infection and recrudescence are very unlikely, some patients suffer fevers, fatigue, sweats, body pain, and the feeling of poor health long after the acute illness, apparently as a post-infectious syndrome as they present no evidence of persistent infection (Ramsey et al., 2002).

ANAPLASMA PLATYS INFECTION OF DOGS Canine *A. platys* infection is associated with cyclic thrombocytopenia.

Control and Prevention of Anaplasmoses and Cowdriosis

Anaplasmosis and heartwater (cowdriosis) of cattle cause economic losses by affecting meat and dairy production; therefore, much attention and effort have been paid to controlling these two diseases in animals. Anaplasmosis and ehrlichioses in animals could be controlled and prevented by arthropod control, chemoprophylaxis, immunization with vaccines, and maintenance of *Anaplasma*-free herds (Kocan et al., 2000b). Ticks can be controlled by application of acaricides; however, repeated application of acaricides could result in environmental pollution and development of acaricide-resistant tick populations. Tetracyclines are used extensively in the United States for anaplasmosis control. Chemotherapy can prevent clinical anaplasmosis but does not prevent cattle from becoming persistently infected with *A. marginale*. Outbreaks of anaplasmosis or heartwater disease can occur when carrier cattle are imported. Thus, in areas where anaplasmosis or heartwater disease is not endemic, the diseases have been effectively controlled by avoiding importation of infected animals (Kocan et al., 2000b).

Vaccine for Anaplasmosis

Vaccines have been developed for prevention of deaths of animals caused by anaplasmosis and heartwater, although they do not prevent infection. Vaccine studies for these diseases in animals will lead to the development of vaccines for use against ehrlichioses in humans. Both live and killed vaccines for anaplasmosis have been used in the United States. The antigens for vaccines previously were prepared from *Anaplasma*-infected bovine blood before *A. marginale* could be cultivated in cell culture. Live vaccines employ *A. centrale*, a low virulence strain, or attenuated *A. marginale*. After vaccination with live *A. centrale* or attenuated *A. marginale*, cattle develop mild-to-inapparent infections and become persistent carriers, which protects them against clinical anaplasmosis upon challenge exposure with *A. marginale* (De Waal, 2000; Kocan et al., 2000b). The fact that live vaccines containing *A. centrale* and the attenuated *A. marginale* do not provide effective cross-protection in widely separated geographic areas limits their broad use (Kuttler et al., 1984; Brizuela et al., 1998).

Anaplasma marginale outer membrane proteins have been evaluated as a vaccine (Palmer et al., 1986a). Current research for a vaccine is focused on development of a recombinant Msp vaccine of *A. marginale*. Cattle immunized with recombinant Msp-1 are partially protected against *A. marginale* challenge, and monoclonal antibodies to Msp-1 neutralize *A. marginale* in vitro (Palmer et al., 1986a). The neutralization-sensitive epitope is conserved among different geographic isolates and is located on a 28- or 29-amino acid tandem repeat of the Msp-1a (Allred et al., 1990). The number of tandem repeats varies between isolates of *A. marginale*. Peripheral blood mononuclear cells obtained from calves immunized with purified outer membranes of *A. marginale* proliferate in response to antigenic stimulation with Msp-1, Msp-2 or Msp-3 (Brown et al., 1998).

Genus Ehrlichia

Phylogeny

Ehrlichia diverged from a common ancestor of *Anaplasma* with which they share many biologic similarities (Figs. 1 and 5).

Taxonomy

The genus *Ehrlichia* includes *E.* (formerly *Cowdria*) *ruminantium*, *E. canis*, *E. chaffeensis*, *E. ewingii* and *E. muris* (Dumler et al., 2001).

Habitat

Ehrlichia are obligately intracellular bacteria that reside in a cytoplasmic vacuole in their tick host's midgut or salivary gland cells or in the vertebrate host's monocyte/macrophages (*E. chaffeensis*, *E. muris* and *E. canis*), neutrophils (*E. ewingii*), or endothelium (*E. ruminantium*).

Ehrlichia have evolved mechanisms to avoid the intracellular host defenses such as blocking the Jak1, Jak2, and Stat 1 transduction pathways by which IFN-γ limits availability of iron to ehrlichiae and downregulates NF-κ B activation of reactive oxygen species-mediated killing (Barnewall and Rikihisa, 1994; Barnewall et al., 1999).

Isolation

Some *Ehrlichia* have been isolated from their hosts in antibiotic-free cell culture employing continuous lines of monocytes/macrophages, e.g., DH82 cells. Others including *E. ewingii* have been passaged only in their animal hosts.

Identification

Ehrlichia are identified by determining the DNA sequence of particular genes such as 16S rRNA, *groEL*, *p28* and *gp120*.

Preservation

Ehrlichia are fragile, obligately intracellular bacteria that are best preserved within frozen living cells that can be thawed in a viable state.

Physiology: Adherence to the Host Cell

Ehrlichia enter the skin through a tick bite. The most common adhesin and receptor interaction is lectin-carbohydrate recognition (Ofek et al., 2002). Many bacterial adhesins are lectins, a class of sugar-binding proteins that link the bacteria to carbohydrate moieties of glycoproteins or glycolipids on the mammalian host cells. In some cases, bacterial surface polysaccharide or lipopolysaccharides bind to cognate lectins on the host cell surface (Ofek et al., 2002). *Ehrlichia* does not have detectable LPS. Of particular interest as a potential adhesin is the ehrlichial surface gp120 of *E. chaffeensis* and gp140 of *E. canis*. The evidence supporting gp120 as an adhesin is that recombinant *E. coli* expressing gp120 attaches to and then enters HeLa cells (Popov et al., 2000), which are nonphagocytic cells. The host cell receptors for *Ehrlichia* have yet to be identified. Upon attachment to the host cell, the organisms of *Ehrlichia* are phagocytosed by the host cell. Inhibition of lysosomal fusion by *Ehrlichia* may be critical for intracellular survival and replication of these organisms (Wells and Rikihisa, 1988; Barnewall et al., 1997; Mott et al., 1999). *Ehrlichia chaffeensis* lives in an early endosomal compartment, which fuses with transferrin-rich endosomes (Wells and Rikihisa, 1988; Barnewall et al., 1997; Mott et al., 1999).

Genetics

P28 Outer Membrane Proteins and Persistent Infection by Ehrlichia The 28-kDa protein is an outer membrane protein of *E. chaffeensis* (Yu et al., 1993). Twenty-two genes homologous to the *p28* gene have been found in a single locus of both *E. chaffeensis* and *E. canis* (Yu et al., 2000b; Ohashi et al., 2001). The *p28* multigene family has been found in all members of genus *Ehrlichia*, including *E. chaffeensis*, *E. canis*, *E. muris*, *E. ewingii* and *E. ruminantium* (Sulsona et al., 1999; Yu et al., 1999; Yu et al., 2000b; Gusa et al., 2001; Ohashi et al., 2001). The *p28* genes are homologous to the *msp-2* genes on the basis of both nucleotide and amino acid sequences;

however, *p28* genes are structurally very different from *msp-2* genes. Members of *p28* gene family are more diverse than those of the *msp-2* gene family. The *p28* gene can be classified into four groups. Genes close to each other physically have higher homology than genes physically more distant (Yu et al., 2000b). No highly conserved nucleotide sequences could be found when all *p28* genes were aligned, indicating no selective pressure to keep the genes from mutating. The homology of *p28* genes ranges from 19 to 80%. There are three to four hypervariable regions among the *p28* genes in a single group. Unlike the *msp-2* genes, all *p28* genes are complete genes, i.e., no genes are truncated at their ends (Yu et al., 2000b). With a proper promoter, all the *p28* genes should be expressed. Moreover, most *p28* genes are actively transcribed in cell culture (Long et al., 2002). A monoclonal antibody recognizing the recombinant p28 protein of *E. chaffeensis* reacts with a group of 25- to 30-kDa proteins by Western blot, indicating that the multiple proteins are expressed by the *p28* gene family (Yu et al., 1993). Analysis of the clinical isolates from different geographic locations reveals an absence of gene recombination among the *p28* genes (Long et al., 2002). Thus, recombination of the *p28* genes is not important for antigenic variation of *E. chaffeensis*; however, antigenic variation of the p28 protein may be the result of differential expression of *p28* genes.

Glycoproteins of Ehrlichia A 120-kDa glycoprotein (gp120) of *E. chaffeensis* is an immunodominant outer membrane protein (Yu et al., 1997; McBride et al., 2000). The protein is differentially expressed on the dense-core cell, but not on the reticular cell of *E. chaffeensis* (Popov et al., 2000b). The *E. chaffeensis* gp120 contains nearly identical tandem repeats with 80 amino acids each (Yu et al., 1997). The number of repeats varies among *E. chaffeensis* isolates from two to five with corresponding differences in the molecular size of this surface glycoprotein (Chen et al., 1997; Yu et al., 1997; Sims et al., 2000; Standaert et al., 2000). A 140-kDa glycoprotein (gp140), the *E. canis* analog of gp120, contains 14 tandem repeats of 36 amino acids each (Yu et al., 2000c). The *E. canis* gp140 and the *E. chaffeensis* gp120 are O-linked glycoproteins (McBride et al., 2000). The carbohydrate content of the glycoproteins comprises approximately 50% of their molecular mass.

Ecology

The life cycle of *Ehrlichia* includes two hosts: a tick and a vertebrate host. The organisms multiply in the tick midgut and salivary glands (Groves et al., 1975). Ticks transmit these bacteria in the course of feeding on mammals. The bacteria are transmitted in ticks from stage to stage (transstadially) when the tick molts or are passed from infected to noninfected ticks via vertebrate hosts that develop ehrlichemia. All species of *Ehrlichia* cause persistent infection in their natural mammalian hosts. Thus, mammalian hosts serve as a reservoir.

Ehrlichia ruminantium causes persistent infection in sheep, cattle, African buffalo and other wild African ruminants (Andrew and Norval, 1989). *Ehrlichia chaffeensis* has been documented to cause persistent infection in dogs (Breitschwerdt et al., 1998) and deer (Dawson et al., 1994a). Chronic canine ehrlichiosis is the most notorious persistent ehrlichial infection (Harrus et al., 1998). Owing to the homology between the *msp-2* genes of *Anaplasma* and the multiple copies of the ehrlichial *p28* multigene family, *p28* is postulated to be involved in the antigenic variation of *E. chaffeensis* that allows it to evade the host immune system (Reddy et al., 1998).

Ehrlichia ruminantium is transmitted by *Amblyomma* ticks. *Amblyomma variegatum* is the most important vector of *E. ruminantium* in Africa, and *A. hebraeum* is probably the only natural vector in most parts of southern Africa. Other *Amblyomma* ticks that have been proven as field vectors of heartwater include *A. lepidum* in the Sudan, *A. astrion* on the islands of Sao Tome and Principe, and *A. pomposum* in Angola. Other species, including five African species (*A. cohaerens*, *A. gemma*, *A. tholloni*, *A. sparsum* and *A. marmoreum*; Walker and Olwage, 1987), have been proven to be capable of transmitting heartwater in the laboratory, but have not been implicated in field outbreaks of the disease. *Amblyomma marmoreum* and *A. sparsum* have been imported into Florida and are of great concern because both species may play an important role in the establishment and maintenance of *E. ruminantium* (Burridge et al., 2000). In contrast, American *Amblyomma* species (*A. maculatum*, *A. americanum* and *A. cajennense*) are not competent for transmitting *E. ruminantium* (Mahan et al., 2000). The mammalian hosts of *E. ruminantium* include cattle, sheep, goats, and a variety of nondomesticated animals (Deem, 1998).

The proven vector for *E. chaffeensis* is the lone star tick, *A. americanum* (Anderson et al., 1993; Wolf et al., 2000), but *Dermacentor variabilis* and *Ixodes pacificus* have also been found infected in nature (Kramer et al., 1999). Human monocytotropic ehrlichiosis (HME) has been reported in the south-central and southeastern states and California. The mammalian hosts of *E. chaffeensis* are deer and wild and domesticated canines

(Dawson et al., 1994a; Dawson et al., 1994b; Dawson et al., 1996; Lockhart et al., 1997; Breitschwerdt et al., 1998; Murphy et al., 1998a; Kocan et al., 2000a). *Anaplasma platys* DNA has been amplified from *R. sanguineus* (Inokuma et al., 2000). *Ehrlichia ewingii* is transmitted by *A. americanum* (Anziani et al., 1990; Wolf et al., 2000). The vector for *E. canis* is the brown dog tick, *R. sanguineus* (Groves et al., 1975; Smith et al., 1976). Wild and domestic canids are the natural reservoir of *E. canis*.

Epidemiology

The striking concept that is becoming clear is that human monocytotropic ehrlichiosis (HME) is a very common disease rather than a rare entity (Olano et al., 1999). The geographic distribution of HME corresponds to that of the lone star tick (*A. americanum*). Following the report of the first case of HME in 1987, more than 700 cases were recorded at the United States Centers for Disease Control and Prevention (CDC) by 1997. Passive reporting of cases suggests that even in the states with the greatest number of cases, the incidence is low (e.g., 0.5 cases per 100,000 population in Arkansas). In contast, prospective outpatient clinic-based surveillance reveals a rate of 10–100 cases per 100,000 population in an endemic area of southeastern Missouri and southwestern Illinois, which is in an area where the *E. chaffeensis* zoonotic cycle involving white-tailed deer and lone star ticks is likely very similar to that in much of the rural southern states. Cases occur between April and September with 68% in May, June and July.

Ehrlichiosis ewingii is also transmitted by the lone star tick and has been diagnosed in Missouri, Oklahoma and Tennessee, although its distribution is likely throughout the range of the vector. A high proportion (70%) of patients have clear evidence of immunocompromise. Canine infections, of course, have the same geographic occurrence.

Canine monocytotropic ehrlichiosis is widespread around the world where the brown dog tick, *Rhipicephalus sanguineus*, occurs.

Ehrlichia ruminantium is widely distributed in sub-Saharan Africa and the West Indies where African *Amblyomma* ticks occur naturally or have been introduced.

Disease

Human Monocytotropic Ehrlichiosis The most severe human ehrlichiosis yet identified is human monocytotropic ehrlichiosis (HME). Between 40 and 63% of patients require hospitalization for an illness that has a median of 23 days of duration (Fishbein et al., 1994; Everett et al., 1994; Olano et al., 1999; Standaert et al., 2000). Despite the dramatic efficacy of doxycycline treatment, the case fatality rate is 2.5–3% (McQuiston et al., 1999). More than 80% of patients report tick exposure. Generally 7–10 days after tick bite inoculation of *Ehrlichia chaffeensis*, the patient presents with fever, malaise and headache often accompanied by myalgia. The clinical manifestations worsen progressively with anorexia (66%), nausea (48%), vomiting (37%), rash (36%), abdominal pain (26%), diarrhea (25%), lymphadenopathy (25%) and confusion (20%). Life threatening effects of severe HME are respiratory failure owing to interstitial pneumonia and adult respiratory distress syndrome, meningoencephalitis, hypotension, acute renal failure, coagulopathy and hemorrhage. Patients with HME usually develop leukopenia, thrombocytopenia, and elevated levels of serum hepatic transaminases. Conditions of immunocompromise such as acquired immunodeficiency syndrome pose a risk for overwhelming infection, with *E. chaffeensis* leading to death in a high proportion of patients (Paddock et al., 2001). The higher age-specific incidence for older patients suggests the importance of host factors in the disease. It should be noted, however, that children can also become infected and even develop severe illness (Schutze and Jacobs, 1997). It is becoming clear that this is a very common disease rather than a rare entity (Olano et al., 1999).

Canine Monocytic Ehrlichiosis The course of canine infection with *E. canis* is generally described as comprising three sequential stages: acute illness, subclinical phase, and chronic illness often leading to death. The acute illness varies in severity, with some dogs experiencing life-threatening illness but most animals suffering only mild-to-moderate fever, anorexia, and diminished activity (Reardon and Pierce, 1981a). Laboratory evaluation reveals thrombocytopenia and leukopenia that continue through the subclinical phase when the animal appears healthy. The duration of the subclinical phase is variable, lasting from months to years even. It is generally believed that particular breeds of dog, e.g., German shepherds, are more susceptible to a severe course and fatal outcome. Although genetic analysis of *p28* and *gp140* genes indicates that a single clone of *E. canis* is widely distributed in the United States, the possibility that some strains possess virulence factors that others lack cannot be excluded. The mechanism of thrombocytopenia during the acute and subclinical stages includes both splenic sequestration and anti-platelet antibodies (Harrus et al., 1999). The terminal phase of chronic canine mono-

cytotropic ehrlichiosis involves bone marrow hypoplasia (Reardon and Pierce, 1981b). Some animals die because of hemorrhage, e.g., massive epistaxis; others die with opportunistic infections.

Heartwater Disease Heartwater (cowdriosis), caused by *E. ruminantium*, is an acute and often fatal infectious disease of cattle, sheep, goats and certain wildlife species and is associated with endothelial infection in the brain and with pericardial effusion. Heartwater can cause mortality rates of up to 90% in susceptible domestic ruminant species. Its distribution in sub-Saharan Africa, the islands of the South Atlantic and Indian Oceans and the Caribbean Sea is associated with the presence of the tick vectors of the genus *Amblyomma*. The risk of spread of heartwater from the Caribbean is of concern to livestock production on the North and South American mainland, where totally susceptible domestic and wild animal populations exist (Mahan et al., 2000).

Prevention of Ehrlichioses

Human monocytotropic ehrlichiosis can be avoided only by preventing tick bite or removal of tick vectors prior to transmission. Canine monocytic ehrlichiosis has been controlled by acaricide elimination of ticks and use of tetracycline prophylaxis in the outbreak setting. There are no vaccines for canine or human ehrlichioses; however, substantial effort has been devoted to the prevention of heartwater and the development of a vaccine against *E. ruminantium*.

To prevent heartwater disease in southern Africa, animals are usually intentionally infected with *E. ruminantium* and then treated with antibiotics. The method suffers several disadvantages including deaths of the infected animals and difficulty in standardization of the vaccine (Mahan et al., 1999). Chemically inactivated or lysed *E. ruminantium* derived from bovine endothelial cell culture protects goats, sheep and cattle against homologous and heterologous challenge. Cattle immunized with inactivated vaccine develop a mild disease after challenge. In immunized animals, *E. ruminantium* is presumably controlled by cellular immunity mediated by IFN-γ and TNF-α. CD_4^+. T-cell lines from cattle vaccinated with the inactivated vaccine secrete IFN-γ when stimulated with *E. ruminantium* antigens. CD_4^+ and γ δ T cells expressing high levels of TNF-α, IFN-γ, and IL-2 receptor α chain were detected in cattle vaccinated with the live vaccine. IFN-γ has been shown to inhibit *E. ruminantium* growth in vitro, and its administration in mice can prevent death by an ordinarily lethal challenge. Both live and inactivated vaccines induce T-cell responses to outer membrane proteins of *E. ruminantium* including Map-1 (the homolog of the p28 protein family) and Map-2, suggesting that Map-1 and Map-2 play roles in stimulating immunity to control *E. ruminantium*. DBA/2 mice immunized with a *map-1* DNA vaccine are partially protected against lethal challenge. Splenocytes from the immunized mice produce IFN-γ and IL-2 when stimulated with *E. ruminantium* lysates or recombinant Map-1 antigen.

Genus Wolbachia

Taxonomy

Wolbachia pipientis is included in the Anaplasmataceae family (Dumler et al., 2001).

Habitat

Wolbachia strains are dimorphic with small, irregular, rodlike (0.5–1.3 μm in length) and coccoid (0.25–0.5 μm in diameter) forms. *Wolbachia pipientis* grows in the cytoplasm of its host cell in a membrane-bound vacuole of host origin. *Wolbachia* are endosymbionts of arthropods and nematodes. *Wolbachia* are estimated to infect 16% of insect species. *Wolbachia* are obligately intracellular, and they are maternally inherited. *Wolbachia pipientis* has been observed mainly in the cytoplasm of cells in the reproductive organs of the mosquito. Occasionally, *W. pipientis* has also been found in Malpighian tubules, muscle and nervous tissue (Stouthamer et al., 1999).

Ecology

To ensure a steady stream of progeny, *Wolbachia* have evolved strategies to boost their own reproductive success by increasing that of infected female hosts. *Wolbachia* manipulate arthropod reproduction by cytoplasmic incompatibility (CI), parathenogesis, and feminization. In most insect species, *W. pipientis* induces CI that results in embryonic death. CI occurs when a *Wolbachia*-infected male insect mates with a female insect that is either uninfected or infected with a different *Wolbachia* strain. In a CI cross, the sperm can successfully enter the eggs, but paternal chromosomes are unable to properly decondense and fuse with maternal chromosomes before entering the first mitotic division (Lassy and Karr, 1996). Thus, the maternal chromosomes enter mitosis before the paternal chromosomes, and the paternal chromosomes are eliminated, which renders the developing embryo haploid (Lassy and Karr, 1996; Callaini

et al., 1997). These embryos eventually die in diploid species and some haplodiploid mite species, whereas they develop into normal (haploid) males in other haplodiploid species such as wasps (Stouthamer et al., 1999). The effect of CI on the population of insects is that *Wolbachia*-infected females have a higher fitness than uninfected females.

Parthenogenesis occurs when infected females produce all-female offspring without fertilization by a male (Stouthamer et al., 1999). Parthenogenesis occurs only in haplodiploid hymenopteran species through a modification of the first mitotic division. Hymenoptera exhibits arrhenotoky, in which males arise from haploid eggs and females arise from diploid eggs. In infected eggs, the first mitotic division is aborted in the anaphase, leading to a diploid nucleus in an unfertilized egg (Stouthamer et al., 1999).

Feminization of *Wolbachia* occurs in aquatic insects such as an isopod, which diverts infected genetic males of the isopod into a phenotypic female (intersexed; Stouthamer et al., 1999). Feminization in the woodlouse *Armadillidium vulgare* is caused by suppression of the formation of the androgenic gland, which produces androgenic hormone that induces male differentiation. Without androgenic hormone, individuals normally develop into females.

Other effects of *Wolbachia* on insects are male-killing and modification of fecundity and fertility (Stouthamer et al., 1999), which enhances the spread of *Wolbachia* in the insect populations. The effects of *Wolbachia* on insects may result in speciation of insects owing to mating incompatibility of related species.

Wolbachia and Helminthic Diseases

Although *Wolbachia* does not cause infection in mammals, a recent discovery indicated that the host inflammatory response in the cornea was due to the lipopolysaccharide of *Wolbachia* in a filarial nematode (Saint et al., 2002). Although *Wolbachia* are parasites in most invertebrates, they may live mutualistically with nematodes because *Onchocerca ochengi*, a filarial nematode in cattle, die when their bacteria are destroyed. In other species, the females simply become sterile (Zimmer, 2001). The fact that elimination of *Wolbachia* is lethal or harmful to the parasite provides a new approach to treat some parasitic infections. For example, the antibiotic doxycycline has been used effectively to treat river blindness (Hoerauf et al., 2000). Embryogenesis of filarial nematodes is completely dependent on the presence of *Wolbachia*; thus antibiotics can be used effectively to sterilize the adult female worm (Hoerauf et al., 2000).

Applications

Wolbachia may be used as a genetic tool to transform pests and manipulate the vectors of the diseases. For example, if a gene encoding resistance to *Plasmodium* is transformed in the *Wolbachia* genome and these *Wolbachia* are established in a malaria vector, the mosquito might not become infected with *Plasmodium*. The recent discovery that *Wolbachia* contains bacteriophages (Masui et al., 2001) may provide a key genetic tool to transform *Wolbachia*.

Genus Neorickettsia

Taxonomy

The genus *Neorickettsia* includes *N. risticii*, *N. sennetsu* and *N. helminthoeca*.

Habitat

Neorickettsia organisms are obligately intracellular bacteria that reside within a cytoplasmic vacuole in the vertebrate host. They normally reside in a trematode parasite.

Genetics

Heterogeneity of the surface antigens has been observed between strain 90-12 of *N. risticii*, which was isolated from a vaccinated horse suffering from clinical Potomac horse fever, and strain 25-D, which was originally isolated in 1984 (Vemulapalli et al., 1995). Strain 90-12 is more virulent for mice and horses than strain 25-D. Mice immunized with strain 25-D are only partially protected against challenge with strain 90-12, whereas mice immunized with strain 90-12 are completely protected against challenge with strain 25-D. Sera from the strain 25-D-infected horses neutralize the homologous strain but do not neutralize strain 90-12; however, sera from strain 90-12-infected horses neutralize both strains. Although most antigens are similar between the two strains, there is a significant difference between them in the 85- and 50-kDa antigens. The 85-kDa antigen is specific to strain 90-12, and the 50-kDa antigen is present only in strain 25-D; however, the 85-kDa protein crossreacts with a 50-kDa antigen (Vemulapalli et al., 1995). Different type tandem repeats are present in both the 85- and 50-kDa protein genes, and eight identical repeat motifs are found in both genes; however, the numbers and positions of the repeats differ between the genes. The difference in sizes of the 85- and 50-kDa proteins is caused by the variable sequences. The crossreactivity of the

recombinant proteins confirmed the presence of conserved epitopes between these two antigens. Mice immunized with the recombinant 85-kDa protein are protected against homologous and heterologous strains, whereas mice immunized with the 50-kDa protein are protected against only the homologous strain (Vemulapalli et al., 1995).

Ecology

Neorickettsia risticii is maintained in nature in a complex aquatic ecosystem. Aquatic insects are likely to play an important role in the epidemiology of Potomac horse fever (PHF). A broad intermediate host range has been found for the trematodes that are the reservoir host for *N. risticii. Neorickettsia risticii* has recently been detected in trematode stages found in snail secretions and in metacercariae found in immature and adult aquatic insects including caddisflies (Trichoptera), mayflies (Ephemeroptera), damselflies (Odonata and Zygoptera), dragonflies (Odonata and Anisoptera), and stoneflies (Plecoptera; Chae et al., 2000). Transmission of *N. risticii* to horses can occur through accidental ingestion of insects such as caddisflies containing *Neorickettsia*-infected metacercariae (Madigan et al., 2000). The trematode infection rate varies between 40 and 93.3% in large snails (shell size >15 mm) and between 0 and 13.3% in small snails (<15 mm). A study in California indicates that the highest trematode infection rate for large and small snails is observed in September, and the lowest infection rate for large snails is recorded in June and October (Chae et al., 2000).

Physiology

Neorickettsia possibly enter the bloodstream through the wall of the gastrointestinal tract after ingestion. After entering the body, these organisms attach to the target cell surface through an adhesin and a receptor. The evidence that organisms in the family Anaplasmataceae attach to the host cell through an adhesin of the organism and a host cell receptor came from the study of *N. risticii* (Messick and Rikihisa, 1993; Messick and Rikihisa, 1994). Proteinase- or paraformaldehyde-treated *N. risticii* lost the ability to bind to P388D1 cells, indicating that the ehrlichial ligand and host cell receptor are likely surface proteins. The binding to, internalization of, or proliferation of *N. risticii* in P388D1 cells is not affected by cytochalasin D, a microfilament-disrupting agent. Monodansylcadaverine, a transglutaminase inhibitor, does not affect the attachment of ehrlichiae to host cells, but suppresses internalization and proliferation of *N. risticii*. Thus, *N. risticii* is internalized by the P388D1 cells through receptor-mediated endocytosis (sensitive to monodansylcadaverine) and not through phagocytosis (sensitive to cytochalasin; Messick and Rikihisa, 1993; Messick and Rikihisa, 1994). *Neorickettsia risticii* has a different fate when it enters the host cell through an Fc-receptor. *Neorickettsia risticii*-specific IgG antibody-opsonized *N. risticii* is killed by P388D1 cells when it enters the cell through an interaction with Fc-receptor (Messick and Rikihisa, 1993; Messick and Rikihisa, 1994). *Neorickettsia risticii* inhibits phagolysomal fusion by a process that appears to require neorickettsial protein synthesis (Wells and Rikihisa, 1988).

Disease

Potomac Horse Fever Equine neorickettsial colitis was first recognized in Maryland. The disease has been documented in Pennsylvania, New Jersey, New York, Ohio, Idaho, Connecticut (Palmer et al., 1986b), California, South America (Uruguay and southern Brazil; Dutra et al., 2001), and Europe. The disease is seasonal, manifesting as sporadic colitis in unstressed horses. The incidence rate is generally low. Horses on pasture, as well as those stabled, can be affected. Clinical signs vary from fever and depression to severe diarrhea and laminitis. Occasionally, horses develop profound ileus and severe colic (Palmer et al., 1986b). The consistent clinical and hematologic features of horses experimentally infected with *N. risticii* include fever, depression, anorexia and leukopenia. Diarrhea develops in 73% of the horses. Rickettsemia (i.e., the *N. risticii* titer in the blood) reaches a maximum at the peak of fever. Mortality is 9%. There are no significant differences in clinical and hematologic features between horses that survive and those that die of *N. risticii* infection (Dutta et al., 1988).

Sennetsu Neorickettsiosis In 1953 in Japan, *N. sennetsu* was isolated from the blood, bone marrow, and lymph node of a patient with signs, symptoms, and laboratory data suggestive of infectious mononucleosis (Tachibana, 1986). The typical clinical manifestations are chills, fever of two weeks duration, headache, myalgia, and enlarged preauricular and posterior cervical lymph nodes (Misao and Kobayashi, 1955). Hepatosplenomegaly occurs in fewer than half of the patients. After early leukopenia, absolute lymphocytosis develops with 10% or more atypical lymphocytes.

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Prokaryotes (2006) 5:529–546
DOI: 10.1007/0-387-30745-1_21

CHAPTER 3.1.20

The Genus *Coxiella*

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Introduction

Coxiella burnetii is a bacterial obligate intracellular parasite and the causative agent of human Q fever. The Australian physician Edward Derrick used the term "query (Q) fever" in 1935 to describe an outbreak of febrile illness of unknown etiology among abattoir workers in Queensland, Australia (Derrick, 1937). Although Derrick was successful in transmitting a febrile disease to guinea pigs using patient blood or urine, he was unsuccessful in visualizing or isolating the causative agent. Consequently, he sent infected guinea pig liver to Burnet and Freeman, who reproduced symptoms of the disease in various laboratory animals. Impression smears of spleen harvested from infected mice revealed large, intracellular vacuoles containing small rod-shaped organisms that resembled typical rickettsiae; consequently, the organism was named *Rickettsia burnetii* (Burnet and Freeman, 1937). Coincident with the Australian studies, a tick transmission study of Rocky Mountain spotted fever was being conducted in Hamilton, Montana, by Davis and Cox (Davis and Cox, 1938). They established a febrile illness in guinea pigs that did not mimic spotted fever in presentation. The causative agent, referred to as the "Nine Mile agent," was filterable and failed to grow on axenic media. A laboratory worker on the project became infected with the Nine Mile agent and developed symptoms of Q fever as described by Derrick and Burnet. This led to a collaboration between the two groups and a series of experiments that demonstrated that the Nine Mile and Australian Q-fever agents (*R. burnetii*) were the same microorganism. *Rickettsia burnetii* subsequently was reclassified as *Coxiella burnetii* in recognition of the efforts of Cox and Burnet in identifying the new bacterial pathogen (Philip, 1948).

Phylogeny

Because of an obligate intracellular nature, similar staining characteristics, and carriage by ticks, *C. burnetii* was historically classified within the α-1 subgroup of the *Proteobacteria* in the Rickettsiales order, the Rickettsiaceae family, and the *Rickettsia* tribe, which included the genus *Rickettsia* and formerly the genus *Rochalimaea* (*Rochalimaea* has now been reclassified as the genus *Bartonella*). A revised classification of *C. burnetii* will be described in the 2nd edition of *Bergey's Manual of Systematic Bacteriology*, where the organism will be taxonomically placed in the domain Bacteria, phylum *Proteobacteria* phy. nov., class "Gammaproteobacteria," order "Legionellales," family "Coxiellaceae." The other resident of the "Coxiellaceae" family is *Rickettsiella grylii*, an intracellular parasite of crickets (Roux et al., 1997), and the remaining genus within the order "Legionellales" is *Legionella*. Indeed, *C. burnetii* displays both phenotypic and genetic similarities to *L. pneumophila* (Drancourt and Raoult, 1994), including intracellular growth within a membrane-bound vacuole and marked sequence similarities for a variety of genes including *mip* (macrophage infectivity potentiator), a protein associated with increased survival within macrophages (Cianciotto et al., 1995; Mo et al., 1995), and components of a type IV secretory apparatus (Segal and Shuman, 1999). *Coxiella burnetii* is also distinguished from *Rickettsia* on a phenotypic level by displaying pronounced extracellular stability and resistance to chemical and physical disruption, by acid activation of metabolism, by an impressively diverse animal host range, and by an aerosol route of infection (Baca and Paretsky, 1983).

Isolates of *C. burnetii* derived from a variety of geographic areas and various hosts exhibit a considerable degree of phylogenetic homogeneity when examined by 16S rRNA gene sequencing and DNA-DNA hybridization, supporting the designation of a single species with the genus (Stein et al., 1993; Vodkin et al., 1986b). Interestingly, when *C. burnetii* total genomic DNA is examined by restriction fragment length polymorphism (RFLP) analysis, considerable heterogeneity in banding patterns is observed (Heinzen et al., 1990; Hendrix et al., 1991; Mal-

lavia, 1991). This apparent paradox may reflect, in part, the presence of repetitive DNA elements in the *C. burnetii* chromosome (Heinzen et al., 1991; Hoover et al., 1992). The RFLP analysis of genomic DNA resulted in the subdivision of *C. burnetii* isolates into six genomic groups (I to VI; Heinzen et al., 1990; Hendrix et al., 1991; Mallavia, 1991). Evidence suggests that *C. burnetii* isolates carry a single linear chromosome ranging from approximately 1,500 to 2,400 kilobases (kb; Willems et al., 1996; Willems et al., 1998). Most *C. burnetii* strains harbor 1 of 4 autonomously replicating plasmids, termed "QpH1," "QpRS," "QpDV" and "QpDG" (Mallavia, 1991). These plasmids range from 36–42 kb in size and share a common 30-kb "core" region along with unique regions (Mallavia, 1991; Samuel et al., 1985). In some isolates, plasmid sequences are integrated into the chromosome (Mallavia, 1991; Savinelli and Mallavia, 1990; Willems et al., 1997). There is an association of plasmid types with specific genomic groups. For example, QpHI is associated with genomic groups I, II and III, whereas QpRS is associated with genomic group IV (Mallavia, 1991). Common and unique plasmid sequences have been used with the polymerase chain reaction (PCR) to detect *C. burnetii* in clinical samples and as an epidemiologic tool (Willems et al., 1993). The absolute conservation of chromosomally integrated or autonomously replicating plasmid sequences among all C. burnetii isolates examined to date suggests that these sequences play a critical role in *C. burnetii* biology.

Habitat

Coxiella burnetii has a broad host range that includes arthropods (primarily ticks), fish, birds and a variety of mammals (Baca and Paretsky, 1983). The far-ranging zoonotic relationships and geographic distribution of *C. burnetii* are described in an extensive review by Babudieri (1959). The organism has a worldwide distribution with the exception of Antarctic regions and New Zealand (Babudieri, 1959; Hilbink et al., 1993). In both wild and domestic animals, *C. burnetii* does not appear to cause overt disease with the exception of sheep and goats where it can cause abortions (Palmer and Key, 1983). Infection of cattle also has been associated with reproductive problems, including infertility and low birth weight (Ho et al., 1995). All infected mammals shed organisms in feces, urine, milk, and birth products (Babudieri, 1959). During natural acute infection of animals, *C. burnetii* can be isolated from the lung, spleen, liver and blood (Maurin and Raoult, 1999). Chronic infection of females also targets the uterus and the mammary glands (Babudieri, 1959). Of particular significance is the tropism of *C. burnetii* for placental tissue, where the parasite load can reach concentrations $\geq 10^9$ organisms per gram (Babudieri, 1959). Unlike rickettsial agents, *C. burnetii* is remarkably resistant to desiccation and ultraviolet radiation and therefore can persist in contaminated soils for extended periods (Babudieri, 1959). Inhalation of contaminated aerosols is the primary route of human infection with *C. burnetii* (Maurin and Raoult, 1999), and an extremely low intraperitoneal infectious dose of 2–4 organisms has been demonstrated in the guinea pig model of Q fever (Moos and Hackstadt, 1987). Thus, *C. burnetii* represents an occupational hazard, particularly for individuals involved in animal husbandry operations where large numbers of organisms can be shed into the environment during parturition. In arthropods, *C. burnetii* replicates in the midgut or stomach (Babudieri, 1959). Regardless of the host and cell type, *C. burnetii* is an obligate intracellular bacterium that replicates in a membrane-bound vacuole having characteristics of a phagolysosome (Heinzen et al., 1996c).

Isolation

A handful of culture methods are available for isolation of *C. burnetii* from clinical samples or reservoir hosts. Isolation generally requires biosafety level 3 (BSL-3) laboratory practices as *C. burnetii* is notoriously infectious and one of the most common causes of laboratory-acquired infections (Harrison et al., 1990). As an obligate intracellular bacterium, *C. burnetii* requires a viable eukaryotic host cell for propagation, which can be in the form of laboratory animals (primarily guinea pigs and mice), embryonated hen eggs, or tissue culture cells (Maurin and Raoult, 1999). The use of animals for isolating *C. burnetii* is troublesome owing to the additional requirement of a BSL-3 animal care facility. *Coxiella burnetii* is shed in feces and urine of infected animals; consequently, infectious aerosols are generated that represent an occupational hazard for laboratory workers. Guinea pigs develop an illness comparable to that in humans and are particularly useful for obtaining isolates from contaminated samples. Typically, guinea pigs are injected intraperitoneally with a clinical sample and sacrificed about a week later. During acute infection, necrotic foci can be observed in the spleen, liver, bone marrow and other organs. Spleen homogenates are most commonly used for recovery of *C. burnetii* (Heggers et al., 1975).

Cell culture has supplanted animal and embryonated eggs as the method of choice for isolating

C. burnetii. A variety of primary and continuous cell lines support growth of the organism (Baca and Paretsky, 1983). Raoult and coworkers (Raoult et al., 1990b) have developed an especially efficient method of isolating *C. burnetii* from clinical samples. The technique employs infection of human embryonic lung (HEL) fibroblasts grown on a 12-mm round cover slip in a 3.7-ml shell vial. The HEL monolayers are inoculated with 1 ml of clinical sample and vials are centrifuged at 700 × *g* for 1 h at room temperature to promote adherence and internalization of *C. burnetii*. The *C. burnetii* in the coverslip preparations of infected cells are observed by immmunofluorescence or Gimenez staining (Gimenez, 1964) after 5–7 days of incubation. This procedure has been successfully used to isolate *C. burnetii* from a number of clinical specimens and has markedly increased the number of strains available for study (Maurin and Raoult, 1999; Raoult et al., 1990b).

Identification

Several methods are employed to visualize and identify *C. burnetii* within infected tissues and cell cultures. Conventional stains used to visualize *C. burnetii* include Giemsa, Macchiavello, and Gimenez stains, which color organisms purple, red, and bright red, respectively (Baca and Paretsky, 1983). Despite a prototypic Gram-negative cell envelope, the organism stains variably with the Gram stain (Baca and Paretsky, 1983). Infected cells display small (0.3 to 1.0 μm), highly pleomorphic coccobacilli within membrane-bound vacuoles. These vacuoles can grow to encompass nearly all of the cell cytoplasm with minimal cytopathic effect. The pleomorphism of *C. burnetii* results from the production of distinct morphological forms that are part of an incompletely defined biphasic developmental cycle (Heinzen et al., 1999).

Because of the notorious efficiency of aerosol transmission of *C. burnetii*, isolation and cultivation of the organism present an occupational risk to clinical laboratory workers and generally require BSL-3 level containment. Lengthy culture periods are often required and the recovery success rate is variable. Nonetheless, the procedure is conducted by trained personnel in select laboratories, often in combination with immunofluorescence to aid in unequivocal identification of *C. burnetii* as the infectious agent (Muhlemann et al., 1995; Musso and Raoult, 1995; Raoult et al., 1990b). Direct and indirect immunofluorescence using either monoclonal or polyclonal antibodies directed against *C. burnetii* antigens is effective in detecting the organism directly within tissue samples, particularly infected cardiac valves (Baumgartner et al., 1988; Brouqui et al., 1994; Raoult et al., 1994). A capture-enzyme linked immunoassay using a genus-specific monoclonal antibody directed against *C. burnetii* lipopolysaccharide (LPS) detects as few as 2,500 *C. burnetii* particles within clinical samples (Thiele et al., 1992).

Amplification of genus-specific DNA sequences using PCR has rapidly supplanted the use of radiolabeled probes as the nucleic acid method of choice for identifying *C. burnetii* strains in clinical samples and cell culture (Mallavia et al., 1990; Stein and Raoult, 1992; Willems et al., 1993; Willems et al., 1994). Chromosomal genes employed as targets include those encoding 16S rRNA (*rrs*), citrate synthase (*gltA*), and iron-containing superoxide dismutase (*sodB*). Another target of PCR amplification is a unique chromosomal repetitive transposon-like sequence with a copy number of about 19 (Willems et al., 1994). This strategy provides a sensitive and specific detection method that has been used to diagnose *C. burnetii* infections in humans (Maurin and Raoult, 1999; Willems et al., 1994). Unique and conserved *C. burnetii* plasmid DNA sequences also have been successfully employed to identify and strain type various isolates (Willems et al., 1993).

Structure

Coxiella burnetii displays a prototypic Gram-negative cell wall structure when observed by transmission electron microscopy (TEM; McCaul and Williams, 1981a). However, the agent does not stain with the Gram stain consistently and organisms are typically visualized using the Gimenez staining method (Gimenez, 1964). *Coxiella burnetii* is a small (0.3 to 1.0 μm), highly pleomorphic coccobacillus. The pleomorphic nature of the organism is due to the generation of morphologically distinct cell forms that arise as part of a biphasic developmental cycle (Heinzen, 1997a; McCaul, 1991a; McCaul and Williams, 1981a).

The cell wall peptidoglycan is biochemically similar to that of other Gram-negative bacteria. Although the glycan portion is sensitive to digestion by lysozyme, treatment does not result in dissociation of the sacculus structure (Amano and Williams, 1984b). This enhanced rigidity has been attributed to the presence of peptidoglycan-associated, protease-resistant, cell wall proteins (Amano and Williams, 1984b).

Coxiella burnetii undergoes a lipopolysaccharide (LPS) phase variation that is similar to that observed in the Enterobacteriaciae. Transition from a smooth (full-length O-side chains) phase I to a rough (truncated O-side chains) phase II LPS structure occurs upon repeated passage of

the organism in an immunoincompetent host such as embryonated eggs or tissue culture cells. Plaque-purified phase II organisms are avirulent for guinea pigs (a fever animal model of *C. burnetii* infection), whereas phase I *C. burnetii* causes disease and is always associated with naturally infected mammals and ticks (Hackstadt, 1990). A strain displaying a semi-rough-type LPS has intermediate virulence for guinea pigs (as evidenced by fever production) but lacks persistence in the spleen (Moos and Hackstadt, 1987). The genetic lesion(s) accounting for phase variation has not been precisely determined, but likely involves the loss of chromosomal regions carrying LPS biosynthesis genes (O'Rourke et al., 1985; Vodkin and Williams, 1986a).

Phase I and phase II *C. burnetii* are indistinguishable by TEM, and their intracellular growth characteristics are similar. However, the LPS of phase variants is biochemically and antigenically distinct (Amano and Williams, 1984a; Schramek and Mayer, 1982). The aldose sugar component of phase I LPS is chemically more complex, having at least nine different sugars as compared to two in phase II LPS (Schramek and Mayer, 1982). Two phase I sugars, L-virenose and dihydro-hydroxystreptose, are branched sugars that have not been previously described in LPS (Schramek et al., 1985). It has been suggested that these exotic sugars may afford *C. burnetii* protection from lysosomal digestion (Schramek et al., 1985). The central core regions of *C. burnetii* LPS contain a heptose-like moiety and a 2-keto-3-deoxyoctonate (KDO)-like component that is different from enteric KDO (Amano and Williams, 1984a; Hackstadt et al., 1985). *Coxiella burnetii* lipid A contains a complex and heterogeneous mixture of branched fatty acids (Wollenweber et al., 1985). The unusual biochemical makeup of *C. burnetii* LPS probably accounts for the 100- to 1,000-fold less toxicity in a mouse lethality assay when compared to LPS of *Escherichia coli* or *Salmonella typhimurium* (Amano et al., 1987).

Surface iodination experiments reveal a complex assortment of *C. burnetii* surface proteins with predominant labeling of proteins in the 12–14, 27–30, and 60 kDa range (Hackstadt, 1985; Hackstadt, 1988; Williams et al., 1984b; Williams and Stewart, 1984a). Although full-length LPS of phase I organisms is known to sterically block iodination of certain surface proteins (Hackstadt, 1988), the overall protein profile of phase variants appears identical, and phase-specific proteins have not been identified (Hackstadt, 1988; Williams et al., 1984b; Williams and Stewart, 1984a). The cloning of genes encoding four *C. burnetii* surface proteins has allowed a more detailed examination of their potential functions. Macrophage infectivity potentiator (Mip) is a peptidylprolyl isomerase (PPIase) localized to the cytoplasm, periplasmic space and outer surface of *C. burnetii* (Mo et al., 1995; Mo et al., 1998). Although the function of Mip in *C. burnetii* has yet to be defined, *L. pneumophila mip* mutants are attenuated in their ability to infect and survive in macrophages (Cianciotto and Fields, 1992). A second periplasmic and outer-surface-localized enzyme, Com-1, is a homologue of the disulfide bond-forming enzymes DsbA and DsbC (Hendrix et al., 1993). These enzymes are required for folding of virulence determinants in *Shigella flexnerii* and *E. coli* (Watarai et al., 1995). An immunogenic lipoprotein designated P2 (Williams et al., 1990) and a plasmid-encoded surface protein termed "E'" also have been described (Minnick et al., 1991).

Coxiella burnetii has an impressive ability (greatly surpassing that of bacterial vegetative cells) to survive in the extracellular environment and to resist physical and chemical disruption (Babudieri, 1959; Scott and Williams, 1990). Viable organisms can be recovered after heat treatment at 63°C for 30 min, exposure to a 10% salt solution for 180 days at room temperature, or sonication in distilled water for 30 min (Babudieri, 1959; Heinzen, 1997a). The remarkable resistance of *C. burnetii* is attributed to a small, resistant cell form that is part of a biphasic developmental cycle (Heinzen, 1997a; McCaul and Williams, 1981a). Two morphologically distinct cell types, termed "large-cell-variants" (LCVs) and "small-cell-variants" (SCVs), are part of this cycle. They can be isolated with relative purity by exploiting differences in their buoyant densities (Heinzen et al., 1996b; Wiebe et al., 1972). Preparations highly enriched for SCV also can be obtained by procedures that exploit the sensitivity of LCV to physical disruption (Heinzen, 1997a; McCaul et al., 1981b).

The LCV can reach a length exceeding 1.0 μm and is similar to a typical Gram-negative bacterium in possessing a clearly distinguishable outer membrane, periplasmic space, and cytoplasmic membrane (Heinzen, 1997a; McCaul et al., 1981b). The LCV is more pleomorphic than the SCV, with a thinner cell wall and a dispersed nucleoid (Heinzen, 1997a; McCaul et al., 1981b). Both LCV and SCV divide by binary fission (Heinzen, 1997a). The SCV is typically between 0.2 and 0.5 μm in size, rod-shaped, and very compact. The visible periplasmic space is replaced with an electron-dense region bounded by the cytoplasmic and outer membranes. The most distinctive ultrastructural characteristic of the SCV is the electron-dense, condensed chromatin (Heinzen, 1997a; McCaul et al., 1981b). A subpopulation of the SCV, called the "small, dense cell," has been described that displays extreme tolerance to breakage by high pressure

(20,000 lb/in^2), a procedure that destroys typical SCVs (McCaul et al., 1991b).

An electron-dense, membrane-bound, polar body termed a "spore-like particle" (SLP; Schaal et al., 1987) has been visualized by TEM within LCV (Heinzen, 1997a; McCaul et al., 1981b). Evidence obtained by McCaul and Williams (1981a) resulted in a model for *C. burnetii* development that includes both vegetative morphological differentiation of SCV to LCV and sporogenic differentiation of SLP within the LCV. In the postulated developmental cycle, it was proposed that SLPs are released from degenerating LCV to act as the infectious precursor of SCV. The SLPs are approximately 130–170 nm in diameter and are bounded by a limiting membrane. The particle is occasionally observed in cells morphologically classified as LCV and usually occurs in a polar location. From the center outward, the SLP is believed to be comprised of a dense core, a system of membranes, peptidoglycan and an outer membrane (McCaul, 1991a). One report indicates that SLPs contain DNA (McCaul and Williams, 1990). Whether the SLP is a developmental progenitor of the SCV remains to be proven. The biochemical spore marker, dipicolinic acid, is not detected in *C. burnetii* and traditional spore stains have little affinity for the SLP (McCaul, 1991a). Moreover, the SLP has not been purified to homogeneity and tested for infectivity.

Although the SLP has not been purified and biochemically characterized, analysis of purified SCV and LCV cell lysates and immunogold TEM of infected cells clearly demonstrate that SCV and LCV have a different protein composition (Heinzen, 1997a; Heinzen et al., 1996b; Heinzen et al., 1996c; Heinzen et al., 1999; McCaul et al., 1991b; Seshadri et al., 1999). Two SCV-specific DNA-binding proteins designated "Hq1" and "ScvA" have been identified and their encoding genes cloned (Heinzen and Hackstadt, 1996a; Heinzen et al., 1996b). Both proteins are very basic with high isoelectric points. The ScvA protein is only 30 amino acids in length and has no homologues in the protein database. The Hq1 protein is 117 amino acids in length and exhibits 34% and 26% identity with eukaryotic histone H1 and the histone-like protein Hc1 of *Chlamydia trachomatis*, respectively. Hc1 is thought to play a role in condensing chlamydial chromatin during reticulate-to-elementary body differentiation (Hackstadt et al., 1991). Immunogold TEM demonstrates an abundance of ScvA in association with the condensed chromatin of the SCV (Heinzen et al., 1996b), and both ScvA and Hq1 bind DNA in vitro (Heinzen and Hackstadt, 1996a; Heinzen et al., 1996b). These observations led to speculation that ScvA and Hq1 are integral components of the compact SCV nucleoid structure (Heinzen and Hackstadt, 1996a; Heinzen et al., 1996b). Binding of SCV genomic DNA by one or both of these proteins could serve a protective role by stabilizing the chromosome or inducing topological changes that alter gene expression. Several proteins also are differentially synthesized by the LCV. Immunoblotting with specific antibodies demonstrates that the translation elongation factor EF-tu is only detectable in lysates of LCV, whereas EF-ts is present at a fourfold higher concentration in LCV than in SCV (Seshadri et al., 1999). This observation led to the hypothesis that SCVs are functional equivalents of stationary phase growth forms, whereas LCVs represent log phase cell forms (Heinzen et al., 1999). This hypothesis seems unlikely with the recent discovery that synthesis of the stationary phase stress response sigma factor RpoS is dramatically upregulated in the large cell variant (R. Seshadri and J. E. Samuel, submitted). Expression of a major outer-membrane protein termed "P1" with porin activity is also upregulated in the LCV when compared to the SCV (McCaul, 1991a; McCaul et al., 1991b; Seshadri et al., 1999; S. Varghees and J. E. Samuel, submitted).

Cultivation

A milestone event in the study of *C. burnetii* and other rickettsiae occurred in 1941 with the successful propagation of these organisms in embryonated hen's eggs by Cox (1941). This method of propagation is still used and can yield up to 0.2 gram (wet weight) of purified *Coxiella* from one dozen yolk sacs (Baca and Paretsky, 1983). A drawback of this procedure is that it generates a significant amount of contaminated waste that must be carefully decontaminated. The protocol involves inoculation of the yolk sac of 5–7-day-old embryos with continued incubation for 10–12 days. The organism replicates to high densities in the yolk sac membrane.

Both continuous and primary tissue culture cell lines have been used to propagate *C. burnetii*. These include primary chick and mouse embryo fibroblasts and continuous cell lines like Vero (African green monkey kidney epithelial), L-929 (murine fibroblast), and J774.1 and P388D1 (both murine macrophage-like cells; Baca and Paretsky, 1983). Organisms are typically harvested when large vacuoles filled with *C. burnetii* are observed throughout the monolayer. Cells are scraped from culture flasks, and *Coxiella* are released by mechanical disruption or by gentle sonification (Baca and Paretsky, 1983).

Purification of *C. burnetii* from yolk sacs or tissue culture cells is a laborious and time-consuming procedure that involves a series of

differential centrifugation steps followed by density gradient centrifugation through sucrose or Renografin (Samuel et al., 1983; Weiss et al., 1975). The hydrophobic, truncated LPS of phase II *C. burnetii* results in tenacious adherence of organisms to host material, which consequently results in lower yields than that obtained for phase I organisms (Baca and Paretsky, 1983; Heinzen et al., 1999). Guinea pigs are the preferred laboratory animal for amplification and purification of *C. burnetii* isolates, although they are not as widely used since the development of cell culture methods. Necrotic foci are observed in the liver, spleen, and heart, and organisms are typically harvested from enlarged infected spleens 1–2 weeks following infection (Heggers et al., 1975).

Physiology

Our current understanding of the unique biology of *C. burnetii* is limited because of restrictions imposed by its obligate intracellular nature, the lack of workable genetic systems, and the relative paucity of laboratories engaged in research on the organism, which requires BSL-3 containment. However, some advances have been made in characterizing the physiologic requirements and capabilities of *C. burnetii*. The organism undergoes luxurious growth in the phagolysosome of host cells with an estimated doubling time of 8–12 hours (Baca and Paretsky, 1983). Replication occurs despite the presence of toxic factors that are normally considered bactericidal such as acid hydrolases and defensins. Minimal cytopathic effects are noted on infected host cells. *Coxiella burnetii* has evolved a unique adaptation to the phagolysosome, as the organism is an acidophile that has an absolute requirement for the moderately acidic pH (~4.5–5) found in the vacuole to activate its metabolism (Hackstadt and Williams, 1981a). An acidic environment is thought to provide a proton motive force that sufficiently energizes the cytoplasmic membrane and transporters of metabolites such as glutamate and proline (Hackstadt and Williams, 1983b; Hendrix and Mallavia, 1984). In vivo, *C. burnetii* replication can be inhibited by treatment of host cells with lysosomotropic amines (Hackstadt and Williams, 1981a) or bafilomycin A1 (Heinzen et al., 1996c), agents that result in alkalinization of the phagolysosome. Like more extreme acidophiles, *C. burnetii* maintains an intracellular pH near neutrality under optimal conditions (Hackstadt, 1983a). When host-cell free *C. burnetii* organisms are incubated at ~pH 4.7 without a metabolizable substrate such as glutamate, the intracellular pH is 5.88, whereas in the presence of glutamate, the intracellular pH rises to 6.7 (Hackstadt, 1983a). Thus, intracellular pH homeostasis of *C. burnetii* is at least, in part, an energy-requiring process. Moreover, intracellular ATP pools are unstable at pH 4.5 in the absence of glutamate, though they are stable at pH 7 with or without glutamate. These unique metabolic properties of *C. burnetii* represent a "biological strategy" (Hackstadt and Williams, 1981a) for maintaining extracellular stability while promoting intraphagolysosomal metabolism and replication.

A metabolic lesion(s) that would account for *C. burnetii*'s obligate parasitism has not been identified. The Embden-Meyerhof-Parnas (McDonald and Mallavia, 1971), oxidative pentose phosphate (McDonald and Mallavia, 1970), gluconeogenesis (McDonald and Mallavia, 1971), TCA cycle (Hackstadt and Williams, 1981b), nucleotide synthesis (Christian and Paretsky, 1977), and protein synthesis and catabolic (Zuerner and Thompson, 1983) pathways are all functional in *C. burnetii*. The mechanism of nutrient acquisition by the organism is undefined but likely involves trafficking and fusion of nutrient-laden autophagic and/or heterophagic vesicles with the parasite-containing phagolysosome where metabolites are hydrolyzed to provide precursors for *C. burnetii* metabolism (Heinzen and Hackstadt, 1997b; Heinzen et al., 1996c). Breakdown of extra- and intracellular high molecular weight compounds by lysosomal enzymes is known to generate high concentrations of monosaccharides, nucleosides, amino acids, fatty acids, sulfates, and phosphates in vesicles of the lysosomal pathway (Barrett, 1984). A vesicle-mediated nutrient delivery system is supported by the observation that low molecular weight fluorescent molecules do not passively diffuse into the *C. burnetii*-containing phagolysosome when microinjected into the cytosol of infected cells (Heinzen and Hackstadt, 1997b). *Coxiella burnetii* has a growth requirement for iron (Howe and Mallavia, 1999). Replication of the organism in J774.1 murine macrophage-like cells upregulates transferrin receptor synthesis with a coincident increase in the intracellular iron concentration. Moreover, growth is inhibited when the intracellular iron chelator desferrioxamine is added to the culture medium.

The physiologic relevance of LCV and SCV developmental forms is undefined. The results of a few studies suggest that LCVs are more metabolically and replicatively active than SCVs (Howe and Mallavia, 1999; McCaul, 1991a; McCaul et al., 1981b). Thus, they may play a more important role than SCVs in amplification and spread of *C. burnetii* within the infected host. A proposal was made that SCVs are simply the equivalent of stationary phase growth forms,

whereas LCVs represent log-phase cell forms (Heinzen et al., 1999). This idea is contradicted by a recent observation that the stationary phase stress response sigma factor RpoS is dramatically upregulated in the LCV (R. Seshadri and J. E. Samuel, submitted). The hardiness of the SCV is well documented (Heinzen et al., 1999). Thus, this cell form may survive the degradative enzymes and peptides of the phagolysosome for extended periods, and it is likely the cell form that is responsible for long-term extracellular survival and natural aerosol transmission of the agent. Because *C. burnetii* organisms do not actively lyse host cells and are transmitted by desiccated infected tissues, a sustained supply of resistant cell forms is critical to their survival. In the laboratory, both LCV and SCV are infectious for in vitro and in vivo models (Heinzen, 1997a; Wiebe et al., 1972), and infection by either cell form eventually results in phagolysosomes harboring a mixture of cell types (Wiebe et al., 1972). The observation that LCVs are infectious in vitro may have little relevance to natural transmission and infection because the fragile LCVs likely do not persist extracellularly in an infectious form for extended periods.

The environmental conditions that drive *C. burnetii* development are unknown, but two obvious candidates are pH and nutrient availability. Fluctuations in phagolysosomal pH may directly trigger pH-sensitive signal transduction systems in the outer membrane of *C. burnetii*, leading to up- or downregulation of developmental genes. *Coxiella burnetii* encodes at least one adaptive sensory kinase, but the environmental stimuli to which it responds are unknown (Mo and Mallavia, 1994). The metabolic status of the host may also drive development. Although not yet tested, the parasitic burden imposed by bacterial growth late in the infectious cycle probably inflicts nutritional stress on the host. Heavily infected host cells that are degenerating may reduce trafficking of nutrient-laden vesicles to the *C. burnetii*-containing vacuole (Heinzen et al., 1996c). This, in turn, may drive development of *C. burnetii* to a population dominated by SCVs, the cell types most likely to survive extracellularly.

Genetics

The genome size of *C. burnetii* isolates ranges from 1,600 to 2,400 kb (Willems et al., 1996; Willems et al., 1998), with a G+C content of 43 mol% (Tyeryar et al., 1973). Evidence suggests that there is one linear chromosome (Willems et al., 1998). Approximately 2% of the coding capacity of *C. burnetii* is carried on a moderately sized plasmid that is maintained at 1 to 3 copies per cell (Samuel et al., 1983). There are four described plasmid types (QpH1, QpRS, QpDG, and QpDV), ranging from 32.6 to 51 kb, associated with specific genomic groups that share a common 30-kb "core" region along with unique regions (Mallavia, 1991). Some isolates do not harbor an autonomously replicating plasmid, but instead have approximately 18 kb of plasmid-like sequences integrated into the chromosome (Savinelli and Mallavia, 1990; Willems et al., 1997). Interestingly, these isolates have all been obtained from Q fever endocarditis patients. The absolute maintenance of autonomously replicating or chromosomally integrated plasmid sequences in all *C. burnetii* isolates examined to date suggests a critical role for this DNA in some aspect of Coxiella biology and/or virulence. Unfortunately, the complete nucleotide sequence of QpH1 (36 kb) did not provide meaningful insight into the biological role of this molecule (Thiele et al., 1994b).

A repetitive DNA element (IS*1111*) resembling an insertion sequence is present at 19 copies in the *C. burnetii* Nine-Mile strain chromosome and comprises 1–2% of the coding capacity (Hoover et al., 1992). Nucleotide sequencing of three copies of this sequence reveals an approximate size of 1,400 bp with small inverted repeats flanking a single open-reading frame predicted to code for a protein with transposase-like properties (Hoover et al., 1992). If IS*1111* can be demonstrated to function as a mobile genetic element, it is intriguing to speculate that it may have utility as a genetic tool in *C. burnetii*.

Despite numerous technical obstacles, genetic transformation of *C. burnetii* has now been accomplished. This advance was aided by cloning a 5.8-kb *C. burnetii Eco*RI chromosomal fragment that autonomously replicates in *E. coli* (Chen et al., 1990; Suhan et al., 1994). Sequence analysis of this region also predicts that it functions as a *C. burnetii* chromosomal origin of replication (Chen et al., 1990; Suhan et al., 1994). A shuttle vector called "pSKO(+)1000" was constructed containing the *C. burnetii* replicon, a ColE1 replicon, and a *bla* gene coding for β-lactamase (Suhan et al., 1996). Electroporation of *C. burnetii* with pSKO(+)1000 and subsequent selection with ampicillin yielded transformants that carried both integrated and autonomously replicating forms of pSKO(+)1000, although the latter form was found to be unstable (Suhan et al., 1996). Using a similar approach, green fluorescent protein has now been expressed in *C. burnetii* (Lukacova et al., 1999). Employing this technology, gene inactivation via allelic exchange and genetic complementation should soon be possible in *C. burnetii*.

Epidemiology

Q fever is a zoonotic disease, and *C. burnetii* is maintained in extensive reservoirs in mammal, bird and tick species (Babudieri, 1959). The organism has a worldwide distribution excluding Antarctic regions and New Zealand (Babudieri, 1959; Hilbink et al., 1993). Wild rodents appear to be a significant wild reservoir (Webster et al., 1995), but domestic animals are most frequently associated with outbreaks of human disease (Marrie, 1990). Sheep, goats and cattle are often chronically infected with *C. burnetii* with no significant disease except for occasional late-term abortions (Palmer and Key, 1983). Abortion results from massive infection of trophoblast cells of the placenta (Babudieri, 1959). Infection of cattle has also been associated with reproductive problems including infertility and low birth weight (Ho et al., 1995). Dairy cows appear to harbor chronic infection at a higher rate than sheep and may be the most significant source of human infection in some areas (Enright et al., 1957). Household pets, especially cats during parturition, have been directly associated with several urban outbreaks (Marrie et al., 1988). Ticks appear to be the only significant arthropod host of *C. burnetii* and acquire the organism by feeding on a bacteremic animal. However, the role of ticks in the natural cycle of *C. burnetii* remains to be defined (Maurin and Raoult, 1999).

The primary mode of transmission of *C. burnetii* to humans is by direct inhalation of contaminated aerosols originating from infected placenta or amniotic fluid or contaminated wool (Maurin and Raoult, 1999; Tigertt et al., 1961). Heavily infected sheep placental tissue, for example, contains up to 10^9 bacteria per gram, and all infected mammals shed organisms in feces, urine, milk, and birth products (Babudieri, 1959). Adding to the insidious nature of *C. burnetii* is its remarkable resistance to desiccation and ultraviolet radiation, allowing survival in contaminated soils and fomites for extended periods, and an aerosol infectious dose of approximately 10 organisms (Babudieri, 1959; Tigertt et al., 1961; Wedum et al., 1972). Thus, *C. burnetii* represents an occupational hazard for individuals involved in animal husbandry operations such as abattoirs and dairy herd operations, where large numbers of organisms can be shed into the environment during parturition (Derrick, 1937). Although Q fever is endemic within some dairy herds in the United States, ingestion of contaminated milk and milk products is currently not a major source of infection (Enright et al., 1957). This is largely due to the modification of pasteurization protocols in the mid-1950s to ensure thermal inactivation of *C. burnetii* (Baca and Paretsky, 1983; Enright et al., 1957). In situations where pasteurization is not employed, and where goats substitute for cows as a source of dairy products, transmission via ingestion can cause significant disease (Benson et al., 1963; Fishbein and Raoult, 1992).

Rare person-to-person transmission of Q fever has been documented. These cases typically occur in association with medical procedures that expose uninfected individuals to contaminated aerosols originating from infected individuals, such as during an abortion of an infected fetus (Raoult and Stein, 1993) or an autopsy of an infected cadaver (Marmion and Stoker, 1950). Some have proposed that Q fever can be sexually transmitted (Kruszewska et al., 1996; Kruszewska and Tylewska-Wierzbanowska, 1993; Kruszewska and Tylewska-Wierzbanowska, 1997). Experimentally infected male mice have been shown to sexually transmit the disease to female mice (Kruszewska and Tylewska-Wierzbanowska, 1993), and *C. burnetii* has been isolated from bull semen (Kruszewska and Tylewska-Wierzbanowska, 1997). Circumstantial evidence also exists for sexual transmission of Q fever among humans (Kruszewska et al., 1996). Only two suspected instances of natural transmission of Q fever via tick bite have been reported (Beaman and Hung, 1989; Janbon et al., 1989). Thus, unlike other rickettsial diseases, tick transmission of Q fever appears to be of minor significance.

Q fever is a common laboratory acquired infection (Pike, 1979). Infections typically occur in research laboratories where *C. burnetii* is being propagated and purified. Q fever outbreaks have also occurred in facilities that conduct experimental research on sheep, a significant animal reservoir of *C. burnetii* (Hall et al., 1982; Meiklejohn et al., 1981).

Pathogenicity

The disease manifestations of Q fever in humans can be separated into acute and chronic illnesses. Following aerosol ingestion and an incubation period of 1–3 weeks, acute disease commonly presents as flu-like illness with hallmark cyclic fever, preorbital headache and myalgia (Maurin and Raoult, 1999). The initial target is the alveolar macrophage, although the organism can subsequently disseminate to replicate within a variety of tissues. Clinically the illness falls within the group of fever syndromes of unknown origin and is not commonly recognized and diagnosed (Fournier et al., 1998). Lung involvement also can lead to atypical pneumonia, which is normally clinically mild (Maurin and Raoult, 1999). Late in the incubation period, a transient bacteremia occurs in most patients, leading to

hematogenous spread to other organs. The liver is the most common site of disseminated infection, where disease normally manifests as a granulomatous hepatitis (Maurin and Raoult, 1999). In many geographic regions, a high percentage of the population (10–20%) has serological evidence of previous infection (Fournier et al., 1998), indicating that disease manifestations of Q fever are often subclinical or mild (Fournier et al., 1998). Indeed, outbreak surveillance reports indicate that approximately 60% of seroconverting individuals can be asymptomatic (Dupuis et al., 1987). Approximately 2% of patients experiencing acute disease will require hospitalization (Maurin and Raoult, 1999).

Clinical manifestations of Q fever lasting longer than 6 months have been classified as chronic disease (Fournier et al., 1998). It is estimated that chronic disease will develop in about 5% of patients that experience acute disease (Fournier et al., 1998). The heart is the most common site of chronic infection, accounting for 60–70% of chronic infections, and endocarditis is the most common clinical manifestation (Maurin and Raoult, 1999). The liver, arteries, and bones also can be involved (Fournier et al., 1998). Clinically, endocarditis presents as a subacute, blood-culture negative, cardiac insufficiency (Raoult et al., 1990a). Over 90% of patients who develop Q fever endocarditis have evidence of previous cardiac valve defects (Maurin and Raoult, 1999). The other predisposed population is the immunocompromised, which lack a vigorous T cell response (Fournier et al., 1998).

The long-term risk of developing vascular disease after infection with *C. burnetii* recently was described in a report by Lovey and co-workers (Lovey et al., 1999). They evaluated a Swiss cohort of 2,044 people exposed to a large outbreak of Q fever in 1983. They established that the 12-year mortality rate was significantly higher for those that were acutely infected in 1983. Moreover, the 12-year risk of developing arteriovascular disease, such as cerebrovascular accident or cardiac ischemia, was significantly higher among those who had been acutely infected than among those who had not been infected (7% versus 4%). Interestingly, an increased risk of development of endocarditis was not documented. These data suggest that acute infection with *C. burnetii* may lead to long-term vascular inflammation and may point to a latent *C. burnetii* infection comparable to that noted for *Chlamydia pneumonia* and other infectious agents.

Serological procedures are the methods of choice in specific diagnosis of acute and chronic Q fever, although culture and molecular techniques are conducted in specialized settings (Fournier et al., 1998). The early standard serologic assay used in diagnosis was the complement fixation test (Peter et al., 1987). This assay detects both phase I and II antigens (i.e., purified organisms of both phases) and is quite specific but less sensitive than alternative assays. Immunofluorescence (IFA), in a microimmunofluorescence format, is currently the preferred method with high specificity and sensitivity. A four-fold rise in IFA titer between acute and convalescent sera is considered diagnostic of Q fever (Maurin and Raoult, 1999). If a single serum sample is used, then IgG and IgM phase II titers of ≥200 and ≥50, respectively, are considered 100% predictive for acute fever, whereas an IgG phase I titer of ≥1,600 is considered 100% predictive of chronic Q fever (Maurin and Raoult, 1999). Immunofluorescence has also proven valuable in diagnosis of Q fever endocarditis by detecting the organism directly within infected cardiac valves (Baumgartner et al., 1988; Brouqui et al., 1994; Raoult et al., 1994). ELISA, immunoblotting and microagglutination assays also have been used for serosurvey and diagnostic evaluation of infection (Maurin and Raoult, 1999). Molecular methods employing PCR amplification of *C. burnetii*-specific DNA sequences from clinical samples and infected cell culture have shown utility in clinical diagnosis. An especially specific and sensitive method of detection uses a chromosomal repetitive transposon-like sequence as a PCR target. This procedure has been used in the diagnosis of Q fever in humans (Maurin and Raoult, 1999; Willems et al., 1994).

Acute Q fever is normally a self-limiting infection that usually spontaneously resolves within two weeks. Intervention with antimicrobial agents effectively minimizes clinical symptoms (Maurin and Raoult, 1999). Several tetracyclines (especially doxycycline) are the preferred antibiotic therapy for acute Q fever (Maurin and Raoult, 1999). Lincomycin, erythromycin, co-trimoxazole, and especially fluoroquinolones also are efficacious (Maurin and Raoult, 1999). Chronic infections are more refractory to antibiotic therapy. Q fever endocarditis, the most common chronic manifestation of *Coxiella* infection, has a mortality rate exceeding 50% (Maurin and Raoult, 1999). Although a good initial clinical response can result from prolonged therapy with tetracyclines, cessation of therapy frequently results in disease relapse and death (Maurin and Raoult, 1999). Combination therapy with doxycycline and a fluoroquinolone (perfloxacin or ofloxacin) shows promise in reducing mortality, but relapse rates remain high (Levy et al., 1991). Treatment with a combination of doxycycline and chloroquine, a drug that raises the pH of the phagolysosome (the intracellular niche of *C. burnetii*),

significantly reduces the relapse rate when compared to a combined doxycycline-ofloxacine regime (Maurin et al., 1992a; Raoult et al., 1999). Alkalinization of the phagolysosome is thought to increase the bactericidal activity of doxycycline (Maurin et al., 1992a). Although an efficacious formalin-inactivated whole cell vaccine against Q fever exists (Q-Vax), it is recommended only for individuals where Q fever is considered an occupational hazard. It is not licensed for use in the United States. In Australia, where Q fever is a particular problem, Q-Vax is used but requires skin testing for prior exposure before vaccination (Marmion et al., 1984; Marmion et al., 1990). Vaccinees that have had previous exposure to *C. burnetii* can develop a serious immune reaction at the site of inoculation.

Immunity

Infection of humans with *C. burnetii* induces robust humoral and cell-mediated immune responses (Maurin and Raoult, 1999). The combined response in acute disease patients usually abrogates clinical symptoms, resulting in a self-limiting illness, and typically confers long-lived protection against repeated infection. Occasionally, infection remains subclinical and persistent, and patients develop chronic disease. Chronic infection in the form of endocarditis usually involves suppression of components of an effective cell-mediated immune response and/or previous valvular damage (Maurin and Raoult, 1999).

Antibody develops against *C. burnetii* antigens during the second week of infection and proceeds with typical immunoglobulin class switching from IgM to IgG, developing increasing avidity for antigen (Guigno et al., 1992). Antibody that reacts with antigens isolated from phase II organisms develops first, followed several weeks later with antibody reacting with antigens from phase I organisms. The role of antibody in control of *C. burnetii* replication is not fully understood, but at least two functions have been reported. Specific antibody facilitates opsonized uptake by monocytes/macrophages. Immune-specific serum also lyses infected macrophages by an antibody-dependent cell-cytotoxicity mechanism (Koster et al., 1984). In addition, normal serum kills phase II bacteria but not phase I bacteria by the alternative complement pathway (Vishwanath and Hackstadt, 1988).

A vigorous cell-mediated immune response is necessary for effective clearance of *C. burnetii* infection. Athymic mice are attenuated in the ability to clear *C. burnetii* from spleen and blood when compared with euthymic litter mates (Kishimoto et al., 1978). *Coxiella burnetii* infection and antigens are potent stimulators of a delayed-type hypersensitivity response and macrophage activation (Waag, 1990). Chronic disease patients have a marked suppression of T cell proliferation in response to antigen-specific stimulation (Koster et al., 1985a; Koster et al., 1985b). A variety of immunosuppressive disease states, including HIV infection, cancer, lymphoma, chronic renal failure and pregnancy, have been associated with a predisposition for the development of chronic Q fever (Raoult et al., 1992; Raoult and Stein, 1993).

Although cytokines contribute significantly to the control of *C. burnetii* replication, they can also exacerbate chronic disease manifestations. Activated macrophages and monocytes effectively kill intracellular *C. burnetii* (Kishimoto et al., 1977). A key role for interferon-γ (IFN-γ) in stimulating host cells to inhibit *C. burnetii* replication was first demonstrated in infected mouse L929 cells and subsequently confirmed in various phagocytic cells (Turco et al., 1984). Recent studies have focused upon the interplay between several cytokine signals and the ability to effectively control *C. burnetii* replication. One model postulates that IFN-γ induces TNF-α expression by infected peripheral blood mononuclear cells (PBMC) causing the infected cells to die via an apoptotic pathway (Dellacasagrande et al., 1999). This would be a novel pathway for control of intracellular replication inasmuch as monocytes are normally resistant to induction of apoptotic death by TNF-α (Wallach et al., 1999). Infection of a mouse macrophage-like cell line (P388D1) with phase I *C. burnetii* induces TNF-α and IL-1 expression whereas infection with phase II *C. burnetii* only induces TNF-α (Tujulin et al., 1999). Chronic disease patients express elevated levels of TNF-α and IL-1β compared to uninfected control individuals. The PBMC from chronic patients produce dramatically elevated levels of IL-10 and TGF-β, which probably contributes to the previously reported immune suppression observed in chronic disease (Capo et al., 1996). Monocytes from these patients are unable to control *C. burnetii* replication compared with those obtained from healthy individuals (Dellacasagrande et al., 2000).

The relative importance of reactive nitrogen and oxygen intermediates (RNI and ROI) in the killing of *C. burnetii* is in question. Monocytes taken from chronic granulomatous disease patients, who are deficient in ROI-mediated killing, effectively clear *C. burnetii* if stimulated with IFN-γ (Dellacasagrande et al., 1999). Moreover, various in vitro studies have demonstrated the lack of measurable superoxide anion (O_2^-) production by human neutrophils following phago-

cytosis of Coxiella (Akporiaye et al., 1990; Baca et al., 1993a; Ferencik et al., 1984; Li et al., 1996). It appears that ingestion of *C. burnetii* fails to stimulate O_2^- release as opposed to parasite directed detoxification of the molecule (Baca et al., 1993a; Baca et al., 1993b; Li et al., 1996). Evidence suggests that *C. burnetii* synthesizes a tyrosine phosphatase that inhibits the oxidative burst and accompanying O_2^- release into the lumen of the phagolysosomal vacuole (Baca et al., 1993b; Li et al., 1996). The enzyme is thought to dephosphorylate and inactivate a critical signal transduction protein necessary for O_2^- production (Li et al., 1996). The role of RNI in control of *C. burnetii* growth is similarly unresolved. A study indicated that IFN-γ treated THP-1 human monocyte-like cells infected with *C. burnetii* do not produce nitric oxide (Dellacasagrande et al., 1999). A caveat of this study is the known difficulty in activating human monocytes in culture (Nathan, 1997). Conversely, IFN-γ inhibits growth of *C. burnetii* in mouse fibroblast L-929 cells. IFN-γ is a known inducer of inducible nitric oxide synthase in this cell line (Turco et al., 1984). It is reasonable to suspect that the reported growth inhibition may be due to subsequent RNI production.

Virulence Determinants and Host-Parasite Interactions

The identification of *C. burnetii* virulence determinants has been hampered by an inability to generate and test defined mutants. However, suitable animal models of acute human Q fever are available, with the guinea pig being the most relevant (Heggers et al., 1975). Guinea pigs challenged intraperitoneally with as few as 10 virulent organisms develop fever within 5 days. Bacteria can be isolated from a variety of tissues, including the spleen, for several months post-infection. Interestingly, a comparison of the relative virulence of isolates from acute and chronic infections found that as few as 10 IFU (inclusion forming units) of acute isolate induce a strong fever response, whereas 10^6 IFU of chronic isolate did not cause detectable fever in guinea pigs, although infection could be confirmed by isolation of these organisms from spleens (Moos and Hackstadt, 1987). In general, most mouse strains remain asymptomatic following infection, although *C. burnetii* can be found in granulomatous lesions in various organs (Scott et al., 1987). A mouse lethality model of human Q fever has been developed in A/J mice. These mice are particularly sensitive to infection by *C. burnetii* because of deficient INF-γ priming of a protective T cell response (Scott et al., 1987). Attempts to model human Q fever endocarditis have employed infection of both rabbits and mice (Atzpodien et al., 1994; Hackstadt, 1990; La Scola et al., 1998). Chronic endocarditis does not spontaneously develop in either experimental animal but requires the artificial induction of lesions on heart valves (rabbits; Hackstadt, 1990; La Scola et al., 1998) or immunosuppression (mice; Atzpodien et al., 1994). Interestingly, in both experimental animals vegetative cardiac lesions containing *C. burnetii* developed after infection with prototypic human acute disease isolates, indicating, at least in these animal models, that acute disease isolates may be capable of causing chronic infection (Hackstadt, 1990; La Scola et al., 1998).

Previous studies showing that acute and chronic disease isolates differed both genetically and biochemically led to the hypothesis that acute isolates were distinct in virulence potential from chronic isolates (Hackstadt, 1986; Hendrix et al., 1991; Mallavia, 1991; Samuel et al., 1985). This hypothesis lost epidemiologic support when isolates from chronic disease patients were identified with genetic markers of acute isolates (Thiele and Willems, 1994a). Nonetheless, the potential to cause acute or chronic disease may be determined by specific factors encoded by different isolates in combination with host factors such as immune status. The potential for dramatic phenotypic differences among these isolates was underscored by a report that the genome size between different isolates may vary by nearly one megabase (Willems et al., 1996).

To date, the only defined virulence determinant of *C. burnetii* is LPS. The organism undergoes a phase (LPS) variation that is similar to that observed in the Enterobacteriaciae. Transition from smooth (full-length O-side chains) phase I to rough (truncated O-side chains) phase II LPS occurs upon repeated passage of the organism in an immunoincompetent host such as embryonated eggs or tissue culture. Phase II organisms are avirulent for guinea pigs, whereas phase I *C. burnetii* causes disease and is always associated with naturally infected mammals and ticks (Hackstadt, 1990). A strain displaying a semi-rough-type LPS has intermediate virulence for guinea pigs (Moos and Hackstadt, 1987). Phase I to phase II transition is likely due to accumulation of point mutations in LPS biosynthesis genes or the loss of chromosomal regions that carry these genes (O'Rourke et al., 1985; Vodkin and Williams, 1986a). *Coxiella burnetii* LPS is weakly pyrogenic when compared to other Gram-negative LPSs (Hackstadt, 1990). Full-length LPS contributes to the virulence of phase I by inhibiting deposition of C3b complement component and subsequent complement-mediated killing by the alternative pathway (Vishwanath and Hackstadt, 1988).

Internalization into host cells occurs by microfilament-dependent, parasite-directed endocytosis (Baca et al., 1993a; Meconi et al., 1998). *Coxiella burnetii* plays a passive role in internalization, as inactivated organisms are internalized at a rate equal to that observed for viable bacteria (Baca et al., 1993a). Upon adherence of virulent phase I *C. burnetii* to THP-1 human monocytic cells, a dramatic reorganization of the actin cytoskeleton occurs, resulting in production of pronounced membrane protrusions (Meconi et al., 1998). Similar cellular effects are not observed upon adherence of avirulent phase II organisms (Meconi et al., 1998). Interestingly, this dramatic cytoskeletal response does not correlate with an increased rate of internalization of phase I over phase II organisms, as the latter are more efficiently internalized (Capo et al., 1999). This may reflect differential engagement of host cell receptors by phase variants. The THP-1 receptor for virulent phase I organisms consists of a complex of leukocyte response integrin $\alpha_v\beta_3$ and integrin-associated protein whereas avirulent phase II organisms additionally engage the CR3 receptor (Capo et al., 1999). The cytoskeletal rearrangements and resultant membrane projections associated with phase I adherence may restrict engagement of the CR3 coreceptor and lower the efficiency of internalization (Meconi et al., 1998). Full-length LPS of phase I also may sterically mask the critical *C. burnetii* CR3 ligand (Hackstadt, 1988). The *C. burnetii* ligand(s) mediating internalization are still unknown.

Studies strongly support the idea that once internalized, *C. burnetii* resides in a vacuole with characteristics of a secondary lysosome. The early parasite-containing phagosome proceeds through the endocytic pathway eventually acidifying to a pH of approximately 4.8 (Akporiaye et al., 1983b; Maurin et al., 1992b). *Coxiella burnetii* requires a moderately acidic pH (~5) to activate its metabolism and thus has evolved to exploit the only intracellular niche that provides this acidic environment (Hackstadt and Williams, 1981a). The *C. burnetii*-containing vacuole fuses with lysosomes as demonstrated by the colocalization of the lysosomal enzymes 5′-nucleotidase (Burton et al., 1971), acid phosphatase (Akporiaye et al., 1983b; Burton et al., 1978; Heinzen et al., 1996c), cathepsin D (Heinzen et al., 1996c), vacuolar type H+ ATPase (Heinzen et al., 1996c), and two predominant lysosomal glycoproteins (LAMP-1 and LAMP-2; Heinzen et al., 1996c). The organism undergoes luxurious growth within this vacuole despite the presence of factors normally considered bacteriocidal (Reiner, 1994). Maturation of the nascent *C. burnetii*-containing phagosome to a mature phagolysosome appears to be delayed (Howe and Mallavia, 2000). At one hour postinfection, the percentage of vacuoles containing viable *C. burnetii* that colocalize with lysosomal markers is roughly one-half that observed for vacuoles harboring inactivated *C. burnetii* (Howe and Mallavia, 2000). It is hypothesized that delayed maturation allows morphological differentiation of the environmentally stable SCV developmental form to the more metabolically active LCV developmental form (Howe and Mallavia, 2000). Supporting this idea is the observation that the SCV-specific DNA-binding protein ScvA is more efficiently degraded when purified *C. burnetii* organisms are metabolically activated at pH 5.5 than at pH 4.5 (the approximate pH of a mature phagolysosome; Howe and Mallavia, 2000). Degradation of ScvA is thought to be a prerequisite for SCV-to-LCV transition (Heinzen et al., 1996b); thus, delayed phagolysosomal maturation would provide optimal pH conditions for ScvA degradation and subsequent SCV-to-LCV morphological differentiation. These data also imply that a product of *C. burnetii* metabolism is responsible for delayed lysosomal fusion.

Strategies for combating the toxic constituents of the phagolysosome are likely important virulence determinants of *C. burnetii*. The biochemically unusual cell envelope of the organism may confer intrinsic resistance to the lysosomal environment (Amano and Williams, 1984a; Amano et al., 1984c; Schramek et al., 1985; Wollenweber et al., 1985). The SCV developmental form is particularly resistant to chemical and physical disruption and consequently may be able to survive the degradative conditions of the phagolysosome for extended periods (Heinzen, 1997a). The SCV also undergoes binary fission and can differentiate into the more metabolically and perhaps divisionally active LCV. This process may occur at a significant rate only when phagolysosomal conditions are more amenable for growth of the LCV (Heinzen, 1997a).

An innate host defense mechanism important in controlling replication of intracellular bacterial pathogens is the sequestration of iron (Britigan et al., 2000). *Coxiella burnetii* appears to resist this host defense by stimulating an increase in transferrin receptor biosynthesis. Inoculation of a J774.1 murine macrophage-like cell line with viable *C. burnetii* corresponds to an upregulation of transferrin receptor synthesis and a concomitant increase in total cellular iron (Howe and Mallavia, 1999). In vivo, *C. burnetti*-induced upregulation may compensate for IFN-γ-induced downregulation of transferrin receptor synthesis (Taetle and Honeysett, 1988). The mechanisms by which *C. burnetii* acquires iron from the host are undefined, but a ferric uptake regulatory protein (Fur) and a periplasmically localized protein

that is transcriptionally regulated by Fur have been identified (J. E. Samuel, unpublished).

Coxiella burnetii likely secretes molecules into the phagolysosomal vacuole that modify the environment. This behavior has been observed for a number of intracellular parasites that reside in membrane-bound vacuoles (Hackstadt, 1998; Small et al., 1994). In vitro studies have demonstrated that *C. burnetii* translocates a variety of proteins during metabolic acid activation in defined medium (Redd and Thompson, 1995). A few *Coxiella* enzymes that are localized to the outer membrane and/or secreted have been hypothesized to play roles in intracellular survival based upon their recognized activities in facultative intracellular bacteria. Macrophage infectivity potentiator (Mip) is a peptidylprolyl isomerase (PPIase) localized to the cytoplasm, periplasmic space, and outer surface of *C. burnetii* and *L. pneumophila* (Cianciotto et al., 1995; Mo et al., 1995; Seshu et al., 1997). Mutants of *L. pneumophila* that do not express Mip are attenuated in their ability to infect and survive in macrophages. A second periplasmic and outer surface-localized enzyme, Com-1, is a homologue of the disulfide bond forming enzymes DsbA and DsbC (Hendrix et al., 1993). These enzymes are required for folding of virulence determinants in *S. flexneri* and *E. coli* (Watarai et al., 1995). The *C. burnetii* com-1 gene complements an *E. coli dsbA* deletion mutant and purified recombinant Com-1 is enzymatically active (Hendrix et al., submitted). *Coxiella burnetii* synthesizes cytoplasmically localized catalase and iron-containing superoxide dismutase (Akporiaye and Baca, 1983a; Heinzen et al., 1992). These proteins presumably play important roles in detoxifying superoxide anion and hydrogen peroxide generated by *C. burnetii* oxidative respiration (Baca et al., 1994). Secreted forms of these enzymes, known virulence factors of other intracellular parasites (Amemura-Maekawa et al., 1999; Bandyopadhyay and Steinman, 1998; St. John and Steinman, 1996), have not been described for *C. burnetii*. However, a protein tyrosine acid phosphatase that blocks superoxide anion production by fMetLeuPhe-stimulated human neutrophils has been partially purified from *C. burnetii* (Baca et al., 1993b; Li et al., 1996). A specific inhibitor of the acid phosphatase dramatically reduces the percentage of L929 cells persistently infected with *C. burnetii* (Baca et al., 1993b). Neutrophils treated with *C. burnetii* acid phosphatase increase tyrosine phosphorylation of a ~44-kDa host protein (Li et al., 1996). Although the identity of this host protein is unknown, it may be involved in regulating the oxidative burst of neutrophils. Indeed, ingestion of *C. burnetii* by macrophage-like cell lines results in a greatly diminished respiratory burst with little production of superoxide anion (Akporiaye et al., 1990; Baca et al., 1993a; Ferencik et al., 1984; Li et al., 1996). Several facultative intracellular bacteria have been shown to block the oxidative burst by expressing an acid phosphatase enzyme, including *Legionella micdadei* (Saha et al., 1985) and *Yersinia pseudotuberculosis* (Bliska and Black, 1995).

Applications

A low infectious dose, extracellular stability, and an aerosol route of infection make *C. burnetii* a potential bioterrorism agent (Mobley, 1995). In response to a growing concern of such an insidious use, the Centers for Disease Control has recently designated *C. burnetii* as a "select agent," placing restrictions on transport.

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Prokaryotes (2006) 5:547–561
DOI: 10.1007/0-387-30745-1_22

CHAPTER 3.1.21

The Genus *Wolbachia*

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Introduction

Numerous invertebrate species form long lasting symbioses with bacteria (Buchner, 1949; Buchner, 1965). One of the most common of these bacterial symbionts is *Wolbachia pipientis*, which has been estimated to infect anywhere from 15–75% of all insect species (Werren et al., 1995a; West et al., 1998; Jeyaprakash and Hoy, 2000; Werren and Windsor, 2000) as well as many species of arachnids, terrestrial crustaceans and filarial nematodes (O'Neill et al., 1997a; Bandi et al., 1998). In most arthropod associations, *Wolbachia* act as reproductive parasites manipulating the reproduction of their hosts to enhance their own vertical transmission. There appears to be little direct fitness cost to the infected host besides the costs arising from the reproductive manipulations. However instances have been reported where *Wolbachia* can be either deleterious (Min and Benzer, 1997; Bouchon et al., 1998) or beneficial (Girin and Boultreau, 1995; Stolk and Stouthamer, 1995; Wade and Chang, 1995; Vavre et al., 1999b; Dedeine et al., 2001) to their hosts.

Wolbachia were first described as intracellular *Rickettsia*-like organisms (RLOs), infecting the gonad cells of the mosquito, *Culex pipiens* (Hertig and Wolbach, 1924), and were later named "*Wolbachia pipientis*" (Hertig, 1936). It was not until the work of Yen and Barr (Yen and Barr, 1971; Yen and Barr, 1973) that *Wolbachia* were implicated in causing crossing incompatibilities between different mosquito populations (Laven, 1951; Ghelelovitch, 1952). When polymerase chain reaction (PCR) diagnostics for *Wolbachia* became available, it became clear that this agent was both extremely widespread and also responsible for a range of different reproductive phenotypes in the different hosts it infected (O'Neill et al., 1992; Rousset et al., 1992; Stouthamer et al., 1993). The most common of these are cytoplasmic incompatibility, inducing parthenogenesis, overriding host sex-determination, and male-killing (O'Neill et al., 1997a). As of the time of this writing, more than 450 different *Wolbachia* strains with unique gene sequences, different phenotypes, and infecting different hosts have been deposited in GenBank and the *Wolbachia* host database (http://www.wolbachia.sols. uq.edu.au).

Phylogeny

Wolbachia pipientis (Hertig, 1936) is the only species of the genus *Wolbachia*, family Anaplasmataceae, order Rickettsiales, class α-proteobacteria (Dumler et al., 2001). Two other species, *Wolbachia persica* (Suitor and Weiss, 1961) and *Wolbachia melophagi* (Nöller, 1917; Philip, 1956), originally in the same genus have since been removed (Dumler et al., 2001). *Wolbachia*'s closest relatives in the Anaplasmataceae are the genera *Anaplasma*, *Ehrlichia* and *Neorickettsia* (Fig. 1). They are all obligate intracellular bacteria that reside in vacuoles of eukaryotic cells (Dumler et al., 2001).

On the basis of the phylogeny of the 16S *rRNA* gene, the genus *Wolbachia* as currently defined is monophyletic (O'Neill et al., 1992; Bandi et al., 1998; Lo et al., 2002). *Wolbachia* have been divided into six supergroups A–F on the basis of 16S *rRNA* and *ftsZ* gene sequences; A, B, E and F are associated with arthropods (Vandekerckhove et al., 1999; Lo et al., 2002), and C and D are associated with filarial nematodes (Bandi et al., 1998; Lo et al., 2002). The phylogenetic relationship between these supergroups is currently not well resolved (Lo et al., 2002; Fig. 2).

The validity of the A and B supergroups that infect insects has been confirmed by phylogenetic analysis of the heat shock operon *groE* (Masui et al., 1997), of the spacer 2 region between the 23S and the 5S *rRNA* coding genes (Van Meer et al., 1999) and of the surface protein gene *wsp* (Zhou et al., 1998; Fig. 3). There is evidence that homologous recombination between different *Wolbachia* strains occurs (Jiggins et al., 2001; Werren and Bartos, 2001) and thereby has the potential to confound the interpretation of phylogenetic data based on the sequences of single genes. The lack of congruency between phylogenetic trees of *Wolbachia*

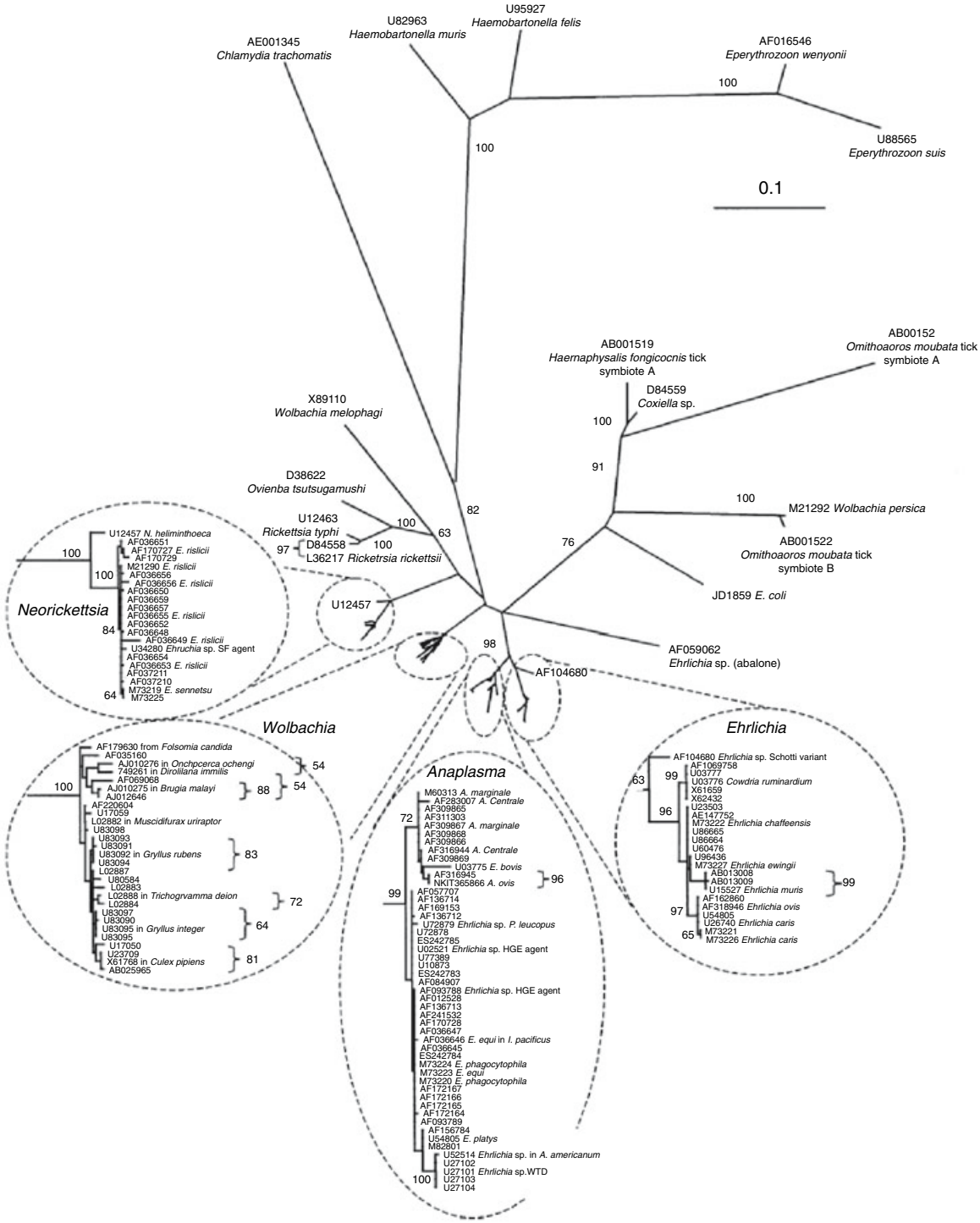

Fig. 1. Phylogenetic tree inferred from the small subunit (16S) rRNA gene sequences of *Ehrlichia*, *Anaplasma*, *Neorickettsia* and *Wolbachia* species, including 455 sites after removal of sites containing a gap in any sequence. The sequence from *Chlamydia trachomatis* (accession no AE001345 [www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=AE001345]) was used as an outgroup. Numbers above internal nodes indicate the percentage of 1000 bootstrap replicates that supported the branch. All bootstrap values are included for clades that were consistently observed using the phylogenetic methods applied (maximum parsimony, minimum evolution, maximum likelihood and majority-rule bootstrap analysis of neighbor-joining trees). The maximum-likelihood tree is shown. Bars, estimated number of substitutions per site; the scales for the figure and insets are the same. From Dumler et al. (2001).

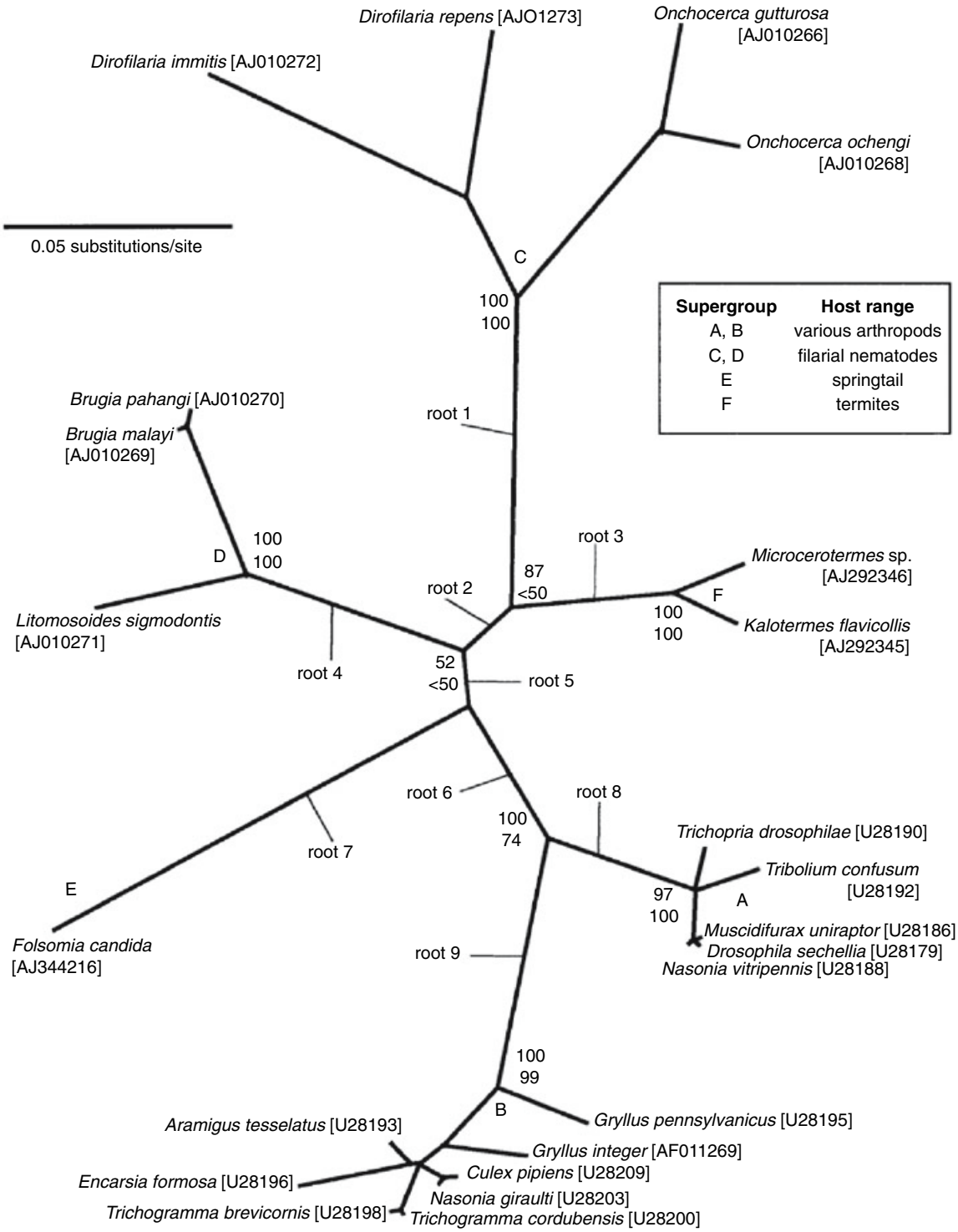

Fig. 2. Unrooted phylogenetic tree of *Wolbachia* endosymbionts of arthropods and filarial nematodes based on *ftsZ*, estimated using Bayesian inference of phylogeny. Posterior probabilities supporting nodes of interest are shown above bootstrap values from a maximum parsimony analysis. Names represent host species. Roots 1-9 indicate positions where the *ftsZ* gene of the outgroup *Anaplasma marginale* was constrained during likelihood estimations to examine the most appropriate root placement. Accession numbers are shown adjacent to each taxon. Each supergroup is labelled with one of the letters A-F. From Lo et al. (2002).

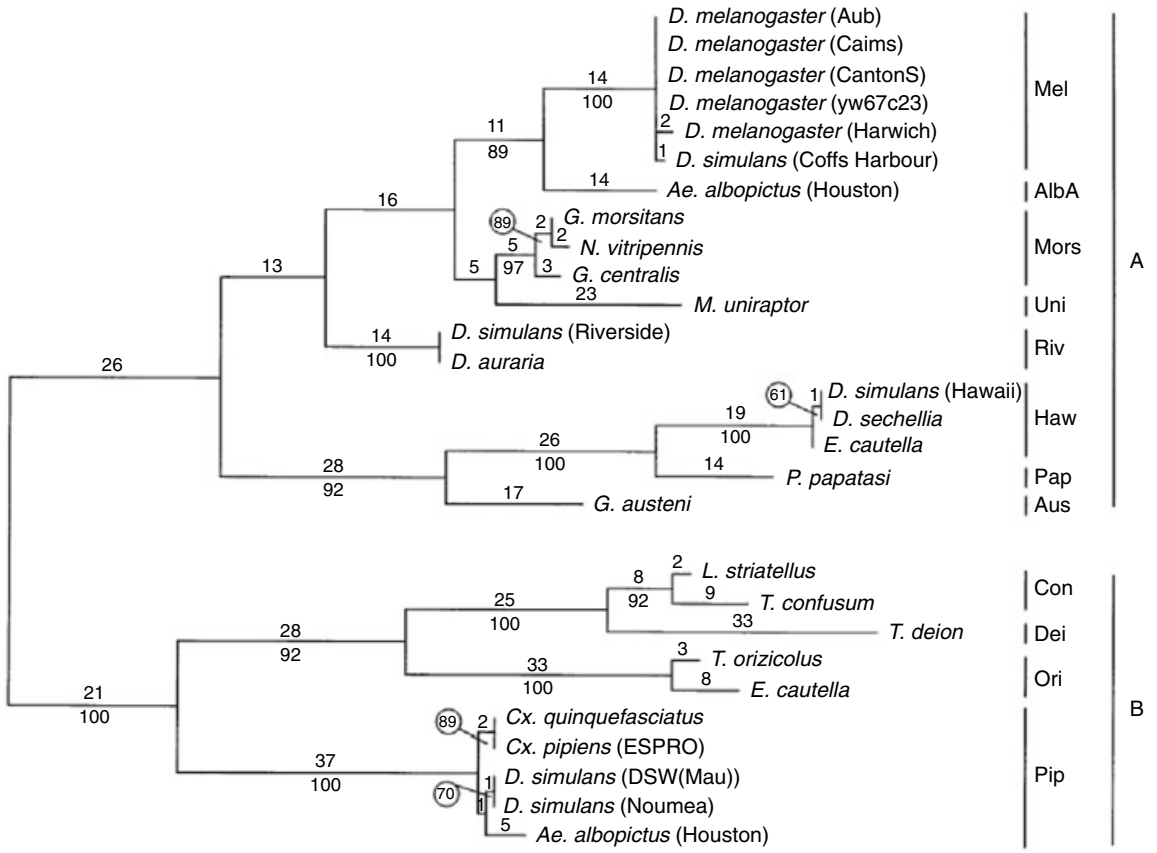

Fig. 3. One of four most parsimonious trees generated from a branch-and-bound search of aligned *wsp* sequences (tree length = 472; CI = 0.64). Tree shown is midpoint rooted. Branch lengths, as determined from the Phylogenetic Analysis using Parsimony (PAUP) table of linkages, are labelled above branches and bootstrap values (500 replicates) are labelled below branches. Bootstrap values less than 50 are not shown. Taxa are labelled as the host from which the *Wolbachia* strain was isolated. From Zhou et al. (1998).

strains and their hosts indicates that horizontal transfer of *Wolbachia* to new host species has occurred on multiple occasions during evolutionary history (O'Neill et al., 1992; Werren et al., 1995b; Vavre et al., 1999a).

Taxonomy

The species name *Wolbachia pipientis* (Hertig, 1936) was originally assigned to the infection in a particular host, the common house mosquito *Culex pipiens*. The name has since been extended to highly similar bacteria in arthropods and filarial nematodes. In most associations, the literature only refers to the genus name "*Wolbachia*." With the rapid discovery of numerous *Wolbachia* strains, a uniform nomenclature system became necessary. The current system was based on an abbreviation style *w*Host (Rousset and de Stordeur, 1994), as proposed in Zhou et al. (1998). This system is now widely accepted and has been further specified by Charlat et al. (2002a). Separate names should be assigned to strains differing in any of the following three traits: *Wolbachia* gene sequences, phenotypic effects on hosts, or the host species infected. Strain names should then consist of a *w* followed by two or three letters to refer to the *Wolbachia* strain and a subscript referring to the host species (e.g., $w\mathrm{No}_{D.sim}$ for the *Wolbachia* infection of *Drosophila simulans* originally identified from Noumea or $w\mathrm{Cer1}_{R.cer}$ for one of the *Wolbachia* infections of *R. cerasi*). Most *Wolbachia* strains of the A and B supergroup have now been assigned strain names on the basis of the sequence of the surface protein gene *wsp* (Zhou et al., 1998). It should be noted that while strain designation in itself makes no assumptions about relatedness and as such can be based upon any consistent genetic or ecological feature, rigorous phylogenetic analysis might require the use of multiple gene sequences to account for potential recombination between strains. The discovery in recent years of a large number of diverse *Wolbachia* strains suggests that the taxonomy of the

Wolbachia pipientis group as a whole will soon need to be re-examined with the possibility that the current species might end up being split into a number of new species, potentially based on supergroup assignments.

Habitat

Wolbachia are obligate intracellular bacteria of invertebrates, with the majority of the currently described hosts living in terrestrial habitats. *Wolbachia* have an impressive host range. Infections have been detected in all major orders of insects, arachnids (such as spiders and mites), terrestrial crustacean species, and filarial nematodes. This extreme diversity of hosts makes *Wolbachia* one of the most ubiquitous intracellular symbionts yet described. Within the host cell, *Wolbachia* is always seen within a vacuole, presumably of host origin (Fig. 4). The nature of this compartment and the extent it is modified by *Wolbachia* have yet to be determined. *Wolbachia* are inherited vertically by transovarial transmission through the cytoplasm of host eggs. As a result *Wolbachia* always infects the female germline of its host. *Wolbachia* is usually lost from the cytoplasm of sperm cells during spermatogenesis (Clark et al., 2002; Veneti et al., 2003) and as such is only maternally inherited. In addition to the germline, a range of other somatic tissues is known to be infected. The extent and diversity of somatic tissues infected vary with host and *Wolbachia* strain (Dobson et al., 1999).

Isolation

Wolbachia's entire life cycle is dependent on the cytoplasmic environment of the host. The bacteria cannot yet be cultivated on cell-free media and can only be maintained in individual hosts or cell lines (O'Neill et al., 1997b; Dobson et al., 2002; Noda et al., 2002). *Wolbachia* infections have been established in a variety of insect cell lines including those originating from *Aedes albopictus* (O'Neill et al., 1997b; Dobson et al., 2002; Noda et al., 2002), *Drosophila melanogaster*, *Spodoptera frugiperda* (Dobson et al., 2002), and *Heliothis zea* (Noda et al., 2002). *Wolbachia* can also be maintained in a mammalian cell line originating from mouse connective tissue (Noda et al., 2002). *Wolbachia* strains can be artificially transferred between host species by embryonic microinjections (Nigro, 1991; Boyle et al., 1993; Braig et al., 1994) or inoculation with crushed tissues from pupae (Williams et al., 1993), ovaries, and fat- and nervous tissues (Bouchon et al., 1998). Transinfection experiments have enabled the comparison of different *Wolbachia*-host interactions and provided a means to determine what aspects of the association are regulated by either host or symbiont (Clancy and Hoffmann, 1997; Poinsot et al., 1998; McGraw et al., 2001; Riegler et al., 2004). Transinfection experiments have also been used to segregate multiple infections from a single host (Charlat et al., 2002b; Riegler et al., 2004) or establish multiple infections within a single host (Rousset et al., 1999).

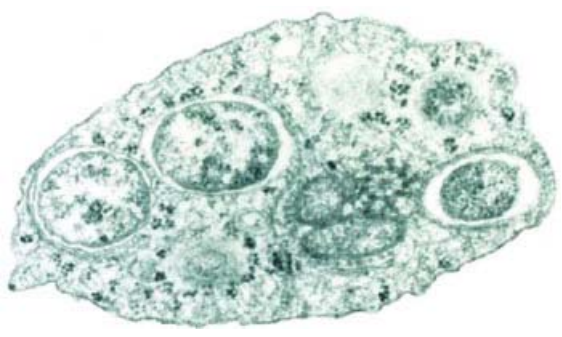

Fig. 4. Transmission electron micrograph of *Wolbachia* (arrow) within a developing spermatid of the moth *Ephestia cautella*. Courtesy of Scott O'Neill.

Identification

Wolbachia are coccoid or bacilliform in morphology, 0.8–1.5 μm long (Hertig, 1936). These Gram-negative bacteria have two cell membranes and are enclosed within a vacuole (Fig. 4). Several techniques have been utilized to visualize *Wolbachia* within host tissue. *Wolbachia* can be readily stained by Giemsa (Hertig, 1936) or general DNA-binding fluorochromes such as DAPI (4′,6-diamino-2-phenylindole dihydrochloride; O'Neill and Karr, 1990; Bressac and Rousset, 1993). Monoclonal and polyclonal antibodies have been developed for the outer membrane proteins of several species of the order Rickettsiales (Ohashi et al., 1998) and specifically also for *Wolbachia* (Kose and Karr, 1995; Dobson et al., 1999; Masui et al., 2001). *Wolbachia* have also been successfully detected using in situ hybridization techniques with *Wolbachia* specific DNA probes (Heddi et al., 1999). Several polymorphic genes of *Wolbachia* have been isolated and characterized for a wide range of strains. *Wolbachia* specific PCR primers have been designed and are commonly used to identify *Wolbachia* infections in total host genomic DNA extracts (Table 1). The best primers to detect a wide range of different *Wolbachia* strains are those in the 16S primer-set, which can be used for strains from all supergroups described so far (O'Neill et al., 1992; Bandi et al., 1998; Vandekerckhove et al.,

Table 1. List of diagnostic *Wolbachia* primers.[a]

Gene		Primer 5′-3′	Super-group	References
16S	16S*f*	TTGTAGCCTGCTATGGTATAACT	A, B	O'Neill et al., 1992
	16S*r*	GAATAGGTATGATTTTCATGT	A, B	
	FILf	TATATAGCTTGCTATAGTGTA	C	Sironi et al., 1995
	FILr	TCGAACAGGCATAATTTCCA	C	
	Bsymbf	ACGAGTTATAGTATAACT	D	Taylor et al., 1999b
	Bsymbr	CCTTCGAATAGGAATAAT	D	
ftsZ	*ftsZunif*	GGYAARGGTGCRGCAGAAGA	A–F	Lo et al., 2002
	ftsZunir	ATCRATRCCAGTTGCAAG	A–F	
	ftsZf1	GTTGTCGCAAATACCGATGC	A, B	Werren et al., 1995
	ftsZr1	CTTAAGTAAGCTGGTATATC	A, B	
wsp	*81F*	TGGTCCAATAAGTGATGAAGAAAC	A, B	Zhou et al., 1998
	691R	AAAAATTAAACGCTACTCCA	A, B	
	WSPintF	TAGYTACTACATTCGCTTGCA	C, D	Bazzocchi et al., 2000
	WSPintR	CCAAYAGTGCYATAAAGAAC	C, D	

[a]Primers are for the 16S *rRNA* gene, the cell cycle gene *ftsZ* and the outer surface protein gene *wsp*, used for the detection of the six different *Wolbachia* supergroups A–F.

1999). However the polymorphism in the 16S gene is low, which makes the detection of multiple infections in individuals and the characterization of single strains difficult. For the latter, primers designed to amplify the cell cycle gene *ftsZ* (Holden et al., 1993; Werren et al., 1995b; Bandi et al., 1998; Lo et al., 2002) and the outer surface protein gene *wsp* (Braig et al., 1998; Zhou et al., 1998) are more suitable.

Preservation

Because of their intracellular biology, *Wolbachia* cannot easily be preserved and are best maintained in cultures of their hosts. Some of *Wolbachia*'s hosts undergo diapause phases to survive extreme conditions. Under such circumstances *Wolbachia* are usually retained in most associations. However loss of infections during diapause has been observed in a few cases (Perrot-Minnot et al., 1996). *Wolbachia* can also be kept in cell lines (O'Neill et al., 1997b; Dobson et al., 1999; Noda et al., 2002), where they can be stored at –80°C (O'Neill et al., 1997b). Treatment with antibiotics, particularly with tetracycline or rifampicin, in combination with high temperature is commonly used for removing *Wolbachia* infections (Dobson and Rattanadechakul, 2001a; Fenollar et al., 2003b; Volkmann et al., 2003). Rearing insects under nutritional stress has been reported to reduce the efficiency of maternal transmission (Sinkins et al., 1995; Clancy and Hoffmann, 1998).

Genomics

Wolbachia strains have small streamlined genomes comprised of a single circular chromosome. No plasmids are known to occur in *Wolbachia*. The genome size among the members of different *Wolbachia* supergroups varies considerably. Strains of the nematode-associated C and D supergroup have a chromosome size of 0.9–1.1 Mb, whereas members of the insect-associated A supergroup have chromosome sizes of 1.3–1.6 Mb (Sun et al., 2001) and 1.8 for B supergroup (Fenollar et al., 2003a). The reduced size in nematode *Wolbachia* correlates with their observed obligate mutualism and concordant evolution with their hosts (Bandi et al., 1998; Casiraghi et al., 2001).

The genome of the *Wolbachia* strain *w*Mel$_{D.\,mel}$ that naturally infects *Drosophila melanogaster* has recently been sequenced (Wu et al., 2004). This has revealed a striking number of repetitive elements within the *Wolbachia* genome. Over 14% of the chromosome is comprized of repeat sequences, many of them transposable elements. This high level of repetitive DNA is unique for a streamlined intracellular genome. Associated with this high level of repetitive DNA is the common occurrence of translocations and inversions between *Wolbachia* strains (Sun et al., 2003; Wu et al., 2004). Initial data indicate that the presence of repetitive DNA on the *Wolbachia* chromosome is an extremely sensitive marker for discriminating between different *Wolbachia* strains (M. Riegler, personal communication). The genome sequence of *Wolbachia* has also revealed a striking number of genes encoding ankyrin repeat domains. These domains, which mediate protein-protein interactions, are found in over 23 predicted genes of unknown function within the *w*Mel$_{D.mel}$ genome. It is currently hypothesized that they may play a major role in the interaction of *Wolbachia* with its various eukaryotic hosts (Wu et al., 2004).

Ecology

The majority of *Wolbachia* infections in arthropods are physiologically benign. Two strains have been reported as virulent so far, the *popcorn* infection in *D. melanogaster*, *w*MelPop$_I$ (Min and Benzer, 1997), and *w*Vul in *Porcellio dilatus* (Bouchon et al., 1998). Some *Wolbachia* associations have evolved obligate mutualisms with their hosts, as is seen in C and D supergroup infections of filarial nematodes (Bandi et al., 2001). In some insect associations, *Wolbachia* have positive effects on fertility (Girin and Boultreau, 1995; Vavre et al., 1999b) and sperm competition (Wade and Chang, 1995) and are essential for oogenesis (Dedeine et al., 2001). *Wolbachia* can also counteract the deleterious effects of certain mutations in *D. melanogaster* by rescuing oogenesis defects (Starr and Cline, 2002). In most associations, *Wolbachia* manipulate host reproduction, thereby favoring their own dispersal into host populations, a characteristic that leads to the most general definition of *Wolbachia* as reproductive parasites. These reproductive manipulations include cytoplasmic incompatibility, thelytokous parthenogenesis, male-killing and feminization (O'Neill et al., 1997a; Stouthamer et al., 1999).

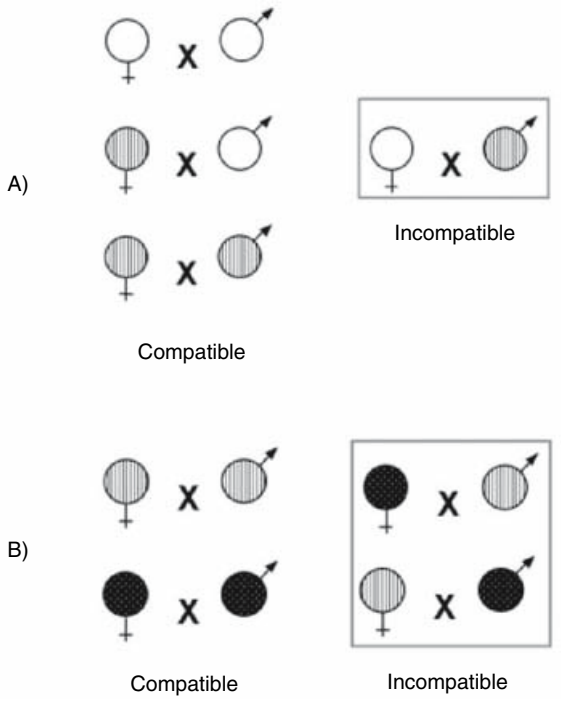

Fig. 5. Schematic illustration of cytoplasmic incompatibility. A) Unidirectional incompatibility is expressed when *Wolbachia* infected males (shaded) mate with uninfected females. These matings produce few viable offspring. All other crosses are compatible. B) Bidirectional incompatibility occurs when insects carrying different *Wolbachia* strains mate. In this case only crosses between individuals infected by the same *Wolbachia* strain are compatible.

Cytoplasmic Incompatibility

Cytoplasmic incompatibility (CI) is the most commonly described phenotype associated with *Wolbachia* infection and has been observed in many insect, mite and crustacean species (Hoffmann and Turelli, 1997). CI arises when infected males mate with females that are either uninfected or infected with a different strain of *Wolbachia* (Fig. 5). The result is a failure of the male pronucleus to successfully complete karyogamy during fertilization of the female eggs. This leads to embryonic lethality in diploid species (O'Neill and Karr, 1990) and either to production of males (Breeuwer and Werren, 1993) or embryonic lethality in haplodiploids (Vavre et al., 2000). Crosses between infected females and uninfected males or between individuals infected with the same strain of *Wolbachia* are fully compatible. The molecular basis of CI is not yet known. Cytological analyses suggest that *Wolbachia* influences proteins involved in host cell cycle regulation (Tram and Sullivan, 2002). *Wolbachia* delay the entry of male pronuclei into the first mitotic divisions either by a direct inhibition of the enzymatic machinery that drives cells into mitosis or indirectly through activation of cell cycle checkpoints (Tram et al., 2003).

A two-component model has been suggested to explain CI (Werren, 1997; Poinsot et al., 2003). *Wolbachia* modify the sperm of infected males during spermatogenesis (modification, or "mod function"), leading to embryonic death unless a related *Wolbachia* is present in the egg and restores viability (rescue, or "resc function"). *Wolbachia* strains can be classified according to their mod and resc capability. For example, a mod+ resc+ strain can induce CI as well as rescue the modification of the same strain, whereas a mod– resc+ strain is incapable of modifying sperm but can rescue sperm modified by closely related *Wolbachia* strains. The mod/resc model also explains other CI relationships such as bidirectional incompatibility between individuals infected with a different *Wolbachia* variant (Fig. 5) or unidirectional incompatibility between individuals infected with a variable number of different *Wolbachia* strains. In the latter case, multiple infections express unidirectional CI when males carry an additional CI inducing strain that is not present in the infected females (Merot et al., 1995; Rousset and Solignac, 1995; Perrot-Minnot et al., 1996; Dobson et al., 2001b; Riegler and Stauffer, 2002).

Thelytokous Parthenogenesis

Hymenoptera and some species of other insect orders possess a haplo-diploid sex determination system (Normark, 2003). Haploid embryos generally develop into males and diploid embryos develop into females. Females can control the laying of unfertilized and fertilized eggs, which then develop into males and females, respectively. Thus sexual haplodiploids are able to produce male offspring parthenogenetically and female offspring sexually (i.e., reproduce by arrhenotoky). Some *Wolbachia* strains are known to interfere with this system during the stage of chromosome segregation and enable females to produce parthenogenic female offspring (i.e., reproduce by thelytoky). In *Wolbachia* infected eggs, two haploid nuclei fuse at the end of the first mitotic division or during interphase before the second division (Gottlieb et al., 2002). Embryos thus develop into homozygous diploid females (Stouthamer and Kazmer, 1994). Thelytokous parthenogenesis induced by *Wolbachia* has been discovered in a variety of parasitoid species (Stouthamer, 1997). Parthenogenesis in the collembolan *Folsomia* has also been assumed to be induced by a *Wolbachia* strain (Vandekerckhove et al., 1999).

Feminization

Wolbachia infections in some terrestrial crustaceans are known to cause feminization (Rigaud, 1997; Bouchon et al., 1998). A similar phenotype has also been detected in a butterfly species (Kageyama et al., 1998; Kageyama et al., 2002; Kageyama et al., 2003). Both groups, crustaceans and butterflies, have unique ZZ or WZ sex chromosome sets with males being the homogamous and females the heterogamous gender (Rigaud, 1997; Kageyama et al., 2002). Feminizing *Wolbachia* strains induce genetic males to develop into functional phenotypic females, thereby providing an opportunity for the symbiont to be maternally transmitted to the next generation. In the isopod *Armadillidium vulgare*, *Wolbachia* prevents the formation of the androgenic gland and also changes the reaction of the host to androgenic hormone activity (Rigaud, 1997).

Male Killing

Wolbachia strains are known that exclusively kill male embryos in some host species. These "male-killing *Wolbachia*" have been found in Coleoptera (Majerus et al., 2000), Lepidoptera (Jiggins et al., 1998) and Diptera (Hurst et al., 2000). In infected populations, male-killing bacteria cause a female biased sex ratio and are thereby theorized to allow a more beneficial resource allocation that increases the reproductive fitness of female hosts and the bacteria they transmit (Hurst et al., 1997). A stable infection equilibrium balanced by resistance factors of the hosts is necessary to prevent the eradication of the infected populations (Randerson et al., 2000).

The type and level of expression of the various *Wolbachia*-induced phenotypes are determined by a mixture of strain and host genotypes. The most intriguing observations so far are switches from one phenotype to another. A switch from CI to a male-killing phenotype was documented after the transfer of *Wolbachia* between two moth species (Sasaki et al., 2002). Another switch in phenotype originally reported as a change from feminizing in one butterfly host species to male-killing in another butterfly species (Fujii et al., 2001) has since been resolved as overlapping feminizing and male-killing phenotypes in the original host (Kageyama and Traut, 2004). It has also been shown that the strength of CI in *Drosophila melanogaster* is dependent on male age (Reynolds and Hoffmann, 2002) and host genotype (McGraw et al., 2001; Reynolds et al., 2003). In many transfer experiments the attenuation or exacerbation of phenotypic effects suggests a strong involvement of host factors (McGraw et al., 2002; Riegler et al., 2004).

The strategy of being a reproductive parasite is not restricted to *Wolbachia*. Other microorganisms are also known to induce biased reproductive phenotypes in arthropods and crustaceans. Feminizing microsporidia are known in amphipod crustaceans (Bulnheim and Vavra, 1968), and male killers are known from the γ-proteobacteria as well as *Spiroplasma* and Bacteroidetes in beetles (Hurst et al., 1997), flies (Williamson et al., 1999), and wasps (Werren et al., 1986). Recently, *Cardinium hertigii*, a member of the Bacteroidetes group, has been shown to cause feminization (Weeks et al., 2001), parthenogenesis (Zchori-Fein et al., 2001; Zchori-Fein et al., 2004) and CI (Hunter et al., 2003) in its arthropod hosts.

The variety of *Wolbachia*-induced reproductive manipulations suggests that these infections impact the population genetics of their hosts. Most apparent is an indirect impact on other cytoplasmic factors, such as the mitochondrial genome. *Wolbachia* infections that cause unidirectional CI and spread into uninfected populations over generations also favor the spread of the infected mitochondrial haplotype and can cause a replacement of original mitochondrial haplotypes (Turelli and Hoffmann, 1991; Ballard et al., 1996). A linkage of *Wolbachia* strains with mitochondrial haplotypes has been shown in the case of CI-inducing strains (Turelli et al.,

1992), as well as in *Wolbachia* associations with other phenotypes such as feminization (Grandjean et al., 1993) and male killing (Jiggins, 2003).

However, CI does not lead to a reduction of nuclear gene flow between populations as long as it is unidirectional (Caspari and Watson, 1959). A few exceptions are seen in island situations (Telschow et al., 2002a). Alternatively, bidirectional incompatibility if complete can inhibit genetic exchange between host populations. In this context the reproductive isolation promoted by *Wolbachia* infections has been seen as a factor promoting speciation in infected hosts (Werren, 1998; Bordenstein et al., 2001; Telschow et al., 2002b).

The strict vertical inheritance of *Wolbachia* has been questioned after the finding of dissimilarities between phylogenetic trees of *Wolbachia* strains and their hosts (O'Neill et al., 1992; Werren et al., 1995b; West et al., 1998; Vavre et al., 1999a). Although efficient horizontal transfer between infected and uninfected individuals of the same parasitoid species has been observed (Huigens et al., 2000) and horizontal transfer of the *Wolbachia* infection of a fly species to a parasitoid wasp described (Heath et al., 1999), it is likely that the limiting factor for efficient horizontal transfer between species is the establishment of the infections in the germline and in the populations of the new host species (Heath et al., 1999; Riegler et al., 2004).

Disease

Despite the fact that *Wolbachia* can reach quite high densities in infected hosts, they do not appear to induce an innate immune response from their hosts (Bourtzis et al., 2000). Moreover most infections do not appear to reduce physiological host fitness appreciably (Hoffmann et al., 1990; Hoffmann et al., 1994; Hoffmann et al., 1998; Giordano et al., 1995; Turelli and Hoffmann, 1995; Bourtzis et al., 1996; Clancy and Hoffmann, 1997; Poinsot and Merot, 1997). Few *Wolbachia* infections cause disease. The best documented is the *popcorn* infection *w*MelPop$_{D.mel}$, which presumably by overreplicating drastically reduces the lifespan of the host *Drosophila melanogaster* (Min and Benzer, 1997). This strain shows a similar virulence phenotype when transferred into the related host species *D. simulans* (McGraw et al., 2002), suggesting that the virulence determinants are encoded by the genome of this particular *Wolbachia* strain. Another virulent *Wolbachia* strain has been described from isopods. Massive symbiont proliferation, followed by necrosis of the nervous tissues, was observed after the artificial transfer of a *Wolbachia* strain naturally infecting *Armadillidium* to *Porcellio dilatatus* (Bouchon et al., 1998).

In recent years *Wolbachia* has been implicated in the inflammatory pathogenesis of human filariasis. It is hypothesized that bacterial toxins released from the *Wolbachia* that infect the filarial nematode induce an inflammatory response in the mammalian host (Taylor and Hoerauf, 1999a; Taylor, 2003). This response has been thought to be largely mediated by lipopolysaccharide (LPS) released from *Wolbachia*. Curiously the genome sequence of the *Drosophila* infecting strain *w*Mel$_{D.mel}$ shows that this *Wolbachia* strain, like many intracellular symbionts, does not contain an intact pathway for LPS biosynthesis (Wu et al., 2004), suggesting either that nematode *Wolbachia* have gained this capability or that some other mechanism may be responsible for the observed pathogenesis.

Applications

The potential applied use of *Wolbachia*-mediated incompatibilities to control insect pests and associated diseases was suggested even before it was understood that *Wolbachia* was the etiological agent responsible for cytoplasmic incompatibility (Laven, 1967; Yen and Barr, 1971). Since then, *Wolbachia* has been proposed as a method to directly suppress pest populations, to modify the ability of insects to transmit disease agents, to enhance the mass production of beneficial insects used for biological control, and in recent years, as a new target for the control of filariasis.

Host Population Suppression

The application of CI as a means to suppress pest insect populations has been considered since its discovery (Laven, 1967; Boller et al., 1976; Brower, 1980). Analogous to the Sterile Insect Technique (SIT; Krafsur, 1998), an inundating release of incompatible males in natural populations should decrease or inhibit successful fertilization of wild females and thereby suppress wild populations. Field experiments with the mosquito *Culex pipiens* have shown promising results (Laven, 1967). However, this technique has a few drawbacks such as immigration of fertilized females (Curtis et al., 1982) and the risk of releasing compatible females along with males. An isolated population, combined with a reliable sexing technique, which guarantees the release of only males is essential for the success of population suppression. A combination of CI and SIT, whereby insects could be irradiated at lower doses and then sterilized in a conventional SIT program, has been suggested. *Wolbachia*

would induce the crossing sterility and the irradiation would prevent the release of fertile infected females (Arunachalam and Curtis, 1985; Shahid and Curtis, 1987). The use of cytoplasmic incompatibility, or the Incompatible Insect Technique (IIT; Blümel and Russ, 1989), has been suggested for a range of pests of agricultural and medical importance (Laven, 1967; Boller et al., 1976; Brower, 1980).

Modulating Insect-Transmitted Disease

In addition to direct population suppression, *Wolbachia*-based strategies could be used to interfere with the ability of insect populations to transmit pathogens, either through modifying their population age structure or by spreading genes into populations that block transmission of pathogens. In the first instance it has been proposed that overreplicating virulent *Wolbachia* strains (Min and Benzer, 1997; McGraw et al., 2002) could be used to skew population age-structure towards younger individuals and in so doing reduce the ability of the insect population to transmit disease agents such as dengue virus (Sinkins and O'Neill, 2000; Brownstein et al., 2003; Rasgon et al., 2003).

Alternatively *Wolbachia* could be used to drive refractoriness genes located either in themselves or in other maternally inherited factors such as mitochondria, viruses or inherited nutritive symbionts through host populations by a hitch-hiking effect (Beard et al., 1993; Curtis and Sinkins, 1998; Turelli and Hoffmann, 1999; Sinkins and O'Neill, 2000). The drive of nuclear genes into host populations using *Wolbachia* is less feasible, as there is no linkage between infections and the host nuclear genome. However, transformation of organisms with constructs that contain the genes involved in the induction of *Wolbachia* phenotypes together with the desired genes has been suggested (Curtis and Sinkins, 1998; Turelli and Hoffmann, 1999; Sinkins and O'Neill, 2000).

Wolbachia as a Target for Filariasis Control

Recent work suggests that *Wolbachia* endosymbionts of filarial nematodes are a major source of the inflammatory response observed in humans suffering from lymphatic filariasis (Taylor et al., 2000) and onchocerciasis (Saint Andre et al., 2002). Antibiotic therapy trials have led to long-term reductions of *Wolbachia* and interruption of nematode embryogenesis (Hoerauf et al., 2002). Similar antibiotic therapy strategies are currently being tried for other filarial diseases and should open new ways of controlling human filarial infection and disease (Taylor and Hoerauf, 2001).

Wolbachia as a Fitness Enhancer in the Rearing of Beneficials

Associations have been reported where *Wolbachia* clearly have favorable effects on their hosts. *Wolbachia* is known to increase fitness parameters in hymenopteran parasitoids (Girin and Boultreau, 1995; Stolk and Stouthamer, 1995; Vavre et al., 1999b). Similarly, male-killing bacteria are seen in the context of a better resource allocation in lady bird beetles (Hurst et al., 1997). Both groups of insects are commercially used in biological control. Hence, *Wolbachia*'s potential of favoring the reproduction of their hosts could become an important trait for the rearing of beneficials. This aspect has been discussed in the comparison of sexually and parthenogenetically reproducing *Trichogramma* lines (Stouthamer, 1993; Silva et al., 2000; Tagami et al., 2001; Tagami et al., 2002). *Wolbachia* infected thelytokous lines seem to perform better than uninfected arrhenotokous counterparts (Silva et al., 2000).

Acknowledgments. We thank Jeremy Brownlie, Sylvain Charlat, Molly Hunter, Inaki Iturbe-Ormaetxe, Wolfgang Miller and Elizabeth McGraw for valuable comments and stimulating discussions on the subject, as well as reading through earlier versions of the manuscript. We also acknowledge Oxford University Press, the Royal Society, the Society of General Microbiology and the authors J. S. Dumler and N. Loo for granting us permission to reproduce figures 1–3 of published articles.

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Prokaryotes (2006) 5:562–584
DOI: 10.1007/0-387-30745-1_23

CHAPTER 3.1.22

Aerobic Phototrophic Proteobacteria

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Introduction

For decades anoxygenic photosynthesis in purple bacteria has been widely assumed to be an anaerobic metabolic process, active in light and anaerobic conditions (Imhoff, 1988; Imhoff and Truper, 1989; Prince, 1990). However, many obligately aerobic species of a new physiological group of bacteria that have a purple bacterial type of photosynthetic apparatus have been isolated relatively recently. Nowadays, these bacteria are found world-wide in different geographical areas and ecological niches. They have been described as inhabitants of freshwater and marine microbial mats or as free-floating populations in sea water, meromictic lakes, warm and hot water springs, acidophilic drainage waters, and deep ocean hydrothermal environments of so-called black smokers (Shimada, 1995; Yurkov and Beatty, 1998a). Most strains of the aerobic phototrophs isolated so far inhabit a wide variety of eutrophic aquatic environments and seem to comprise a significant part of the aerobic heterotrophic bacterial population. Recently two new species, *Craurococcus roseus* and *Paracraurococcus ruber*, have been isolated from soil (Saitoh et al., 1998). Although this increasingly large group of bacteria is very heterogeneous phylogenetically, morphologically and physiologically, these bacteria share a common novel characteristic, which is the inability to use bacteriochlorophyll for anaerobic photosynthetic growth and the presence of photochemical reactions in cells only under aerobic conditions (Yurkov and Beatty, 1998a).

Until knowledge sufficient to create a taxonomic name has accumulated, convention among scientists in this field is to call this group "the aerobic phototrophic bacteria" (Shimada, 1995; Yurkov and Beatty, 1998a). This chapter refers to these bacteria as "the aerobic phototrophic bacteria."

Taxonomy, Phylogeny and Origin

At present, aerobic phototrophic bacteria are taxonomically classified into seven freshwater (*Sandaracinobacter*, *Erythromonas*, *Erythromicrobium*, *Roseococcus*, *Porphyrobacter*, *Acidiphilium* and *Roseateles*), six marine (*Erythrobacter*, *Roseobacter*, *Citromicrobium*, *Rubrimonas*, *Roseovarius* and *Roseivivax*) and two soil (*Craurococcus* and *Paracraurococcus*) genera (Table 1). The discovery of obligately aerobic heterotrophs that synthesize bacteriochlorophyll *a* aroused immediate interest and stimulated research on their evolution and origin. The most intriguing fact discovered was the inability of the "typical" photosynthetic apparatus of these bacteria to support anaerobic photosynthetic growth. Two major questions arose simultaneously: 1) What is the evolutionary origin of the aerobic phototrophic bacteria in general? and 2) How did the photosynthetic apparatus evolve in these predominantly aerobic heterotrophic microorganisms? Recently created phylogenetic trees show that aerobic phototrophs do not present a homogeneous cluster but are distributed among photosynthetic and nonphotosynthetic species, indicating that some species are not closely related. This situation (similar to that described by the phylogeny of anaerobic phototrophic bacteria) is the basis for the proposal that non-photosynthetic proteobacteria had a photosynthetic ancestor and the photosynthesis genes were lost in some phylogenetic lineages during evolution (Woese, 1987). Available data cannot explain whether the poorly developed and low efficiency photosynthetic apparatus of the aerobic phototrophs is a relict inherited from a "true" photosynthetic ancestor and kept by these bacteria during evolution towards the aerobic respiratory mode of energy generation, or whether the photosynthetic apparatus is a relatively recent evolutionary acquisition, which reoccured in some aerobic heterotrophic species. Although the evolutionary significance of the inability to grow anaerobically photosynthetically despite the presence of a photosynthetic apparatus is unclear, further investigations of aerobic phototrophs will ultimately solve this puzzle and contribute to our understanding of the evolution of photosynthesis.

Today, two independent theories weigh the evolutionary origin of aerobic phototrophic bac-

Table 1. Major properties of the aerobic phototrophic species.

Species	Phylogeny (subclass)	Color	Carotenoid in vivo peaks (nm)	Bchl in vivo peaks (nm)	Cell shape and size (μm)	DNA G+C content (mol%)	Place of isolation
Freshwater:							
Sandaracinobacter sibiricus	α-4	yellow-orange	424, 450, 474	800, 867	Thin, long rods (0.3–0.5 × 1.5–2.5)	68.5	Russia, hot temperature spring bacterial mat
Erythromonas ursincola	α-4	Orange-brown	430, 458, 485	800, 867	Ovoid (0.8–1.0 × 1.3–2.6)	65.4	Russia, Southern Kurily, hot temperature spring caynobacterial mat
Erythromicrobium							
ramosum	α-4	red-orange	466, 478	798, 832, 868	Rods, branched (0.7–1.0 × 1.6–2.5)	64.2	Russia, warm water spring algo-bacterial mat
ezovicum	α-4	red-orange	466,478	798, 836, 868	Long bacilli (0.6–0.8 × 2.7–2.8)	62.5	
hydrolyticum	α-4	red-orange	466, 478	798, 838, 868	Rods, branched (0.7–1.1 × 1.8–2.5)	65.2	
Roseococcus thiosulfatophilus	α-1	pink-red	482, 510, 538	800, 858	Coccoid (0.9–1.3 × 1.3–1.6)	70.4	Russia, hot temperature spring, cyanobacterial mat
Porphyrobacter							
neustonensis	α-4	orange-red	464, 491	799, 869	Pleomorphic (0.4–0.8 × 1.1–2.0)	65–66	Australia, subtropical pond, water surface
tepidarius	α-4	orange	460, 494	800, 870	Ovoid (0.5–0.7 × 0.8–1.4)	65	Japan, brackish hot spring
Acidiphilium							
cryptum	α-1	Pink	n.a.	n.a.	Rods (0.3–1.2 × 0.6–4.2)	67.3	Acid mineral environment
rubrum	α-1	Pink-red	465, 492	792, 864	Rods (0.6 × 2.0)	63.2	Acid mine drainage
multivorum	α-1	Pale pink	525	n.a.	Rods (0.5–0.9 × 1.5–3.8)	66.2–66.3	
Roseateles depolimerans	β	pink	482, 515, 550	800, 870	Rods (0.5 × 2.0)	66.2–66.3	Japan, river water

(Continued)

Table 1. *Continued*

Species	Phylogeny (subclass)	Color	Carotenoid in vivo peaks (nm)	Bchl in vivo peaks (nm)	Cell shape and size (μm)	DNA G+C content (mol%)	Place of isolation
Seawater:							
Erythrobacter							
longus	α-4	Orange	470	800, 869	Rods (0.4–0.5 × 1.0–5.0)	60–64	Japan, high-tidal seaweeds
litoralis	α-4	Red-orange	437, 461, 488	800, 868	Rods (0.2–0.3 × 1.0–1.3)	67	The Netherlands, marine cyanobacterial mats
Roseobacter							
litoralis	α-3	Pink	510	806, 868	Ovoid rod (0.6–0.9 × 1.0–2.0)	59.6 ± 0.5	Japan, high tidal seaweeds
denitrificans	α-3	pink	510	806, 868	Ovoid rod (0.6–0.9 × 1.0–2.0)	56.3–58.1	
Citromicrobium bathyomarinum	α-4	Citron yellow	433, 457, 487	800, 867	Extremely pleomorphic	67.5	Juan de Fuca Ridge, Northeastern Pacific, deep ocean hydrothermal vent environment
Rubrimonas cliftonensis	α-3	pink	n.a.	806, 871	Rods (1.0–1.5 × 1.2–2.0)	74.0–74.8	Australia, West Coast, saline lake water
Roseovarius tolerans	α-3	Pink-red	Around 510	800, 878	Rods (0.7–1.0 × 1.1–2.2)	62.2–63.8	East Antarctica, heliothermal meromictic Ekho Lake
Roseivivax							
halodurans	α-3	Pink	n.a.	803, 873	Rods (0.5–1.0 × 1.0–5.0)	64.4	Australia, West Coast, charophytes of the saline lake
halotolerans	α-3	pink	n.a.	805, 871	Rods (0.5–1.0 × 1.0–5.0)	59.7	Australia, West Coast, epiphytes on the stromatolites
Soil:							
Craurococcus roseus	α-1	pink	n.a.	800, 872	Coccus (0.8 × 2.0)	70.5	Japan, soil
Paracraurococcus ruber	α-1	red	n.a.	802, 856	Coccus (0.8 × 1.5)	70.3–71.0	Japan, soil

Abbreviations: n.a., data not available; and Bchl, bacteriochlorophyll.

Adapted from Yurkov et al., 1994b; 1997; Yurkov and Gorlenko, 1992b; Fuerst et al., 1993; Hanada et al., 1997; Wakao et al., 1993; Wakao et al., 1994; Suyama et al., 1999; Shiba and Simidu, 1982; Shiba, 1991; Yurkov et al., 1999; Suzuki et al., 1999b; Labrenz et al., 1999; Suzuki et al., 1999a; and Saitoh et al., 1998.

teria. One idea is that branching of aerobic phototrophic bacteria from different species of anaerobic phototrophic bacterial ancestors can explain the observed 16S rRNA phylogenetic heterogeneity as well as differences in the photosynthetic apparatus. Thus, aerobic phototrophic bacteria could represent an intermediate phase of evolution from anaerobic purple bacteria to non-photosynthetic aerobes (Woese et al., 1984).

A second possibility is that lateral transfer of photosynthesis genes (Blankenship, 1992; Nagashima et al., 1993; Nagashima et al., 1997b) might have resulted in the acquisition of a photosynthesis gene cluster by nonphotosynthetic aerobes. However, the relatively recent acquisition of multigene-dependent photosynthetic complexes by lateral gene transfer to all aerobic phototrophic bacteria seems to be less likely.

The majority of aerobic phototrophic species is phylogenetically associated with members of the α-subclass of the class *Proteobacteria* (Table 1). *Roseococcus thiosulfatophilus*, *Craurococcus roseus* and *Paracraurococcus ruber* are members of subclass α-1 and are moderately related to *Rhodopila globiformis*, *Thiobacillus acidophilus* and members of the genus *Acidiphilium*. *Erythromicrobium*, *Erythrobacter*, *Sandaracinobacter*, *Erythromonas*, *Citromicrobium* and *Porphyrobacter* are very closely related genera and are clustered in the α-4 subclass, more distant from other aerobic phototrophs. The 16S rRNA sequence data placed *Roseobacter*, *Rubrimonas*, *Roseovarius* and *Roseivivax* in a branch separate from α-4 and α-1 representatives and in a relatively close phylogenetic relationship with purple nonsulfur *Rhodobacter sphaeroides* and *R. capsulatus* within α-3 subclass (Fuerst et al., 1993; Kawasaki et al., 1993; Stackebrandt et al., 1996; Yurkov et al., 1994c; Yurkov et al., 1997; Yurkov et al., 1999; Suzuki et al., 1999b; Labrenz et al., 1999; Suzuki et al., 1999a). A phylogenetic study performed on a psychrophylic (gas-vacuolate) bacterium isolated from polar sea ice, *Octadecabacter*, identified this organism as a close non-phototrophic relative of *Roseobacter* species (Gosink et al., 1997). Recently, the nonphotosynthetic *Sphingomonas* group was included in the α-4 subclass (Yabuuchi et al., 1990), such that the *Erythromicrobium-Erythrobacter-Porphyrobacter* cluster is most closely related to members of the genus *Sphingomonas* (Yurkov et al., 1994c).

Only one species, *Roseateles depolymerans*, has been recently described to belong to the β-subclass of the *Proteobacteria* (Suyama et al., 1999). Because this is a very unique example, the ability of this species to grow anaerobically photosynthetically under different nonstandard growth conditions should be carefully investigated.

Habitat

When the first marine obligately aerobic phototrophic strain Och101 was isolated from different sites of Tokyo Bay (seaweed, seawater, sand and bottom sediments) and later described as the first known species, *Erythrobacter longus* (Shiba and Simidu, 1982), in this group, it was thought that something very rare, unique and extraordinary had been discovered. However, an extensive search for these bacteria during recent years has shown that the obligately aerobic phototrophic bacteria are widely distributed around the world and represent a significant portion of the heterotrophic microbial population in various environments. Ecological niches of the aerobic phototrophs are very diverse; nevertheless, the availability of oxygen seems to be an obligatory requirement. The majority of species has been isolated from aquatic environments with significant amounts of soluble organic matter (Table 1). They are often found accompanying layers of cyanobacteria as well as purple nonsulfur and purple sulfur bacteria in cyanobacterial mats. The mats from which the isolates came were largely composed of the cyanobacteria *Oscillatoria*, *Synechococcus* and *Phormidium*, diatoms and the purple phototrophic bacteria *Thiocapsa roseopersicina*, *Rhodopseudomonas palustris*, *Rhodomicrobium vannielii* and *Rhodopseudomonas viridis* (Yurkov and Beatty, 1998a). Samples of these mats contained up to 106 cells of aerobic bacteria containing bacteriochlorophyll *a* per ml. Some species were isolated from cyanobacterial mats developed in springs with considerably hot temperatures. *Erythromonas ursincola* was isolated from an environment with a temperature of about 40°C and *Roseococcus thiosulfatophilus* from a site with a temperature of 54°C (Yurkov and Gorlenko, 1992a; Yurkov and Gorlenko, 1993a). However, in pure laboratory cultures all of these strains demonstrated typical mesophilic properties and grew optimally at 28–30°C (Yurkov and Gorlenko, 1992a; Yurkov and Gorlenko, 1993a; Yurkov et al., 1992a; Yurkov et al., 1992b). The reasons why and how these obligately aerobic species survive in thermal environments are unclear.

Two moderately thermophilic or thermotolerant representatives, *Porphyrobacter tepidarius* and *Citromicrobium bathyomarinum*, were discovered recently (Hanada et al., 1997; Yurkov et al., 1999). *Porphyrobacter tepidarius* was isolated from bacterial mats in the brackish Usami hot spring (Japan) where the temperature at the sampling site was 42.7°C, the pH was 5.8, and the bacterial mat consisted mainly of a dark green layer of thermophilic filamentous cyanobacteria. This new species grew at temperatures up to 50°C, and optimal growth occurred at 40 to 48°C

(Hanada et al., 1997; Table 2). Aerobic anoxygenic phototrophic strains containing bacteriochlorophyll *a* were discovered in hydrothermal "black smoker" plume waters of the Juan de Fuca Ridge in the Pacific Ocean (Yurkov and Beatty, 1998b). Water samples taken from about 2000 m beneath the ocean surface were found to contain aerobic bacteria producing bacteriochlorophyll *a* in numbers of 20–40 cells/ml, about 30% of the pigmented strains that formed colonies on the rich medium used. The representative strain JF-1 (*C. bathyomarinum*) revealed a broad tolerance to culture conditions such as salinity, temperature and pH. Thus, growth was obtained in a freshwater medium and a medium supplemented with 10% NaCl, at temperatures ranging from 5 to 42°C, and at pH values of 5.5 to 10.0 (Table 2). Therefore, *C. bathyomarinum* is a salt, pH and thermotolerant species (Yurkov et al., 1999). The fact that such a bacterium was found at this location suggests that the ecology and microbial population of the black smoker environment is even more diverse than has been imagined.

The greatest variety of species and the largest numbers of strains have been found in sea, lake and river waters (Table 1). In Tokyo Bay, the proportions of these bacteria among the species that formed colonies (on the medium employed) ranged from 0.9 to 1.1% in the seaweed samples and from 1.2 to 6.3% in the beach sand samples (Shiba et al., 1979). The presence of aerobic heterotrophic bacteriochlorophyll-α synthesizing strains in high proportions (10 to 30% of the total heterotrophic bacterial strains isolated) was described for marine environments on the west and east coasts of Australia (Shiba et al., 1991b) and at the Pacific Ocean inlet English Bay in Vancouver, Canada (V. V. Yurkov et al., unpublished observation).

Several strains of the pelagic bacterium, *Porphyrobacter neustonensis*, were purified from the surface of a freshwater subtropical pond in Australia (Fuerst et al., 1993), and acidophilic heterotrophic bacteria that synthesize bacteriochlorophyll *a* were isolated from an acidic mine drainage system (Wakao et al., 1993). Aerobic phototrophic bacteria were detected in high numbers relative to the numbers of other heterotrophic strains in the North Adriatic Sea, where they comprised 5 to 55% of the total cultured cells (P. Lebaron, personal communication).

Microbial populations in saline lakes and stratified meromictic saline lakes showed a high presence of the aerobic phototrophic bacteria. *Rubrimonas cliftonensis*, *Roseivivax halodurans* and *Roseivivax halotolerans* have been isolated from the charophytes and the epiphytes on the stromatolites of a saline lake located on the west coast of Australia (Suzuki et al., 1999a; Suzuki et al., 1999b). Eight closely related strains of *Roseovarius tolerans* were recovered from various depths of the hypersaline, heliothermal and meromictic Ekho Lake (East Antarctica) which originated from fjords about 5,000–6,000 years ago (Labrenz et al., 1999). Ekho Lake contains many different environments throughout its depths. A total of 135 prokaryotic and 52 eukaryotic morphotypes were discovered. This high morphological diversity prompted the isolation of some 250 bacterial cultures from different depths of Ekho Lake. Prokaryotic photosynthetic primary producers such as cyanobacteria were almost completely absent from the lake and the aerobic phototrophic *Roseovarius* is at present the only photosynthetic bacterial species described from this lake (Labrenz et al., 1999).

Another meromictic lake (Mahoney Lake), which has stratified salinity without outflow, near Penticton in the dry region of South Central British Columbia, was found to be highly enriched in aerobic phototrophic strains. Earlier microbiological investigations of Mahoney Lake were done on the anaerobic anoxygenic phototrophs and revealed an extremely dense population of the purple sulfur bacterium *Amoebobacter purpureus* (Overman et al., 1991). Recent study of the lake's aerobic phototrophic bacterial population has found that this fraction of the community is very rich, diverse and environmentally specific. A large number (more than 30) of taxonomically new strains of the obligately aerobic phototrophic bacteria has been isolated (N. Yurkova et al., unpublished observation).

Some aerobic phototrophic species also occur in highly acidic environments. All species of the *Acidiphilium* genus (Table 1) were reported from acid mine drainage, coal refuse and coal mine drainage. Acidophilic aerobic phototrophs which grow at pH 2.0–6.0 are often found accompanied by *Thiobacillus ferrooxidans* in such environments. *Acidiphilium* is a genuine acidophile that requires high acidity to grow (pH 3.0) (Wakao et al., 1994; Harrison, 1981; Wichlacz and Unz, 1986).

In general, not much research has been done to identify aerobic phototrophic bacteria from soil. A recent report on the isolation from soil of two new genera, *Craurococcus* and *Paracraurococcus*, which are aerobic and bacteriochlorophyll *a*-containing, has established the soil environment as a new econiche for these bacteria (Saitoh et al., 1998).

Isolation and Enrichment

Aerobic phototrophic bacteria are predominantly heterotrophs that require oxygen and

Table 2. Growth and physiological properties of the aerobic phototrophic species.

Characteristic	Species																					
	Ssi	*Emour*	*Emira*	*Emiez*	*Emihy*	*Rocth*	*Pne*	*Pte*	*Acr*	*Aru*	*Amu*	*Roade*	*Elo*	*Eli*	*Robli*	*Robde*	*Cba*	*Rcl*	*Rovto*	*Rovihad*	*Rovihat*	*Crro*	*Pcrru*
Growth at pH	7–9.5	6–9	6–9.5	6–9.5	6–9.5	6–9	7–9	6–9	2–5.6	2–5.6	2–5.6	5–8	6.5–9	6–9	7.5–9.5	7.5–9.5	5.5–10	n.a.	6.9–9	n.a.	n.a.	n.a.	n.a.
Optimal pH	7.5–8.5	7–8	7–8.5	7–8	7–8	7–8	8	6.5–8.5	3–4	3–4	3–4	6.5	7.0	7–8.5	7–8	7–8	6–8	7.5–8	6.2–9	7.5–8	7.5–8	7.5	6.6–6.8
Growth at t (°C)	15–35	15–35	15–35	15–35	15–35	15–35	15–37	30–50	17–42	17–42	17–42	5–43	2–37	15–37	2–30	2–30	4–45	n.a.	3–43.5	n.a.	n.a.	n.a.	n.a.
Optimal t (°C)	25–30	25–30	25–30	25–30	25–30	25–30	20–30	40–48	27–35	27–35	27–35	35	30	25–30	20–30	20–30	20–42	27–30	8.5–33.5	27–30	27–30	28–32	30–34
Utilization of:																							
acetate	+	+	+	+	+	+	−	+	−	−	−	n.a.	+	+	+	+	+	−	+	+	+	−	−
pyruvate	+	+	+	−	+	+	+	−	−	−	−	+	+	+	+	+	−	+	+	+	+	+	+
glutamate	−	+	+	+	+	+	−	+	n.a.	n.a.	n.a.	n.a.	+	+	+	+	+	−	+	+	+	+	+
butyrate	+	+	+	+	−	−	−	+	n.a.	n.a.	n.a.	n.a.	+	+	−	−	+	n.a.	+	+	+	−	−
citrate	−	+	+	+	+	+	−	−	+	+	+	+	−	−	+	+	−	+	−	+	+	−	−
malate	−	+	+	+	+	+	−	−	n.a.	n.a.	n.a.	+	−	−	+	+	−	−	+	+	+	+	+
succinate	−	+	+	+	+	+	+	−	−	n.a.	+	+	−	+	+	+	−	−	+	+	+	−	−
lactate	−	+	+	+	+	+	−	−	−	−	−	+	−	+	n.a.	n.a.	−	−	−	+	+	+	+
formate	−	−	−	−	−	−	−	n.a.	−	−	−	n.a.	+	−	n.a.	n.a.	−	n.a.	−	n.a.	n.a.	−	−
glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+
fructose	+	+	+	+	+	−	n.a.	−	n.a.	n.a.	n.a.	+	n.a.	+	n.a.	n.a.	−	+	n.a.	+	+	+	+
methanol	−	−	−	−	−	−	−	−	−	−	+	n.a.	−	−	−	−	−	−	−	−	−	−	−
ethanol	−	−	+	+	+	−	−	−	−	−	+	n.a.	n.a.	−	n.a.	n.a.	−	+	n.a.	−	−	−	−
yeast extract	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	n.a.	n.a.	n.a.	n.a.	n.a.
Hydrolysis of:																							
gelatin	−	−	−	−	+	−	−	−	n.a.	n.a.	n.a.	+	+	−	+	+	−	−	−	−	+	−	−
Tween-60	+	+	−	−	+	−	+	+	n.a.	n.a.	n.a.	n.a.	+	+	+	+	+	−	+	−	−	+	−
starch	−	−	−	−	+	−	−	+	−	−	−	n.a.	−	−	−	−	+	−	−	−	−	n.a.	n.a.
Reduction of:																							
NO_3 to NO_2	−	−	−	−	−	−	−	n.a.	+	+	+	−	+	−	−	+	−	+	−	n.a.	n.a.	+	+
NO_2 to N_2	−	−	−	−	−	−	−	n.a.	−	−	−	−	−	−	−	−*	−	−	−	n.a.	n.a.	−	−
TeO_3 to Te	+	+	+	+	+	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	+	+	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Oxidase	+	+	+	+	+	+	−	n.a.	+	+	−	+	+	+	+	+	+	n.a.	+	+	+	+	+
Catalase	−	+	+	+	+	+	+	n.a.	+	+	+	−	+	+	+	+	+	n.a.	+	+	+	+	+
Sensitivity to:																							
Chloramphenicol	+	+	+	+	+	+	n.a.	+	n.a.	n.a.	n.a.	n.a.	+	+	+	+	+	+	+	+	+	+	+
Penicillin	−	−	−	−	+	−	+	+	−	−	+	n.a.	+	−	+	+	−	−	+	−	−	+	−
Streptomycin	+	−	−	−	−	+	n.a.	−	−	−	+	n.a.	−	−	+	+	−	+	+	+	+	+	+
Fusidic Acid	−	+	+	+	n.a.	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+	+	n.a.	n.a.	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Polymixin B	−	+	+	+	+	+	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	−	+	+	+	n.a.	−	n.a.	n.a.	−	−

Symbols: −, substrate not utilized, resistant to antibiotic or compound not hydrolyzed; +, substrate utilized, susceptible to antibiotics or compound hydrolyzed.

Abbreviations: T, temperature; n.a., data not available; *Ssi, Sandaracinobacter sibiricus; Emour, Erythromonas ursincola; Emira, Erythromicrobium ramosum; Emiez, Erythromicrobium ezovicum; Emihy, Erythromicrobium hydrolyticum; Rocth, Roseococcus thiosulfatophilus; Pne, Porphyrobacter neustonensis; Pte, Porphyrobacter tepidarius; Acr, Acidiphilium cryptum; Aru, Acidiphilium rubrum; Amu, Acidiphilium multivorum; Roade, Roseateles depolymerans; Elo, Erythrobacter longus; Eli, Erythrobacter litoralis; Robli, Roseobacter litoralis; Robde, Roseobacter denitrificans; Cba, Citromicrobium bathyomarinum; Rcl, Rubrimonas cliftonensis; Rovto, Roseovarius tolerans; Rovihad, Roseivivax halodurans; Rovihat, Roseivivax halotolerans; Crro, Craurococcus roseus; Pcrru, Paracraurococcus ruber.*

[a]Reduces NO_2 to N_2O.

some organic carbon sources. Therefore, no selective medium has been developed to isolate aerobic phototrophic bacteria, and many nonphototrophic microorganisms grow well on agar of rich organic media. A wide variety of media rich in organic components such as yeast extract, peptone, casamino acids, salts of tricarboxylic acids or sugars has been used to isolate pure cultures of different aerobic phototrophic species. Direct inoculation (with dilution) of water samples (for isolating free-floating strains) or homogenized mat/sand samples (for isolating strains found in cyanobacterial communities or on solid surfaces) on to agar plates of rich organic media can serve this purpose (Fuerst et al., 1993; Hanada et al., 1997; Shiba et al., 1979; Shiba et al., 1991b; Shiba and Simidu, 1982; Wakao et al., 1994; Yurkov and Beatty, 1998b; Yurkov and Gorlenko, 1990b; Yurkov et al., 1994b). As a rule, inoculated plates have been incubated in the dark at temperature and pH values similar to those found in the environment from which samples were collected. Pure isolates of pigmented colonies are easily obtainable from streaked agar plates. When a pure culture is obtained, a single colony is transferred into liquid medium and cultivated aerobically in the dark.

Solid agar media most commonly used to isolate the obligately aerobic phototrophic bacteria from natural sites are:

1. Medium PPES-II described for the isolation of *Erythrobacter*, *Roseobacter* and some other marine species (Taga, 1968) consists of: polypeptone, 2.0 g; soytone, 1.0 g; proteose peptone N3, 1.0 g; yeast extract, 1.0 g; ferric citrate, 0.1g; artificial seawater, 700 ml; distilled water, 300 ml; and agar, 2%.
2. PA medium used to isolate many freshwater strains from the hot temperature spring cyanobacterial mats (Yurkov and Gorlenko, 1990b; Yurkov, 1990a) consists of: yeast extract, 0.1g; sodium acetate, 0.5g; trace element solution (Drews, 1983), 1 ml; potato broth, 500 ml; distilled water, 500 ml; and agar, 2%.
3. RO medium used to isolate freshwater and marine species (Yurkov and Gorlenko, 1990b; Yurkov et al., 1994b; Yurkov and Beatty, 1998b) consists of (in grams per liter of distilled water): yeast extract, 1.0; Bacto peptone, 1.0; sodium acetate, 1.0; KCl, 0.3; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.05; NH_4Cl, 0.3; K_2HPO_4, 0.3; for marine species add NaCl, 20.0; and agar, 2%. This medium was supplemented with a mixture of vitamins ([per liter of medium] 20 μg of vitamin B_{12}, 200 μg of nicotinic acid, 80 μg of biotin, and 400 μg of thiamine) and 1 ml per liter of a trace element solution (Drews, 1983).
4. Recommended media to grow acidophilic species (Wakao et al., 1994): basal salts (BS) medium consists of: 0.2% $(NH_4)_2SO_4$; 0.01% KCl; 0.01% K_2HPO_4; 0.01% $MgSO_4 \cdot 7H_2O$; and 0.001% $Ca(NO_3)_2$. The culture media are adjusted to pH 3.0 with H_2SO_4. The BS medium (pH 3.0) is supplemented with 0.5% polypeptone, 0.5% glucose, 0.2% vitamin-free casamino acids plus 0.5% glucose, 0.2% trypticase soy or 0.2% trypticase soy plus 1.0% glucose. These media were designated as BS-P, BS-G, BS-CG, BS-T and BS-TG media, respectively (Wakao et al., 1994).
5. PY agar medium (Fuerst et al., 1993) described for the isolation of *Porphyrobacter neustonensis* (in grams per liter of distilled water) consists of: peptone, 10.0; yeast extract, 5.0; NaCl, 5.0; and agar, 1.5%.
6. PYGV medium was used to isolate *Roseovarius tolerans* from the Antarctic hypersaline lake, Ekho Lake (Labrenz et al., 1999). This medium consists (per liter) of: 0.25 g each of Bacto peptone, Bacto yeast extract and glucose, as well as 20 ml of Hutners's basal salt solution (Cohen-Bazire et al., 1957) and 10 ml of vitamin solution N6 (Staley, 1968). Medium is solidified with 1.8% agar.

Identification

Both morphological and physiological properties are very important for the identification of the aerobic phototrophic bacteria. Selection and differentiation cannot be primarily based on a choice of media or specific culture conditions owing to the heterotrophy and oxygen dependence of these bacteria. Many nonphototrophic heterotrophic microorganisms will outgrow the aerobic phototrophic species when rich organic media solidified with agar are used. However almost all species of the aerobic phototrophs growing in standard aerobic conditions are intensely pigmented by carotenoids. Therefore, the color of bacterial colonies has been used as an initial indication of aerobic phototrophic bacteria, but confirmation still required the demonstrated presence of absorption spectra characteristic of bacteriochlorophyll. Aerobic phototrophic strains are distinguished from other heterotrophic bacteria by the presence of bacteriochlorophyll *a*, as indicated by absorption peaks in the region of 800 to 880 nm in cell suspensions or by an absorption peak of about 770 nm in acetone-methanol extracts of cells (Fig. 1).

A common misconception that has occurred in several recent taxonomic descriptions should be mentioned. Although it is known that phototrophic capacity is still recognized as an important taxonomic marker in bacterial classification, some heterotrophic strains that lack

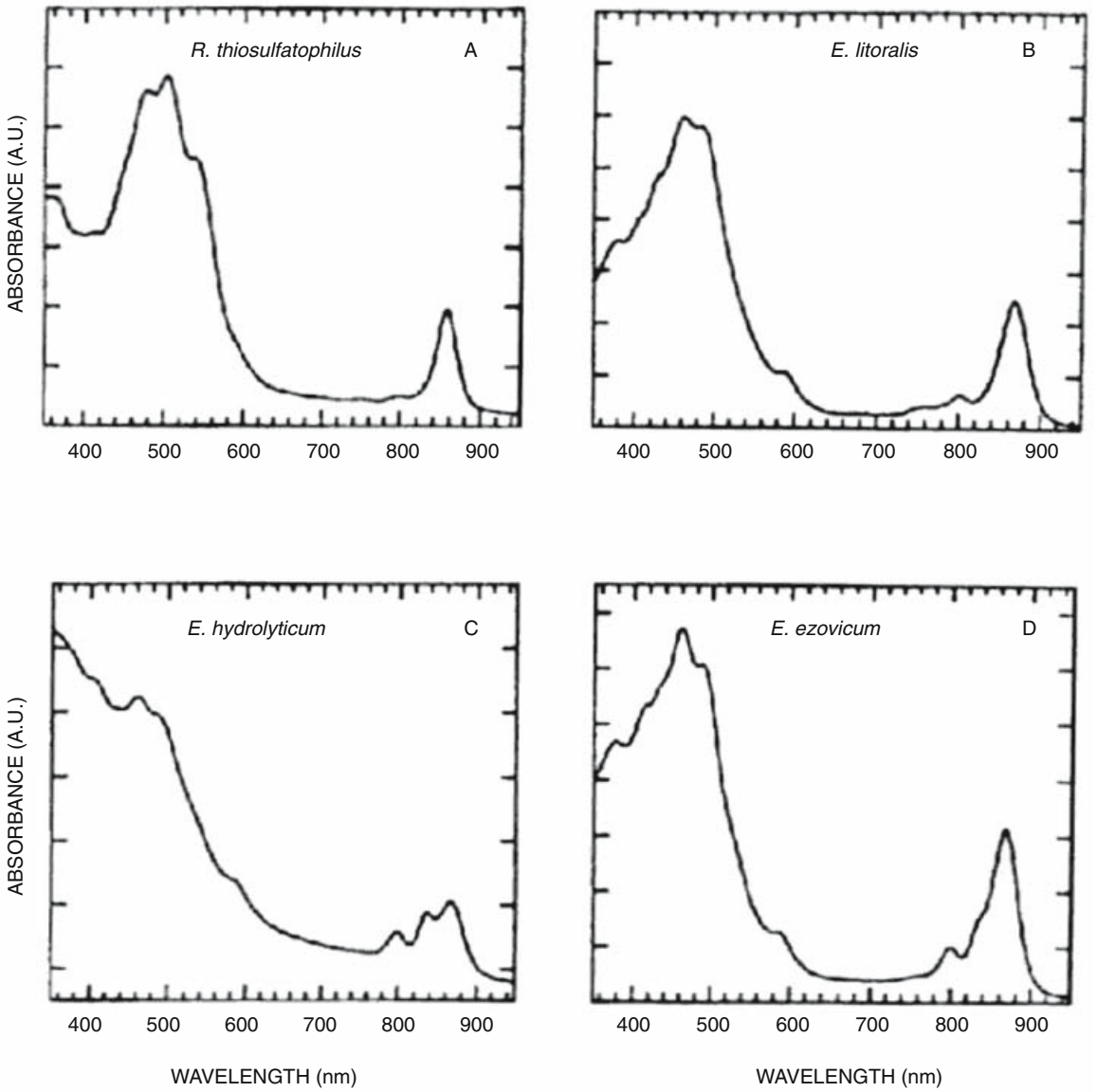

Fig. 1. Absorption spectra of crude membranes isolated from A) *R. thiosulfatophilus*, B) *E. litoralis*, C) *E. hydrolyticum* and D) *E. ezovicum*. Absorption peaks of the bacteriochlorophyll *a* are in the infrared region of 800–870 nm. Absorption peaks of the carotenoids are in the blue and green regions (420 to 550 nm). A.U., absorbance units.

photosynthetic apparatus have been identified as members of the aerobic phototrophic genus *Roseobacter*. Two *Roseobacter* species, *R. algicola* (Lafay et al., 1995) and *R. gallaeciensis* (Ruiz-Ponte et al., 1998), that do not produce bacteriochlorophyll *a* and photosynthetic complexes have been classified as members of the genus *Roseobacter*. *R. algicola* was recently reclassified as *Ruegeria algicola* (Uchino et al., 1998). Another example is the proposal to transfer the obligately aerobic phototrophic *Erythromonas ursincola* to the nonphototrophic genus *Sphingomonas* (Yabuuchi et al., 1999). In all cases the general requirement of bacterial taxonomy has not been followed: If a major phenotypic difference exists between two strains (in this particular case, the presence of photosynthetic apparatus and pigments), they should be in different genera. The only purpose of bacterial taxonomy is to identify and differentiate between microbial genera and species. This differentiation should be based on markers and techniques that are as simple and clear as possible and that can be easily followed by those who do not study taxonomy (microbial physiologists, biochemists or medical microbiologists). Photosynthetic pigments are readily detectable (even visually) in bacterial cells, and therefore photosynthesis is an excellent distinguishing marker.

The major feature that distinguishes the obligately aerobic phototrophic strains from the facultatively anaerobic purple nonsulfur phototrophic strains is the inability of the former to grow photosynthetically under anaerobic conditions. Because this inability to grow anaerobically in the light despite the presence of photosynthetic pigments is of taxonomic significance and importance, the photosynthetic growth should be investigated thoroughly. Different photosynthetic growth conditions such as medium composition, carbon (organic and inorganic) sources, presence and absence of reduced sulfur compounds and light intensities need to be tested.

All species of aerobic phototrophic bacteria synthesize large amounts of carotenoid pigments, which determine the color of the organism (Table 1) and give peaks (Fig. 1) in the blue and green regions (420 to 550 nm) of their absorption spectra (Fuerst et al., 1993; Shiba, 1991a; Shiba and Simidu, 1982; Yurkov and Gorlenko, 1990b; Yurkov and Gorlenko, 1992a; Yurkov and Gorlenko, 1993a; Yurkov et al., 1992c; Yurkov et al., 1994b; Yurkov et al., 1997; Yurkov and van Gemerden, 1993b). The carotenoid composition is species specific, often indicating a large number of different carotenoids of unusual chemical structure (Takaichi et al., 1988; Takaichi et al., 1990; Takaichi et al., 1991a; Takaichi et al., 1991b; Yurkov et al., 1993b).

About 20 different carotenoids have been found in the red-orange colored cells of *Erythromicrobium ramosum* (Yurkov and Beatty, 1998a); the ten predominant ones were purified and structurally characterized (Yurkov et al., 1993d; Table 3) as C_{40} carotenoids and classified into four types: 1) bicyclic carotenoids (β-carotene and hydroxyl derivatives such as zeaxanthin, adonixanthin, caloxanthin and nostoxanthin); 2) the monocyclic carotenoid bacteriorubixanthinal; 3) the acyclic spirilloxanthin; and 4) the polar carotenoid erythroxanthin sulfate. A carotenoid composition similar to that of *E. ramosum* was described in *E. longus* (Takaichi et al., 1991a; Takaichi et al., 1991b; Takaichi et al., 1988; Takaichi et al., 1990) and *E. litoralis* (Yurkov et al., 1994b), with the exception of adonixanthin and 2,3,2′,3′-tetra-hydroxy-β,β,carotene-4-one. Zeaxanthin is a major carotenoid in *Erythrobacter*, whereas in *Erythromicrobium*, zeaxanthin is a minor component and bacteriorubixanthinal, erythroxanthin sulfate and 2,3,2′,3′-tetra hydroxy-β,β, carotene-4-one are the major components (Yurkov et al., 1993d).

Bicyclic carotenoids such as β-carotene and its hydroxyl derivatives were found in *Erythrobacter* and *Erythromicrobium* species, and the color of *Erythromonas* and *Sandaracinobacter* indicates that these bacteria also contain carotene carotenoids, which are rarely present in purple phototrophic bacteria (small amounts of β-carotene were detected in *Rhodomicrobium vannieli*; Britton et al., 1975; Patel et al., 1983). The highly polar carotenoid sulfates have hitherto been found exclusively among the carotenoids of the aerobic phototrophic bacteria (Takaichi et al., 1991b; Yurkov et al., 1993d; Yurkov et al., 1994b) and were recently described as carotenoids having novel structures (Takaichi et al., 1991b).

The carotenoid composition of two other *Erythromicrobium* representatives, *E. ezovicum* and *E. hydrolyticum*, has not yet been analyzed in detail. Nevertheless, in vivo absorption spectra revealed carotenoid absorption peaks at 466 and 478 nm, as in *E. ramosum*, indicating a similar carotenoid composition in these species, which is also apparent in the color of liquid cultures (intensely red-orange) (Yurkov et al., 1997; Table 1).

The carotenoid composition of the pink-red colored *Roseococcus* species is not as rich as of *Erythrobacter* and *Erythromicrobium* species, but nevertheless it is unusual. *Roseococcus thiosulfatophilus* contains mainly two very polar red pigments, C_{30} carotene-dioate (4,4′-diapocarotene-4,4′-dioate) and the respective diglucosyl ester (di[β-D-glucopyranosyl]-4,4′-diapocarotene-4,4′-dioate). Together they contribute 95% of the total carotenoid content (Yurkov et al., 1993d). Such highly polar C_{30} carotenoid glyco-

Table 3. Carotenoids in *Erythromicrobium ramosum* (in order of polarity).

N	Carotenoid	Common name	Chemical formula
1	β,β-Carotene	*β-carotene*	$C_{40}H_{56}$
2	1,1′Dimethoxy-3,4,3′4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene	*Spirilloxanthin*	$C_{42}H_{60}O_2$
3	3-Hydroxy-1′-methoxy-3′,4′-didehydro-1′,2′-dehydro-β,ψ-caroten-19′-al	*Bacteriorubixan-thinal*	$C_{41}H_{56}O_3$
4	3,3′-Dihydroxy-β,β-carotene	*Zeaxanthin*	$C_{40}H_{56}O_2$
5	3,3′-Dihydroxy-β,β-carotene-4-one	*Adonixanthin*	$C_{40}H_{54}O_3$
6	2,3,3′-Trihydroxy-β,β-carotene	*Caloxanthin*	$C_{40}H_{56}O_3$
7	3,2′,3′-Trihydroxy-β,β-carotene-4-one (probable structure)	None	$C_{40}H_{54}O_4$
8	2,3,2′,3′-Tetrahydroxy-β,β-carotene	*Nostoxanthin*	$C_{40}H_{56}O_4$
9	2,3,2′,3′-Tetrahydroxy-β,β-carotene-4-one	None	$C_{40}H_{54}O_5$
10	3,2′,3′-Trihydroxy-β,β-carotene-4-one-3-sulfate	*Erythroxanthin-sulfate*	$C_{40}H_{54}O_7S$

sides have never before been observed in purple phototrophic bacteria, although the same carotenoid and its diglucosylated form had been postulated to exist in *Methylobacterium rhodinum* (formerly *Pseudomonas rhodos*; Kleinig et al., 1979).

The most abundant carotenoid detected in *Roseobacter* sp. is spheroidenone, which is the major carotenoid of anaerobic purple bacteria such as *Rhodobacter* species (Harashima and Nakada, 1983; Shiba, 1991a; Takaichi et al., 1991a).

The only carotenoid of *Acidiphilium rubrum* is spirilloxanthin, which is found in several purple phototrophic bacteria (Wakao et al., 1996).

Typically, cells of aerobic phototrophic bacteria contain small amounts of bacteriochlorophyll relative to the abundance of carotenoids (in contrast to anaerobic purple phototrophic bacteria). For example, the anaerobic phototrophic bacterium *Rhodobacter sphaeroides* may yield about 20 nmol of bacteriochlorophyll/mg dry weight of cells, whereas the bacteriochlorophyll content of obligately aerobic species was found to be: *Erythrobacter longus*, 2.0 nmol/mg dry weight of cells; *Sandaracinobacter sibiricus* and *Erythromicrobium hydrolyticum*, 1.0–4.0 nmol/mg of protein; *A. rubrum*, 0.7 nmol/mg dry weight of cells; *R. thiosulfatophilus*, 0.1–1.0 nmol/mg of protein. Therefore, the ratio of bacteriochlorophyll to carotenoid peaks in whole cells of aerobic phototrophic species is typically about 1 : 8 to 1 : 10 (Harashima et al., 1980; Shimada, 1995; Takemoto and Kao, 1977; Wakao et al., 1993; Yurkov, 1990a; Yurkov and Gorlenko, 1992a; Yurkov et al., 1992c; Yurkov et al., 1994b; Yurkov and van Gemerden, 1993c). With such a low number of photosynthetic units (reaction center + light harvesting system), the absence of an extensive intracytoplasmic system in obligately aerobic species is not surprising. An intracytoplasmic system was not detected in thin-section electron micrographs of *Erythrobacter*, *Erythromicrobium*, *Roseococcus*, *Porphyrobacter*, *Erythromonas* or *Citromicrobium* (Fuerst et al., 1993; Hanada et al., 1997; Yurkov and Beatty, 1998b; Yurkov and Gorlenko, 1992a; Yurkov and Gorlenko, 1993a; Yurkov et al., 1991b; Yurkov et al., 1994b; Yurkov et al., 1996; Yurkov et al., 1997; Yurkov et al., 1999). Occasionally, "chromatophore-like" vesicle structures have been observed in *Roseobacter denitrificans* (Harashima et al., 1982; Shimada, 1995). Thin-section electron micrographs of *S. sibiricus* (formerly *Erythrobacter sibiricum*) revealed rare vesicular or loop-like cytoplasmic invaginations (Yurkov et al., 1991b).

Natural Zn-containing bacteriochlorophyll *a* was discovered in the aerobic acidophilic bacterium *Acidiphilium rubrum* (Wakao et al., 1996). This Zn-containing bacteriochlorophyll (Bchl) *a* is esterified with phytol (Zn-Bchl a_p). Chemical analysis of *A. rubrum* cell extracts yielded a 13 : 2 : 1 molar ratio of Zn-Bchl : Mg-Bchl : bacteriopheophytin, and most of these pigments were determined to be photochemically active (Wakao et al., 1996). Bacteriochlorophyll *a* purified from *E. longus* and *R. denitrificans* was found to be Bchl a_p, which contains phytol as the esterifying alcohol, the most common bacteriochlorophyll *a* form found in purple phototrophic bacteria (Harashima et al., 1980; Harashima and Takamiya, 1989; Kondratieva et al., 1989; Kuntzler and Pfennig, 1973; Wakao et al., 1993). No bacteriochlorophyll *a* esterified to geranylgeraniol, as in the anaerobic phototrophic bacterium *Rhodospirillum rubrum* (Brockmann et al., 1973), has been detected in aerobic phototrophic species. However, the ester moiety of bacteriochlorophyll from most species of *Erythromicrobium*, *Erythrobacter*, *Roseococcus*, *Sandaracinobacter*, *Erythromonas* and other recently described genera has not yet been determined.

Important information can be further obtained from the size and consistency of the colonies on agar plates and microscopic examinations. Shape of the cells, cell width and length, motility and type of flagellation, mode of division, aggregates and capsule formation are important properties that can be obtained from both phase contrast light and electron microscopies. Cell ultrastructure should be observed in ultra thin sections under the electron microscope. The morphology and ultra structure of some representative species of the aerobic phototrophs are shown in Fig. 2 and 3.

DNA-DNA hybridization is used to distinguish between species that highly resemble each other (Yurkov et al., 1991a). Also G+C content of DNA has to be determined to describe a new species. Substrate utilization, vitamin requirement, antibiotic resistance and some other significant physiological properties are required for precise identification (Table 2). Recently a number of additional chemotaxonomic markers (such as quinones, soluble and membrane-bound cytochromes, fatty acids, polar lipids, light-harvesting and reaction center complexes) also have been employed. A summary of information available for species of the aerobic phototrophic bacteria currently described is presented in Tables 1, 2 and 4.

Modern achievements in phylogenetic research based on 16S rDNA sequence analysis used to complement modern approaches in bacterial taxonomy are considered valuable in the phylogenetic assessment of aerobic phototrophic species.

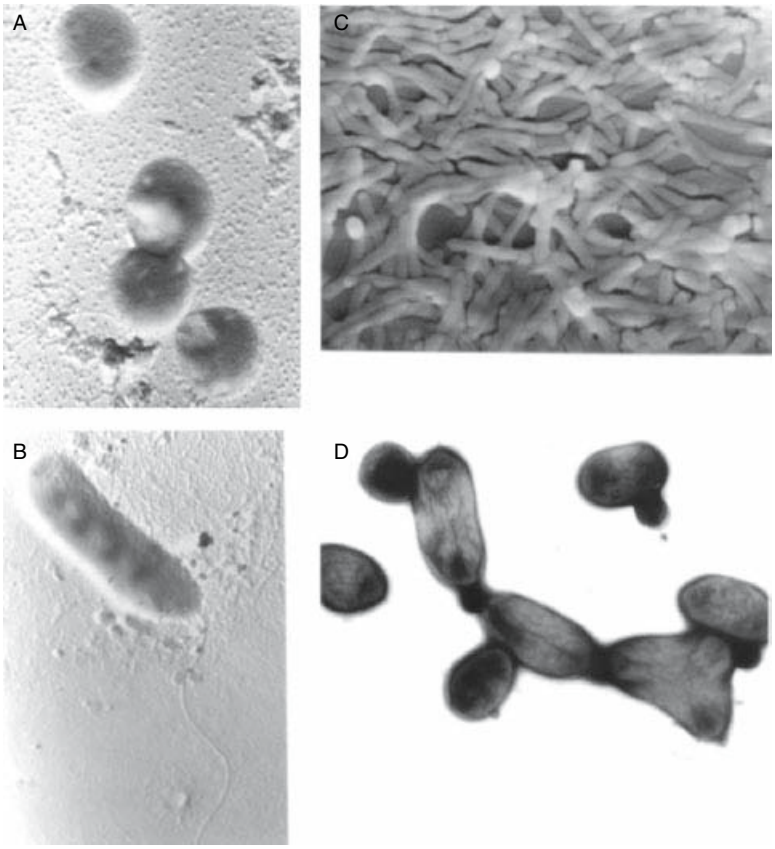

Fig. 2. Elecron micrographs showing the morphological diversity of aerobic phototrophic bacteria. Scanning electron micrographs of carbon shadowed A) coccus cells of *R. thiosulfatophilus*, B) cell of *E. ezovicum* containing a single flagellum, C) *S. sibiricus* long cells showing their distribution in a microcolony. D) Transmitting electron micrograph of negatively stained, highly pleomorphic *C. bathyomarinum*.

Preservation

It has been found that liquid (taken from late logarithmic growth phase) and agar surface cultures of most aerobic phototrophic species remain viable after storage at 4°C for at least two months (Yurkov and Beatty, 1998a). Long-term preservation is possible by storage in liquid nitrogen or freezing at –70°C. For this purpose, dense cell suspensions of liquid cultures (mid-logarithmic growth phase) are supplemented with glycerol (30%) as a cryoprotective agent. Lyophilization also can be used as a method of preservation.

Physiology

Photosynthetic Potential

Although the aerobic phototrophic bacteria and the anaerobic purple phototrophic bacteria have similar photosynthetic apparatus, electron transfer carriers (Garcia et al., 1994; Okamura et al., 1985; Okamura et al., 1986; Yurkov et al., 1994a; Yurkov et al., 1995; Table 4), reaction center and light-harvesting I polypeptides (Liebetanz et al., 1991), efficient photoinduced electron transfer is operative only under aerobic conditions in the aerobic phototrophic bacteria (Garcia et al., 1994; Okamura et al., 1985; Yurkov et al., 1995).

The photochemical activity of the aerobic bacterial photosynthetic apparatus has been analyzed independently in several laboratories using different species and techniques (Garcia et al., 1994; Hanada et al., 1997; Okamura et al., 1984; Okamura et al., 1985; Takamiya et al., 1988; Wakao et al., 1996; Yurkov et al., 1995). The results indicate that the photosynthetic apparatus of aerobic phototrophic bacteria, although it has some peculiarities, is functional in terms of a cyclic electron transfer system.

In species of the genera *Erythrobacter*, *Roseobacter*, *Roseococcus*, *Erythromicrobium*, *Erythromonas* and *Sandaracinobacter*, photoinduced cyclic electron transfer occurs only under relatively oxidized (aerobic) conditions (Garcia et al., 1994; Okamura et al., 1985; Yurkov et al., 1995). Under relatively reduced (anaerobic) conditions, no light-induced reaction center absorbance changes were observed. The lack of photochemistry under anaerobic conditions is consistent with the inability of these bacteria to grow by light-dependent photophosphorylation in the absence of oxygen (Okamura et al., 1986; Shiba, 1991a; Shiba and Harashima, 1986; Shiba

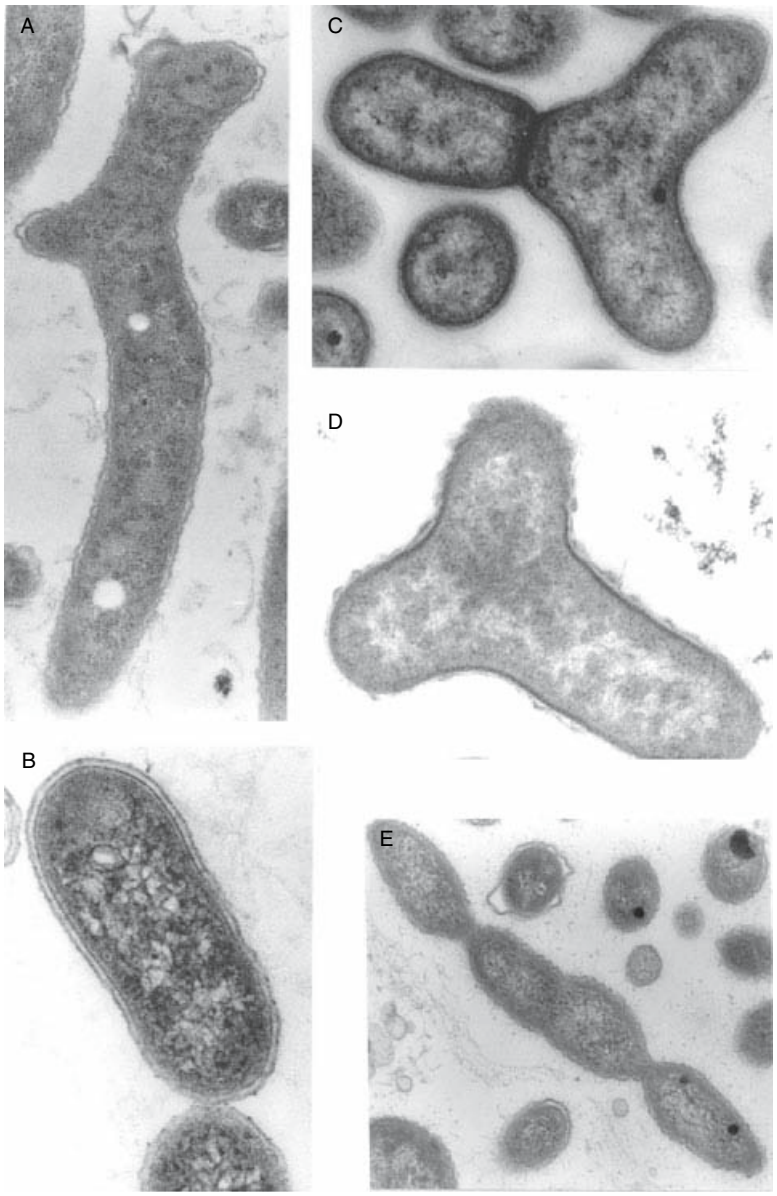

Fig. 3. Electron microscopy of thin sections of aerobic phototrophic species. A) Gram-negative branching thread-like cell of *E. ramosum*. B) Typical Gram-negative cell wall organization in *E. litoralis*. C) A later stage of Y-cell division in *C. bathyomarinum*. One daughter cell is separated by the cell wall from two as-yet-unseparated nascent cells. D) *C. bathyomarinum*, Y-cell presumably preceding division to form three daughter cells. The nucleoid is seen as light zones of the section, distributed in three directions. E) *E. ezovicum* dividing by constrictions.

and Simidu, 1982; Yurkov and Gorlenko, 1990b; Yurkov and Gorlenko, 1992a; Yurkov and Gorlenko, 1993a; Yurkov et al., 1992c; Yurkov et al., 1994b; Yurkov et al., 1997). It was demonstrated that the photosynthetic electron transfer system of *R. denitrificans*, *E. litoralis*, *E. ramosum*, *E. ursincola*, *S. sibiricus* and *R. thiosulfatophilus* is inoperative in anaerobic cells, presumably because of the high mid-point potential (E_m) of the reaction center primary acceptor Q_A. The values of Q_A mid-point potential determined at pH 7.8 were +150, +80, +25 and +5 mV for *E. litoralis*, *E. ramosum*, *E. ursincola* and *S. sibiricus*, respectively. These are much higher values than determined for anaerobic phototrophic bacteria, such as *R. sphaeroides*, *R. rubrum*, *R. tenuis* and *R. viridis*, which yield negative Eh (ambient redox potential) values. The Em values of the primary electron acceptor measured above the pK (pH value above which the midpoint potential of an electron carrier is not affected by pH) for *E. litoralis*, *R. denitrificans*, *S. sibiricus* and *E. ursincola* are equal to –30, –44, –55 and –85 mV, respectively. These values are 65 to 120 mV more positive than those observed in anaerobic phototrophic bacteria (Yurkov and Beatty, 1998a). Therefore, it is likely that the Q_A of aerobic phototrophic bacteria is in the reduced state (i.e., dihydroquinol) under anaerobic conditions and acceptance of an electron from the special pair cannot occur unless an oxidant such as O_2 is provided to maintain Q_A in the quinone form, which is capable of acting as an electron acceptor.

Table 4. Comparative data[a] on the photosynthetic apparatus and electron carriers.

Species	RC	Absorption peaks (nm)		RC-bound cyt *c*, M.W. (kDa)	Number and M.W. (kDa) of soluble cyt *c*	Number and M.W. (kDa) of membrane bound cyt *c*	Ubiquinones (mmol/g dry cells)	
		LHI	LHII				Q_9	Q_{10}
Sandaracinobacter sibiricus	+	867	absent	37.0	1 (14.0)	2 (30.0; 37.0)	0.06	0.71
Erythromonas ursincola	+	867	absent	40.0	3 (6.5; 9.0; 14.0)	4 (14.3; 21.0; 24.0; 40.0)	n.d.	0.11
Roseococcus thiosulfatophilus	+	856	absent	44.0	2 (4.0; 6.5)	4 (21.5; 23.0; 26.0; 44.0)	n.a.	n.a.
Roseobacter denitrificans	+	870	806	42.0	2 (13.5; 14.5)	n.a.	n.d.	+
Erythromicrobium ramosum	+	868	798; 832	absent	2 (8.0; 14.3)	3 (8.0; 26.0; 30.0)	0.09	0.19^M
Erythromicrobium ezovicum	+	868	800; 832	absent	2 (8.0; 14.3)	2 (30.0; 34.0)	0.02	0.3
Erythromicrobium hydrolyticum	+	866	799; 833	absent	1 (14.3)	2 (21.0; 30.0)	0.02	0.01^M
Erythrobacter litoralis	+	868	absent	absent	4 (14.0; 21.5; 24.0; 26.0)	2 (30.0; 35.0)	n.a.	n.a.
Erythrobacter longus	+	870	absent	absent	2 (12.5; 17.0)	n.a.	n.d.	+
Porphyrobacter neustonensis	+	869	absent	absent	1 (n.a.)	n.a.	n.d.	+
Porphyrobacter tepidarius	+	870	absent	absent	1 (n.a.)	n.a.	n.d.	+

Symbols: + means present; and ^{M}, in addition to ubiquinone Q_{10} the methylated form was revealed.

Abbreviations: RC, reaction center; LHI and II, light-harvesting complex I and II; n.d., not detected; and n.a., data are not available.

[a]Data presented for investigated species.

[b]Cultivated under dark-aerobic conditions.

Membranes of aerobic phototrophic bacteria are highly enriched in carotenoids. For example, the molar ratio of bacteriochlorophyll to carotenoid in membranes of *R. thiosulfatophilus* and *E. ramosum* is 1 : 4 and 1 : 7, respectively. However in purified photosynthetic pigment-protein complexes this ratio decreased to 1:1.4 in an enriched reaction center-light harvesting I (RC-LHI) core complex of *R. thiosulfatophilus*, to 1:(0.1–1) in the purified RC-LHI, and to 1:(0.3–1) in the purified LHII complex of *E. ramosum* (Yurkov et al., 1993d; Yurkov et al., 1994a). The RC-LHI core complex of *R. thiosulfatophilus* contained only the C_{30} carotenoid diglucosyl ester (di[β-D-glucopyranosyl]-4,4′-diapocarotene-4,4′-dioate).

Bacteriorubixanthinal is the major carotenoid in the pigment-protein complexes (LH and RC) of *E. ramosum*, along with small amounts of spirilloxanthin (RC-LHI) and zeaxanthin (LHII; Yurkov et al., 1993d).

The quantum yields of singlet energy transfer between carotenoids and bacteriochlorophyll (a light-harvesting function) calculated from comparison of absorption and fluorescence excitation spectra indicate that the majority of the carotenoids in the membrane of *E. ramosum* and *R. thiosulfatophilus* does not contribute to the light-harvesting function (Yurkov et al., 1994a). The function of such large amounts of carotenoids in these cells is unclear. Carotenoids could play a role in scavenging singlet oxygen or free radicals or both, a process observed for several carotenoids in organic solvents (Krinsky, 1979; Krinsky, 1989; Oliveros et al., 1992), or perhaps a role in screening cells from high intensities of blue light.

Cells of *E. longus* are also rich in such photosynthetically uncoupled carotenoids, as more than 70% of the total carotenoids present do not function as light-harvesting pigments (Noguchi et al., 1992).

The effect of light on pigment synthesis in both the aerobic phototrophic and the anaerobic phototrophic bacteria is roughly qualitatively similar, but there are quantitative differences. In common is the light stimulation of growth and inhibition of aerobic respiration, suggesting the operation of a photosynthetic electron transport system that shares components with a respiratory system in both groups (Harashima et al., 1987; Harashima et al., 1982). Differences are described below. In anaerobic phototrophic bacteria high light intensities repress the synthesis of bacteriochlorophyll, whereas moderate and low light intensities stimulate maximal bacteriochlorophyll synthesis (Bauer et al., 1993). In aerobic phototrophic bacteria the influence of light intensities as low as 20 $\mu E/m^2/s$ was found to be strongly inhibitory and abolished bacteriochlorophyll synthesis (Yurkov and van Gemerden, 1993c). Transient light stimulation of growth and complete inhibition of bacteriochlorophyll synthesis were demonstrated in batch culture experiments on *E. longus*, *R. denitrificans*, *E. hydrolyticum* and *S. sibiricus* (Harashima et al., 1982; Harashima et al., 1987; Liebetanz et al., 1991; Yurkov et al., 1993e; Yurkov and van Gemerden, 1993c). However, the clearest results were obtained using chemostat cultures of *E. hydrolyticum* (Yurkov and van Gemerden, 1993c).

The photosynthetic activity of aerobic phototrophic bacteria might seem to be negligible. However, under aerobic alternating light and dark conditions the photosynthetic electron transfer chain might contribute significantly to an increase of the proton gradient and thereby help to maintain an electrochemical potential in the cytoplasmic membranes. This increase could be used for ATP production or for transport of substrates. For example, it is thought that cells spend a large portion of their total energy budget for active transport (Madigan et al., 1997). Therefore, the use of light energy to enhance substrate transport could increase the efficiency of organic substrate consumption for biosynthesis. Such speculations are supported by results obtained with continuous cultures of *E. hydrolyticum* (Yurkov and van Gemerden, 1993c). Thus the simultaneous operation of photosynthesis with heterotrophic respiration may be of advantage in competition with heterotrophs that lack photopigments.

Oxygen Dependence and Heterotrophic Metabolism

Whereas (of the aerobic phototrophic bacteria) only *R. denitrificans* is capable of anaerobic denitrification, and can grow anaerobically with trimethylamine N-oxide (TMAO) as an electron acceptor (Arata et al., 1988; Shioi et al., 1988), the survival of most aerobic phototrophic bacteria seems to depend on the presence of O_2. For example, O_2 stimulates heterotrophic consumption of organic substrates, activates the growth rate and increases other metabolic activities (e.g. thiosulfate oxidation as an additional source of the energy that results in ATP generation in *E. hydrolyticum* and *R. thiosulfatophilus*; Yurkov et al., 1994b). This dependence on O_2 for growth, and thus the synthesis of bacteriochlorophyll and carotenoids, is contrary to the behavior of most species of anaerobic phototrophic bacteria. At present, only the purple nonsulfur species *R. centenum* and *R. sulfidophilum* are known to synthesize a functionally significant photosynthetic apparatus when O_2 concentration is high (Hansen and Veldkamp, 1973; Yildiz et al., 1991). In con-

trast, photophosphorylation and photosynthetic ATP synthesis in cells of *R. denitrificans* were reported to occur only under aerobic conditions. A decrease in O_2 concentration resulted in a decrease in ATP production, regardless of the presence of light (Okamura et al., 1986). Light-induced electron transport through cytochromes and the reaction center (RC) requires active aerobiosis in membrane preparations and intact cells of aerobic phototrophic bacteria (Garcia et al., 1994; Harashima and Takamiya, 1989; Yurkov et al., 1995). Moreover, it seems likely that due to the high mid-point potential of the RC primary acceptor Q_A, oxygen is necessary for aerobic phototrophic bacterial photosynthesis. Thus, these bacteria may have evolved to become aerobes that have retained photosynthesis as an accessory metabolic process that enhances growth under conditions of alternating exposure to light.

Most of the aerobic phototrophic bacteria can oxidize a great diversity of organic carbon sources to support chemotrophic growth (Table 2). Accordingly, media that contain a complex composition of organic sources of carbon such as yeast extract, peptone, casein hydrolysate, potato broth or soytone result in the highest growth yields (Fuerst et al., 1993; Hanada et al., 1997; Shiba, 1991a; Shiba and Harashima, 1986; Shiba and Simidu, 1982; Yurkov and Gorlenko, 1990b; Yurkov and Gorlenko, 1992a; Yurkov and Gorlenko, 1993a; Yurkov et al., 1992c). Most species can metabolize sugars, tricarboxylic acids, fatty acids and amino acids. Some species of *Erythromicrobium* use ethanol in low concentrations as the sole carbon source (Yurkov and Gorlenko, 1993a; Yurkov et al., 1994b). A lipolytic activity was established for many species on the basis of the ability to hydrolyze Tweens (Shiba, 1991a; Shiba and Simidu, 1982; Yurkov and Gorlenko, 1990b; Yurkov and Gorlenko, 1993a; Yurkov et al., 1994b), and some species hydrolyze gelatin or starch (Shiba and Simidu, 1982; Yurkov and Gorlenko, 1993a; Table 2).

The active growth of most aerobic phototrophic species in complex media containing high concentrations of organic compounds (Fuerst et al., 1993; Shiba, 1991a; Shiba and Simidu, 1982; Yurkov and Gorlenko, 1990b; Yurkov and Gorlenko, 1992a; Yurkov and Gorlenko, 1993b; Yurkov et al., 1992b; Yurkov and van Gemerden, 1993b) is in keeping with the high organic matter content of the eutrophic environments from which they were isolated (Fuerst et al., 1993; Shiba et al., 1979; Shiba et al., 1991b; Yurkov, 1990a; Yurkov et al., 1992b; Yurkov and van Gemerden, 1993b). The main exception is *Acidiphilium*, which grows best with relatively low concentration of nutrients (Wakao et al., 1993).

The aerobic phototrophic *Citromicrobium* isolated recently from the presumably oligotrophic environment of "black smoker" plume waters grows on an unusually low (for this physiological group) number of substrates. Glutamate, butyrate and yeast extract are the best carbon sources for *Citromicrobium*, and acetate and glucose support weak growth (Yurkov et al., 1999).

Like the purple nonsulfur bacteria, most species of the aerobic phototrophic bacteria have a highly versatile heterotrophic carbon metabolism. However, these two bacterial groups have different autotrophic growth and CO_2 fixation capabilities. None of the obligately aerobic species has yet been shown to grow autotrophically, and the key enzyme of Calvin cycle (ribulose-bisphosphate carboxylase; RubisCO) has not been found in any species (Shimada, 1995; Yurkov, 1990a; Yurkov and Gorlenko, 1992a; Yurkov and Gorlenko, 1993a; Yurkov et al., 1994b). Experiments on *S. sibiricus* using radiolabeled $^{14}CO_2$ indicated a low level of CO_2 fixation (0.4%) in both illuminated and dark conditions, consistent with heterotrophic CO_2 fixation (Brock et al., 1994; Gusev and Mineeva, 1985) attributed to the enzyme phosphoenolpyruvate (PEP) carboxylase (Yurkov, 1990a). Nonetheless, light stimulation of CO_2 uptake was detected for several species: *E. longus* (Yurkov, 1990a), *R. denitrificans* (Shiba and Harashima, 1986), *S. sibiricus* (Yurkov, 1990a) and *A. rubrum* (Kishimoto et al., 1995). This CO_2 fixation, too low to maintain autotrophic growth, could provide additional organic carbon intermediates for an otherwise essentially heterotrophic metabolism. This view is congruent with the observation that light-stimulated CO_2 uptake occurs in concert with light-activated consumption of acetate and stimulation of growth in chemostat cultures of *E. hydrolyticum* (Yurkov and van Gemerden, 1993c).

All aerobic phototrophic bacteria are able to utilize one or more of the sugars fructose, glucose or sucrose (Table 2). In some cases the ability or inability to use fructose is a specific characteristic of the species. For example, *R. thiosulfatophilus* grows on glucose as the sole carbon source, whereas fructose does not support growth (Yurkov and Gorlenko, 1992a; Yurkov et al., 1994b).

The catabolism of glucose by aerobic phototrophic bacteria was studied by analysis of enzyme activities for two species, *S. sibiricus*, strain RB16-17 (formerly *E. sibiricum*), and *E. longus*, strain OCh101 (Yurkov et al., 1992e). Both species possess glucose-6-phosphate dehydrogenase and 2-keto-3-deoxygluconate-aldolase. The high activity of these two enzymes indicates that glucose utilization is mainly via the Entner-Doudoroff pathway (Madigan et al.,

1997). Low activity of the key enzyme of the Embden-Meyerhof pathway, fructose-diphosphate aldolase, was detected and it was concluded that this enzyme functions in biosynthesis (Yurkov et al., 1992e). No 6-phosphogluconate dehydrogenase was detected in *S. sibiricus*, whereas *E. longus* cells contained trace amounts of 6-phosphogluconate dehydrogenase, suggesting that the pentose monophosphate pathway exists in *E. longus* (Yurkov et al., 1992e).

Aerobic phototrophic bacteria accumulate polysaccharides (e.g., glycogen), polyhydroxyalkoanates and/or polyphosphates (Fig. 4), depending on the growth conditions (Yurkov and Gorlenko, 1992a; Yurkov and Gorlenko, 1993a; Yurkov et al., 1991b; Yurkov et al., 1992b; Yurkov et al., 1994b). Cells of *S. sibiricus* accumulated heavily osmium-stained granules of presumably polyphosphates under nearly all experimental conditions studied: in the light and the dark, with high or low aeration, and growth with acetate, butyrate or sucrose as the sole organic carbon source (Yurkov et al., 1991b). The highest amount of polyphosphate was accumulated in a growth medium supplemented with sucrose. Under such conditions polyphosphate granules occupied about 30–40% of the total cell volume (Fig. 4).

Electron-transparent granules presumed to be polyhydroxyalkoanates (polyhydroxybutyrate, PHB) were found in several species of the genera *Roseococcus*, *Erythromicrobium* and *Sandaracinobacter* (Yurkov, 1990a; Yurkov and Gorlenko, 1992a; Yurkov and Gorlenko, 1993a; Yurkov et al., 1991b; Yurkov et al., 1992c), with *S. sibiricus*, strain RB16-17, as the greatest PHB accumulator. The PHB was formed when cells were grown in media unbalanced for nitrogen (urea as a nitrogen source), as well as during incubation in a medium lacking fixed nitrogen. Replacement of ammonia with nitrate as the source of nitrogen also resulted in pronounced formation of PHB granules. Under these conditions, large PHB granules occupied about 40–50% of the total cell volume, to the extreme of causing cell deformation (Fig. 4). Although storage compounds have been revealed in many aerobic phototrophic bacteria, little is known about the conditions that promote accumulation or consumption of these cytoplasmic inclusions.

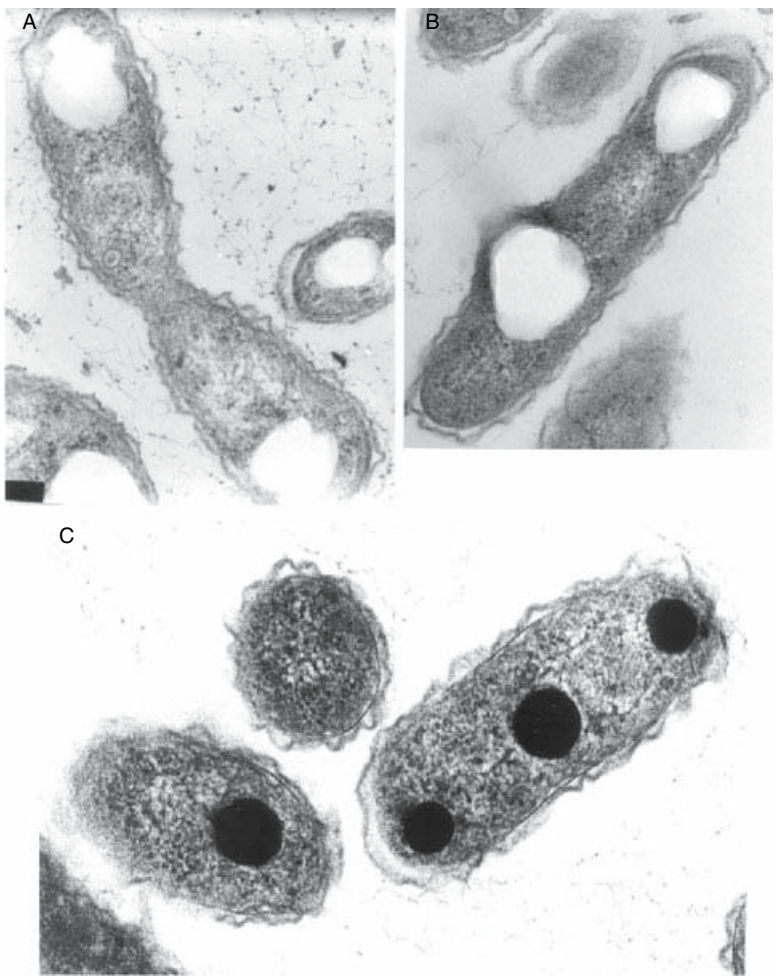

Fig. 4. Intracytoplasmic compounds of *S. sibiricus* revealed by electron microscopy of ultrathin sections. A) and B) Large electron-clear granules of presumed polyhydroxybutyrate (PHB) often occupied up to 40–50% of the total cell volume, deforming the cell shape. C) Electron-dense granules of presumed polyphosphate occupied up to 30–40% of the total cell volume.

Six species, *R. thiosulfatophilus*, *E. ramosum*, *E. hydrolyticum*, *E. ezovicum*, *E. ursincola* and *S. sibiricus* (formerly *E. sibiricum*), were analyzed for the use of sulfide or thiosulfate for growth with these compounds as the sole sulfur source in a defined medium containing acetate as the sole source of carbon. None of these species oxidized sulfide, whereas thiosulfate-oxidizing activity was detected for *E. hydrolyticum*, strain E4(1), and *R. thiosulfatophilus*, strain RB-7. Utilization of thiosulfate by both species was dependent on the presence of an organic carbon substrate and aeration. An increase in aeration increased the growth rate, which in turn provoked faster thiosulfate oxidation and the accumulation of the oxidized sulfur products, tetrathionate or sulfate, in the growth medium of *E. hydrolyticum* or *R. thiosulfatophilus*, respectively (Yurkov et al., 1994b).

Seven species of aerobic phototrophic bacteria (*E. litoralis*, *R. thiosulfatophilus*, *E. ramosum*, *S. sibiricus*, *E. ursincola*, *E. ezovicum* and *E. hydrolyticum*) were recently found to possess a high level of resistance (HLR) to tellurite and the ability to reduce TeO_3^{2-} to metallic tellurium (Yurkov et al., 1996; Table 5 and Fig. 5). The minimal inhibitory concentrations (MIC) of tellurite for aerobic phototrophic bacteria were found to be significantly higher than those determined for other representatives of the α-subclass of the *Proteobacteria*. The highest MIC of tellurite described for *R. capsulatus* and *R. sphaeroides* were 800 and 900 mg/ml, respectively (Moore and Kaplan, 1992; Moore and Kaplan, 1994), whereas MIC values of 2300, 2500 and 2700 mg/ml were found for the aerobic *E. ramosum*, *E. hydrolyticum* and *E. ursincola*, respectively (Yurkov et al., 1996). This reduction of tellurite to the relatively inert metallic tellurium, with accumulation of tellurium as intracellular deposits, seems to be one way bacteria detoxify tellurite (Lloyd-Jones et al., 1991; Moore and Kaplan, 1992; Taylor et al., 1988). However, HLR without metal accumulation was observed for *R. thiosulfatophilus* grown with L-glutamine, succinate, malate, tartrate or acetate and for *E. ezovicum* grown with acetate as the organic carbon source (Table 5). These results imply that tellurium accumulation is not essential to confer TeO_3^{2-} resistance and that another mechanism such as continuous tellurite efflux, complexing or methylation could specify this resistance.

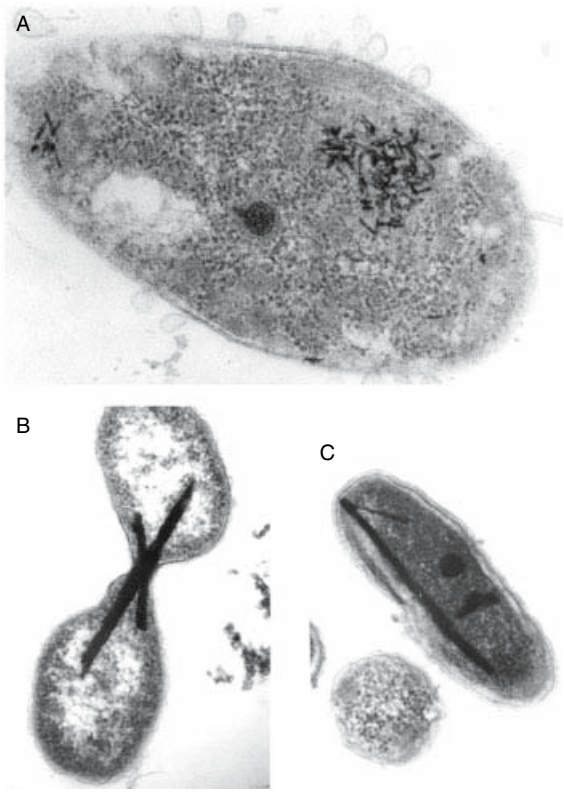

Fig. 5. Electron microscopy of ultrathin sections showing the intracellular deposits of tellurium as a product of tellurite reduction. A) *R. thiosulfatophilus*, relatively small Te crystals similar to Te deposits found in *E. coli* or *Rhodobacter* species. B) Two daughter cells of *E. ramosum* with tellurium crystals apparently interfering with cell division. C) *E. ursincola* accumulates long tellurium crystals.

Genetics

The study of the genetics of aerobic phototrophic bacteria is in its infancy, although the *puf* operon of *R. denitrificans* was cloned and sequenced and showed high sequence similarity (up to 70%) with *Rhodobacter* species (Liebetanz et al., 1991). Recently the *puf* operon of *R. denitrificans* was expressed in a *R. capsulatus puf puc* double-deletion mutant (Kortluke et al., 1997). The strongest expression of the *R. denitrificans puf* operon was observed under the control of the *R. capsulatus puf* promoter, in the presence of *R. capsulatus pufQ* and *pufX* genes, and in the absence of the *R. denitrificans pufC*. Reaction center charge separation and recombination between the primary donor P^+ and the primary acceptor Q_A- were observed in this strain, showing that the reaction center was correctly assembled. However, this strain did not grow photosynthetically under anaerobic conditions. The authors explained the absence of electron transport to Q_B under anaerobic conditions by the presence of M and L subunits from *R. denitrificans* and the H subunit from *R. capsulatus*, which did not interact so as to stabilize the Q_B site or allow electron transport from Q_A to Q_B (Kortluke et al., 1997). The uncertainties surrounding the photosynthetic metabolism of aerobic phototrophic bacteria will undoubtedly be

Table 5. Reduction of K_2TeO_3 by different species of aerobic photosynthetic bacteria: dependence on organic carbon source.

	Eb. litoralis			*E. hydrolyticum*			*E. ursincola*			*E. ramosum*			*E. sibiricum*			*R. thiosulfatophilus*			*E. ezovicum*		
C-source	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
R.O.	+	500	1200	+	500	1200	+	500	1000	+	50	750	+	50	750	+	100	500	+	n.r.	5
Yeast extract	+	250	1200	+	500	2000	+	500	2000	+	250	1500	+	500	1200	+	250	1000	+	n.r.	5
L-glutamine	+	500	1500	+	750	1200	+	1000	2000	+	250	1200	+	100	1000	+	n.r.	1200	+	1000	2000
Succinate	+	750	1200	+	750	1200	+	750	1200	+	500	1200	−			+	n.r.	1200	+	750	2000
Malate	−			+	750	1200	+	500	1200	+	250	1200	−			+	n.r.	1200	+	500	1200
Tartrate	−			±	n.r.	5	−			±	100	500	−			+	n.r.	1200	±	n.r.	5
Acetate	+	750	1200	+	1000	2500	+	250	2700	+	1000	2300	+	500	1200	+	n.r.	1200	+	n.r.	500
Ethanol	±	250	750	+	100	250	−			+	250	1000	−			−			+	n.r.	5

Symbols: +, good growth; ±, weak growth; and −, substrate cannot be utilized.
Abbreviations: R.O.; and n.r., not reduced.
[a]Column 1: Growth without tellurite addition.
[b]Column 2: Tellurite concentration (µg of K_2TeO_3/ml) supporting the highest rate and greatest extent of reduction.
[c]Column 3: Minimal inhibitory concentration (MIC in µg/ml).

clarified by additional molecular biological investigations.

Recently DNA fragments (approximately 3.8 kb) containing *puf* operons and a part of *bchZ* gene obtained from *A. rubrum* and *A. angustum* and fragments (1.5 kb) containing *pufL* and *pufM* of *A. criptum*, *A. multivorum* and *A. organovorum* were amplified and sequenced (Nagashima et al., 1997a). The *puf* operons of *A. rubrum* and *A. angustum* contained *puf B*, *-A*, *-L*, *-M* and *-C* as seen in other purple bacteria with an unknown gene directly upstream of *pufB*. Comparing the deduced amino acid sequences of the *puf* genes of the *Acidiphilium* species with those of other purple bacteria showed that His L168, which is highly conserved in other bacteria, is replaced by a glutamic acid in *Acidiphilium* species. The three-dimensional structures of the reaction centers of *Blastochloris viridis* and *Rhodobacter sphaeroides* suggest that this residue is located close to a special pair of bacteriochlorophylls and may be involved in the stabilization and function of Zn-containing bacteriochlorophyll *a* found in *Acidiphilium* (Nagashima et al., 1997a).

Ecology

In spite of the broad geographical distribution of aerobic phototrophic bacteria in different ecological niches and their presence in high numbers, the ecology and ecological importance of this group of organisms (their role in microbial populations) have not been studied.

Applications

Until now the major application of the aerobic phototrophic bacteria has been as an experimental subject in many areas of microbiology. However, recent discovery of the extremely high resistance to heavy metal oxides and of the ability to reduce these toxic compounds and accumulate them intracellularly in elemental form suggests that these organisms have potential industrial and bioremediation uses.

The maximum size and total amount of tellurium crystals (intracellular product of tellurite reduction) in most of the aerobic phototrophic bacteria were much greater than those observed in *E. coli*, *Pseudomonas* or *Rhodobacter* species (Lloyd-Jones et al., 1994; Moore and Kaplan, 1992; Moore and Kaplan, 1994; Suzina et al., 1995; Taylor et al., 1988; Yurkov et al., 1996). In some cells of *E. ramosum* and *E. litoralis*, the metal crystals occupied 20–30% of the cell volume. The only exception was *R. thiosulfatophilus*, which accumulated small metallic deposits, similar to *E. coli* or *R. sphaeroides* (Yurkov et al., 1996; Fig. 5).

Tellurium (especially of valence IV) is very toxic not only for microbes but also for other organisms, including humans. Therefore, the microbial reduction of soluble Te(IV) to solid Te(0) could be an important mechanism for the removal of this poison from polluted sites. In this context, the development of microbiological methods for environmental remediation of locales contaminated with tellurium oxides could utilize aerobic phototrophic bacteria, which are able to transform very high concentrations of Te(IV) to Te(0). Species of the genera *Erythromicrobium*, *Erythrobacter* and *Erythromonas* were found to be resistant not only to tellurite but also to tellurate, selenite, selenate, arsenate and vanadate (V. V. Yurkov, unpublished observations). Such multi-metal resistance may increase the importance of these bacteria as prospective candidates for the bioremediation of industrial waste waters polluted by a combination of several different heavy metal ions.

Microbial activities are used in the concentration of metals from natural ores and mining tailings with metal levels too low for smelting. Bioleaching by *Thiobacillus ferrooxidans* and related thiobacilli, for example, results in the recovery of up to 70% of the copper in low-grade ores. About 10% of copper in the United States comes from leaching ore through the activity of bacteria such as *Thiobacillus* and *Leptospirillum* species (Prescott et al., 1993). Therefore, tellurium accumulation by aerobic phototrophic bacteria could be useful in tellurium extraction from ores or for recycling of tellurium oxides. Native tellurium is uncommon (an abundance in the lithosphere of 2 parts per billion [ppb]), usually occurring in conjunction with elemental sulfur. Tellurium is present in minerals as tellurides of lead, copper, silver, gold and antimony (Bagnall, 1975). The extraction of tellurium is difficult because of the low content in natural ores, and tellurium compounds are usually obtained as by-products of metal refining processes (Klevay, 1976). Aerobic phototrophic bacteria, which are easy to cultivate and which accumulate high amounts of Te in cells, could be used in microbiological metallurgy for tellurium concentration.

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Prokaryotes (2006) 5:585–589
DOI: 10.1007/0-387-30745-1_24

CHAPTER 3.1.23

The Genus *Seliberia*

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Bacteria of the genus *Seliberia* are recognized by the presence of radial clusters (starlike aggregates) of rod-shaped bacteria, a characteristic screwlike twisting of the rod surface, and the formation of oval or spherical reproductive cells by a budding process—usually at the apical end of rods in a radially arranged aggregate or at a tip of an individual rod (Aristovskaya and Parinkina, 1963; Aristovskaya, 1964). The rods are 0.5–0.7 μm wide and their length varies with the composition of the nutrient medium used (Aristovskaya, 1974). The oval "reproductive" cells, which are found in soil isolates cultivated on appropriate medium, may also occur in the center of the star-shaped aggregates, and these "reproductive" cells have been observed to germinate into rods. A preponderance of oval cells or spherical cells is referred to as the zoogleal stage. The spherical or oval cells of *Seliberia stellata*, a soil isolate, and several seliberia-like helically sculptured rods of aquatic origin are not observed when isolated strains of these bacteria are cultivated on dilute peptone or other routine laboratory maintenance media, rather than on soil extract medium; the rods then divide by transverse asymmetric binary fission. The small, rod-shaped daughter cells, released from the aggregate, are motile by a subpolar flagellum and later may attach to a substrate by means of a polar holdfast. These bacteria are Gram negative. Dichotomous branching is occasionally observed (Aristovskaya and Parinkina, 1963; J. R. Swafford, unpublished observations). The ability to accumulate ferric hydroxide is characteristic.

Habitats

The type species, *Seliberia stellata*, was isolated from soil, specifically from humus-illuvial podzol of the Karelian isthmus (Aristovskaya and Parinkina, 1963), where it was found to be widely distributed. A second species, *S. carboxydohydrogena*, was obtained from an aerotank of a sewage treatment plant (Zavarzin and Nozhevnikova, 1977); since this organism is a carboxydobacterium, capable of oxidizing carbon monoxide, and is also a hydrogen-oxidizing bacterium, it is discussed elsewhere (this Handbook, Chapter 15; see also Meyer, Lalucat, and Schlegel, 1980).

A number of freshwater strains of *Seliberia* (species so far undesignated), assigned to the genus because of their distinctive morphology, have been obtained from ponds, lakes, and streams which are very low in nutrient content rather than eutrophic (Schmidt and Swafford, 1979). Such bacteria have not been found in samples from eutrophic ponds or lakes in our studies—either in transmission electron microscopic surveys of the microbial populations, which would permit their recognition due to their peculiar morphologic characteristics, or in isolation experiments from eutrophic water samples under conditions which should have permitted growth of seliberia-like organisms. Under similar isolation conditions using inocula from oligotrophic water samples and enrichments prepared from them, these bacteria of aquatic origin were readily cultivated and detected. Seliberia-like bacteria have also been observed in stored samples of laboratory distilled water, which are very poor in organic nutrient sources. *Seliberia stellata* and the aquatic seliberia-like bacteria appear to be strict oligotrophs, inhabiting environments where nutritive substances are available only in low concentrations (Aristovskaya and Parinkina, 1963; Schmidt and Swafford, 1979).

Isolation

The isolates assigned to the genus *Seliberia* are oligotrophic and grow slowly on culture media that are very low in nutrients; the final growth yield is sparse. They seem to grow not at all on the somewhat richer nutrient media widely used for the growth of saprophytes, such as nutrient agar (Difco), which contains about 0.8% total nutrient concentration. Soil isolates are able to accumulate ferric hydroxide (Aristovskaya and Parinkina, 1963). These bacteria can be grown as chemotrophic heterotrophs (subcategorized as oligotrophs); with regard to the facultative chemoautotrophs categorized morphologically as *Seliberia carboxydohydrogena*, principles of isolation applicable to carboxide bacteria should be used.

This chapter was taken unchanged from the second edition.

Enrichment

For the oligotrophic soil and water *Seliberia* spp., specific heterotrophic enrichment procedures, which would give a large majority or preponderance of this particular sort of bacterium, are lacking. Many kinds of heterotrophic bacteria will grow or subsist in the very dilute nutrient concentrations that would encourage the development of these helically sculptured bacteria.

Particular enrichment methods for podzol-inhabiting seliberias were not outlined by Aristovskaya and Parinkina (1963), although they mention that these bacteria occurred, in characteristic star-shaped clusters, in great profusion in pedoscopes. Their development in pedoscope samplings of humus-rich soils represents a useful enrichment approach to the seliberias, which could be used as an aid to their isolation. Isolation techniques using pedoscopes have been described by Perfil'ev and Gabe (1969).

To enrich (nonspecifically) for seliberia-like bacteria of aquatic origin, we have successfully used water samples with an oligotrophic character. The water samples of from 250 to 800 ml have been incubated at 24–26°C in glass beakers covered with aluminum foil or plastic film to retard evaporation with (1) no addition of nutrients or (2) addition of very low concentrations of peptone broth (final concentrations of 0.001–0.005% peptone [Difco]) for extended periods of time—a few weeks to several months. Seliberia-like bacteria can usually be found floating at the air-water interface (in the surface pellicle, if heavy microbial growth has occurred). The presence of a dense pellicle may cause difficulties in subsequent isolation attempts, since seliberias are slow-growing organisms, and may occur in the extreme minority in such surface samples. Since seliberias attach firmly (and nonspecifically) to a variety of substrates (each other, other microbes, glass surfaces, for example), one could exploit this characteristic in isolation attempts by inserting glass slides or cover slips (preferably near the surface) into the enrichment.

Isolation of Seliberias from Soil

Organomineral complexes derived from soil humus are suitable for the isolation and cultivation of seliberias (Aristovskaya and Parinkina, 1961, 1963). The preparations of these media contain some somewhat qualitative steps. Brown humic or ulmic acids are extracted from soil (peat-podzolic soil horizon) with 0.1 N NaOH and precipitated from the alkaline extract with HCl. Fulvic acids, which predominate in podzolic zone soils, are separated by filtration and the ulmic acid precipitate is then redissolved in 0.1 N NaOH. The alkaline solution is adjusted to pH 3.5, and a qualitative precipitation with ferric chloride (a few drops of ferric chloride solution per several ml of ulmic acid solution) is performed. The resulting gel is then washed until the filtrate no longer gives a positive test for chloride. The ulmic acid gel is ground with a mortar to a homogeneous consistency. The pH is adjusted to 6.5–7.0. Dilution of the homogenized gel in water to a suitable nutrient concentration for the cultivation of oligotrophs is done qualitatively (1 : 100, 1 : 500, and 1 : 1,000 dilutions serve as a tentative range of nutrient levels in the hunt for oligotrophs such as the *Seliberia* spp.). For solid media, 1.5–2.0% agar is used.

Fulvic acids, which occur in the alkaline filtrate, can be precipitated by adjusting the pH to about 6.0. Filtration, washing, and trituration precede medium preparation, using a procedure similar to the final steps for the ulmic acid medium (Aristovskaya, 1958; Aristovskaya and Parinkina, 1961).

Soil samples known to contain seliberia-like bacteria from preliminary capillary pedoscope observations are streaked on the ulmic or fulvic acid agar media. Slow-growing colonies, particularly organisms whose colonies develop in 4–7 days and show an accumulation of ferric hydroxide, are of interest. However, ferric hydroxide accumulation is mainly characteristic of seliberias when they are growing in mixed cultures. Pure cultures deposit ferric hydroxide when grown on mineral-rich medium in the presence of about 1% CO_2 (Aristovskaya and Parinkina, 1963).

Isolation of Aquatic Seliberias

Surface material from oligotrophic water samples, some of which were enriched with low concentrations of peptone (0.001–0.005%) and incubated at 24–26°C for 2 weeks to several months, is streaked on the following media: (A) 0.2% peptone (Difco), 0.1% yeast extract, 1% Hunter's vitamin-free mineral base (Cohen-Bazire, Sistrom, and Stanier, 1957), 1.2 or 1.5% agar (Difco), and distilled water; (B) 0.02% peptone, 0.01% yeast extract, 0.1% glucose (filter-sterilized), 1% Hutner's base, 1.2 or 1.5% agar (Difco), and distilled water. Incubation should be at 25 or 30°C for 2 or 3 weeks. Colonies growing up rapidly (during the first few days of incubation) should be marked and subsequently ignored, since the seliberias grow slowly. The colonies that appear later are transferred with a fine inoculating needle or a toothpick onto patch plates of medium A. Use of a dissecting

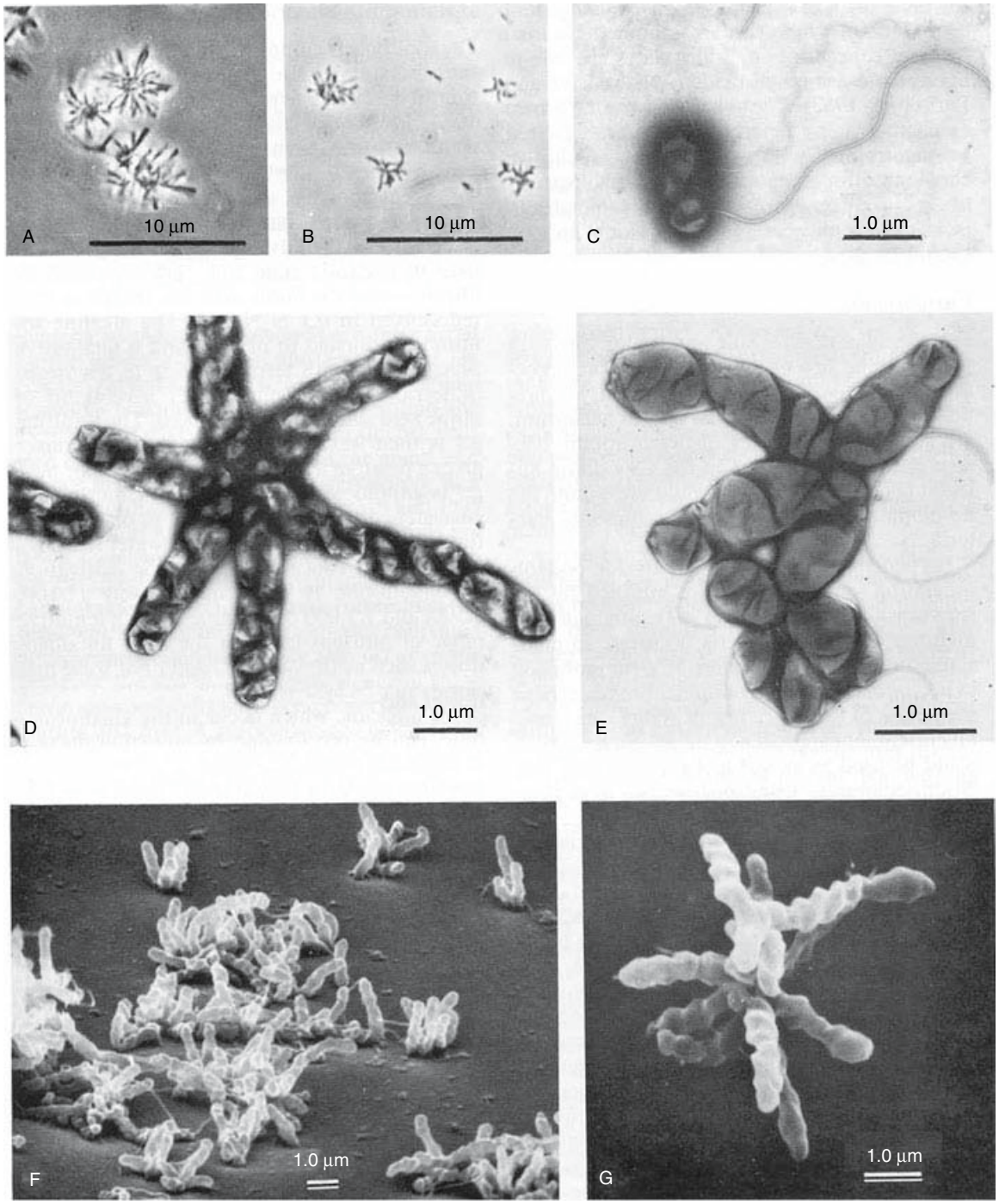

Fig. 1. The morphology of *Seliberia stellata*, a Russian soil strain, and seliberia-like aquatic isolates, as observed with light, transmission, and scanning microscopy. (A) *S. stellata* aggregates, phase-contrast microscopy. (B) An aquatic isolate of seliberia-like bacterium strain ICPB 4133 (Schmidt's strain OMF-1) showing rosettes; phase-contrast microscopy. (C) Negative-contrasted (0.5% Na tungstate) *S. stellata* cell, having two structurally different flagella, grown on peptone–yeast extract solid medium. (D) Star-shaped rosettes of *S. stellata*, showing surface sculpturing, negatively stained. (E) Aquatic isolate (ICPB 4134) with seliberia-like morphotype, grown on a carbon-supported, gold-coated electron microscope grid placed in peptone–yeast extract broth and then stained with 0.5% Na tungstate. Note furrowing of cell surface. (F and G) ICPB strain 4133, grown on glass cover slips, fixed with 2% glutaraldehyde, critical-point dried, and gold coated. Scanning electron microscopy clearly demonstrates attachment of cells at one pole to substrate and (in G) the helical morphology of the cells.

microscope facilitates the selection of appropriate colonies. Phase-contrast microscopy of the organisms from the patch plates (once again concentrating on those requiring several days to give visible growth) has been used to select rosette-forming, asymmetrically dividing, rod-shaped bacteria. From among these, seliberia-like rods, with their helical topography, can be distinguished from bacteriod and fusiform *Caulobacter* spp., *Agrobacterium* spp., and other aggregate-forming bacteria with transmission electron microscopy, using negative-contrast methods. The helical topography of the rods is better demonstrated by scanning electron microscopy (Fig. 1).

Identification

The presence of star-shaped aggregates (Fig. 1A, B) in preparations from seliberia colonies is not uniquely diagnostic of a *Seliberia* sp., since many other bacteria (certain species of *Agrobacterium*, *Pseudomonas*, *Caulobacter*, and *Asticcacaulis*, all rods) form aggregates or rosettes. Seliberias can usually be distinguished from caulobacters, since seliberias do not form stalks, and caulobacter prosthecae can usually be detected with phase-contrast photon microscopy. Apical daughter cells of seliberias are usually shorter than the parent rod (Fig. 1D). Transmission electron micrographs reveal a characteristic wrinkled (helical) surface of the seliberias (Fig. 1D, 1E). However, the tight helical surface of the rods is more apparent in scanning electron micrographs prepared by the critical-point drying technique (Fig. 1F, 1G). When *S. stellata* (one of Aristovskaya's strains, obtained from G. A. Zavarzin) is grown on agar-solidified peptone-yeast extract medium, two distinct kinds of flagella are present on the same cell: a subpolar sheathed flagellum and several thinner, undulating (apparently nonsheathed) lateral flagella (Fig. 1C). When grown in liquid medium, *S. stellata* produces only the sheathed type of flagellum. Nine independently obtained strains of the aquatic seliberia-like bacteria (19 isolates, total) have been examined, and all have a single subpolar sheathed flagellum in the motile stage of growth. Only one aquatic strain has been observed to produce the two distinctive kinds of flagella seen on *S. stellata*, under any cultural conditions so far examined. Very thin pili (40 Å in diameter) are present on both *S. stellata* and on the aquatic seliberia-like strains. One pole of the seliberia cell is adhesive, and a secretion of amorphous material, representing the holdfast, can sometimes be discerned in negative-contrast transmission electron micrographs at one pole of the cell or in the center of a star-shaped aggregate. In liquid medium, cells attached by a pole to a substrate (for example, to a glass cover slip) remain erect (Fig. 1F). Aristovskaya and Parinkina (1963) and Aristovskaya (1964) have described oval or spherical, budding, apical cells, found commonly in *S. stellata*, when grown on soil extract medium, and a zoogleal stage in its life cycle. Deposition of ferric hydroxide may occur particularly when *S. stellata* is cultivated in the presence of other soil organisms in mixed culture or in pure culture, under slightly increased carbon dioxide tension.

All isolates of seliberia-like aquatic bacteria and the one strain of *S. stellata* we have examined are Gram negative. They are somewhat sensitive to penicillin G (growth is inhibited by 1,000 units/ml but not by 10 units/ml), and they are lysed by exposure to lysozyme-ethylenediaminetetraacetate. Growth of the aquatic seliberia-like strains in dilute peptone-yeast extract medium is sparse, white in color, and very adherent to the agar medium; in liquid medium, most of the cells are in large granular aggregates or firmly attached to the glass surface of the culture vessel. Growth of *S. stellata* is relatively dispersed, giving uniform turbidity, although some cell aggregates are visible microscopically. All of the seliberia strains so far examined, including *S. stellata*, are obligately aerobic, although *S. stellata* can carry out denitrification anaerobically. The aquatic seliberia-like bacteria are weak denitrifiers. The possibility that *S. stellata* and the aquatic strains are facultative autotrophs (hydrogen oxidizers or carboxide bacteria) is being investigated.

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SECTION 3.2

Beta Subclass

Prokaryotes (2006) 5:593–601
DOI: 10.1007/0-387-30745-1_25

CHAPTER 3.2.1

The Phototrophic Betaproteobacteria

JOHANNES F. IMHOFF

Introduction

The phototrophic purple β-Proteobacteria are purple nonsulfur bacteria able to perform anoxygenic photosynthesis with bacteriochlorophylls and carotenoids as photosynthetic pigments. Though genetic relationships and chemotaxonomic properties clearly distinguish these bacteria from the phototrophic α-Proteobacteria, both groups share a number of common physiological properties. Owing to their photosynthetic pigments, cell suspensions appear in various colors and have characteristic absorption spectra. Photosynthetic pigments are located in the cytoplasmic membrane and in internal membrane systems (small tubular invaginations of the cytoplasmic membrane) and are bacteriochlorophyll *a* and various types of carotenoids. In most species, the formation of pigments and of the internal membrane systems is repressed under oxic conditions but becomes derepressed at low oxygen tensions.

Phototrophic β-Proteobacteria have high metabolic flexibility. The preferred mode of growth is photoheterotrophically under anoxic conditions in the light. Most of the species also can grow photoautotrophically with molecular hydrogen as photosynthetic electron donor. Growth factors are generally required, most commonly biotin and thiamine, and growth of most species is enhanced by small amounts of yeast extract. Chemotrophic growth under microoxic to oxic conditions in the dark is common to these bacteria. Anaerobic dark growth by fermentation also occurs.

Phylogeny

According to 16S rDNA sequence comparisons, the phototrophic β-Proteobacteria of the genera *Rhodoferax*, *Rubrivivax* and *Rhodocyclus* are well separated phylogenetically from their counterparts of the α-Proteobacteria and belong to different phylogenetic lines within the β-Proteobacteria (Hiraishi, 1994). On this basis, they are highly related to strictly chemotrophic β-Proteobacteria. Such close relationships exist, e.g., between *Rhodoferax fermentans* and *Variovorax paradoxus* and between *Rubrivivax gelatinosus* and *Leptothrix discophora* (see Fig. 1).

The analysis of nucleotide sequences of DNA fragments coding for the L and M subunits of the photosynthetic reaction center showed that sequences of *Rhodoferax fermentans*, *Rubrivivax gelatinosus* and *Rhodocyclus tenuis* were positioned among those of α-Proteobacteria. This contrasts with their phylogenetic relations based on 16S rDNA sequences. The inconsistency was explained by possible horizontal transfer of the genes encoding the photosynthetic reaction center during evolution. Despite this disparity, sequences of the puf genes of *Rhodoferax fermentans* show 77% identity and are most similar to those of *Rubrivivax gelatinosus* (Nagashima et al., 1997).

Taxonomy

Prior to the recognition of their genetic relationship on the basis of 16S rDNA sequence comparison, the phototrophic purple nonsulfur bacteria belonging to the β-Proteobacteria have been included in the Rhodospirillaceae together with the phototrophic α-Proteobacteria (Pfennig and Trüper, 1974). In addition to their clear phylogenetic separation (Gibson et al., 1979; Fox et al., 1980; Hiraishi, 1994), a number of chemotaxonomic properties clearly distinguish the phototrophic β-Proteobacteria from the phototrophic α-Proteobacteria: They have ubiquinone and menaquinone (or rhodoquinone) derivatives with eight isoprenoid units in the side chain (Q-8, RQ-8 and MK-8); they have a "small type" cytochrome c_{551} as typically found in species of the Chromatiaceae and Ectothiorhodospiraceae, but not in phototrophic α-Proteobacteria (Ambler et al., 1979; Dickerson, 1980); and they have a characteristic phospholipid and fatty acid composition with the highest proportions of C-16 fatty acids (16:0 and 16:1) among all phototrophic purple bacteria and correspondingly very low ones of 18:1 (Hiraishi et

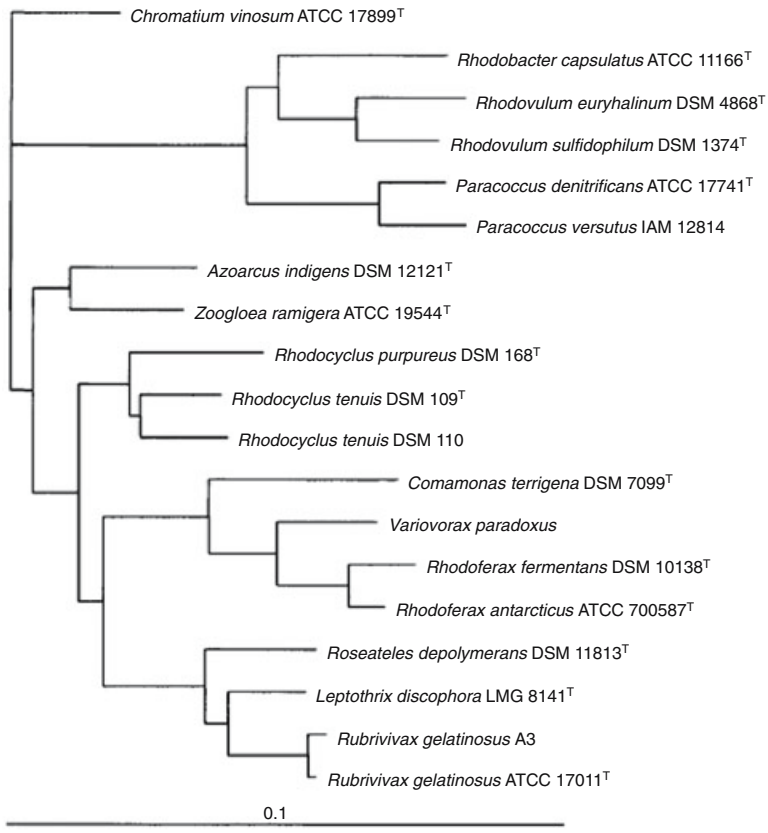

Fig. 1. Phylogenetic tree based on 16S rDNA sequences of phototrophic β-Proteobacteria and some chemotrophic relatives. The bar indicates 10% sequence divergence.

al., 1991; Imhoff, 1984a; Imhoff and Bias-Imhoff, 1995; Imhoff and Trüper, 1989). Their lipopolysaccharides characteristically contain significant amounts of phosphate and amide-linked 3-OH-capric acid (3-OH-C-10) in their lipid A moiety (Weckesser et al., 1995) or 3-OH-C-8:0, which is the major hydroxy fatty acid of *Rhodoferax fermentans* (Hiraishi et al., 1991).

As a consequence, *Rhodospirillum tenue* (Pfennig, 1969) was transferred to *Rhodocyclus tenuis* (Imhoff et al., 1984b). Also *Rhodopseudomonas gelatinosa* was transferred to this genus as *Rhodocyclus gelatinosus* (Imhoff et al., 1984b), but because of its phylogenetic distance to *Rhodocyclus purpureus*, it was assigned later to a new genus as *Rubrivivax gelatinosus* (Willems et al., 1991). Since then *Rhodoferax fermentans* has been described as a new genus and new species (Hiraishi and Kitamura, 1984b; Hiraishi et al., 1991) and *Rhodoferax antarcticus* as another new species of that genus (Madigan et al., 2000).

Rhodoferax fermentans was first known as the "*Rhodocyclus gelatinosus*-like (RGL)" group (Hiraishi and Hoshino, 1984a), because of great phenotypical similarity to *Rubrivivax gelatinosus* (at that time known as *Rhodocyclus gelatinosus*), in particular appearing to resemble *Rubrivivax gelatinosus* subgroup II (Weckesser et al., 1969). More detailed physiological, chemotaxonomic and genetic studies revealed major differences between the RGL group and *Rubrivivax gelatinosus*, and these observations led to the proposal to classify the RGL group as *Rhodoferax fermentans* (Hiraishi et al., 1991). The currently recognized species and genera together with some characteristic properties are listed in Table 1.

The Genus *Rhodocyclus*

At present this genus comprises two species, *Rhodocyclus purpureus* and *Rhodocyclus tenuis* (formerly *Rhodospirillum tenue*). While cells of *Rhodocyclus purpureus* are nonmotile and form a half- or full-circle, those of *Rhodocyclus tenuis* are slender, slightly curved and rapidly motile under optimal growth conditions. Significant differences between the two species also are reflected in their vitamin requirements and substrate utilization. Characteristic properties are summarized in Table 1.

A dichotomy has been observed in *Rhodocyclus tenuis* strains on the basis of the carotenoid composition (Schmidt, 1978), color of cell suspensions and absorption spectra (Biebl, 1973). Some strains assigned to *Rhodocyclus tenuis* have carotenoids of the rhodopinal series and others of the spirilloxanthin series. The type

Table 1. Characteristic properties of species of the genera *Rhodocyclus*, *Rubrivivax*, and *Rhodoferax*.

Characteristic	*Rhodocyclus purpureus*	*Rhodocyclus tenuis*	*Rubrivivax gelatinosus*	*Rhodoferax fermentans*	*Rhodoferax antarcticus*
Cell diameter (μm)	0.6–0.7	0.3–0.5	0.4–0.7	0.6–0.9	0.7
Cell shape	half-circle to circle	curved rods	straight to curved rods	curved rods	curved rods
Motility	–	+	+	+	+
Slime production	–	+	+	–	+
Color	violet, purple-violet	brownish-red or purple-violet	brown	peach brown	peach brown
Major carotenoids	rhodopin rhodopinal	rhodopin rhodopinal lycopene[b]	spheroidene, OH-spheroidene, spirilloxanthin	spheroidene, OH-spheroidene, spirilloxanthin	spheroidene[d]
Growth factors	B_{12}, p-ABA, biotin	none[a]	thiamine, biotin[c]	thiamine, biotin	biotin
Carbon sources:					
Benzoate	+	–	–	–	–
Citrate	–	–	+	–	+
Mannitol	–	–	–	+	+
Sorbitol	–	–	+	+	0
C-10 to C-18 fatty acids	–	+	+	0	0
Gelatine liquefaction	–	–	+	+	0
Fructose fermentation	–	–	–	+	–
Growth with H_2	+	+	+	0	+
N_2-fixation	–	+	+	+	+
Optimum temperature	25–30°C	25–30°C	25–30°C	25–30°C	12–20°C
Major fatty acids:					
C16:0	33–35	33–36	24–35	33–39	0
C16:1	40–45	43–50	35–45	52–54	0
C18:0	<1	<1	1–3	<1	0
C18:1	18	15–18	16–25	5	0
Major 3-OH fatty acid	10:0	10:0	10:0	8:0	0
Major quinones	Q-8 + MK-8	Q-8 + MK-8	Q-8 + MK-8	Q-8 + RQ-8	0
Mol% G + C of DNA	65.1 (HPLC)	64.1–64.8 (HPLC)	71.2–72.1 (HPLC)	59.8–60.3 (HPLC)	61.5 (T_m)
Type strain	DSM 168	DSM 109	ATCC 17011	ATCC 49787	ATCC 700587

Symbols: +, positive in most strains; –, negative in most strains: p-ABA, p-aminobenzoic acid.
Q-8, ubiquinone-8; RQ-8, rhodoquinone-8; MK-8, menaquinone-8 (Hiraishi and Hoshino, 1984; Imhoff, 1984).
[a]Some strains may require vitamin B_{12} (Siefert and Koppenhagen, 1982).
[b]Some strains may contain carotenoids of the spirilloxanthin series and lack rhodopinal (Schmidt, 1978)
[c]Some strains may also require pantothenate.
[d]Although detailed chemical evidence is lacking it appears likely that carotenoids of the spheroidene series are present, including spheroidene, OH-spheroidene and possibly spirilloxanthin.

strain (DSM 109^T) belongs to those strains that synthesize spirilloxanthin, whereas another group of strains (including strain DSM 110) does not form anhydrorhodovibrin and spirilloxanthin but accumulates major amounts of rhodopinal, rhodopinol and lycopenal (Schmidt, 1978). On the basis of 16S rDNA sequences, both of these strains (DSM 109^T and DSM 110) show a close relationship.

The Genus *Rubrivivax*

Rubrivivax gelatinosus has been reported to occur in two distinct morphological forms (Biebl and Drews, 1969). Form I cells are clearly curved, are 0.4–0.7 μm in diameter, and produce less slime during active growth. Form II cells are more or less straight rods, although they sometimes also are bent and produce more slime during active growth, causing sedimentation of the cells in a gelatinous layer. Form I cells utilize a greater variety of carbon sources and have a much shorter doubling time than do form II cells (Weckesser et al., 1969). Furthermore, the lipopolysaccharides of *Rubrivivax gelatinosus* show two different serotypes which do not crossreact with each other (Weckesser et al., 1975). The type strain of *Rubrivivax gelatinosus* was not included in these studies. Further studies are required to clarify the taxonomic status of the strains assigned to this species. Characteristic of *Rubrivivax gelatinosus* is the liquefaction of gelatin, which is catalyzed by an extracellular protease (Klemme and Pfleiderer, 1977). However, gelatin liquefaction is not unique to *Rubrivivax gelatinosus*; about half of the strains with this property have been identified as belonging to *Rhodobacter capsulatus*, whereas other strains with the morphology typical of *Rubrivivax gelatinosus* did not liquefy gelatin (Siefert et al., 1978). The taxonomic status of these strains needs to be clarified. Hydrolysis of gelatin is also catalyzed by *Rhodoferax fermentans* (Hiraishi et al., 1991). Characteristic properties of *Rubrivivax gelatinosus* are summarized in Table 1.

The Genus *Rhodoferax*

Two species of *Rhodoferax* are known. While *Rhodoferax fermentans* has been isolated from sewage and activated sludge (Hiraishi et al., 1991), *Rhodoferax antarcticus* is from an Antarctic microbial mat (Madigan et al., 2000). Both species are facultative photoheterotrophic bacteria that grow anaerobically in the light and aerobically in darkness at full atmospheric oxygen tension. Phototrophic liquid cultures become peach brown, whereas aerobic chemotrophic cultures are colorless or faintly pink. Absorption spectra of phototrophically grown cells or membrane preparations of *Rhodoferax fermentans* show major absorption maxima at around 800 and 850 nm, indicating that the cells contain the core light-harvesting complex (LH I) together with the photosynthetic reaction center and a peripheral light-harvesting complex (LH II). Long wavelength absorption maxima of bacteriochlorophyll in *Rhodoferax antarcticus* cells are at 800, 820 and 866 nm (Madigan et al., 2000), indicating the presence of different pigment/protein complexes. *Rhodoferax antarcticus* is well adapted to grow at low temperatures with an optimum at 12–20°C and the minimum close to 0°C, whereas all other known phototrophic β-Proteobacteria grow best at 25–30°C (Table 1).

Habitats and Ecology

The phototrophic β-Proteobacteria are freshwater bacteria common in stagnant waters exposed to the light that are enriched in organic compounds and nutrients. Sulfide-rich water bodies do not provide favorable conditions for these bacteria because of their inability to use sulfide as an electron donor for growth and their low tolerance to sulfide.

Rubrivivax gelatinosus is widely occurring in natural environments. Together with *Rhodocyclus tenuis*, it is found in the typical freshwater habitats of purple nonsulfur bacteria such as freshwater ponds, sewage ditches and activated sludge. *Rhodocyclus purpureus* appears to be a rare species; it has been isolated only once, from a swine waste lagoon in Ames, Iowa (United States), where it was the dominant phototrophic bacterium (Pfennig, 1978). Strains of *Rhodoferax fermentans* have been isolated from pond water, sewage and activated sludge (Hiraishi et al., 1991), similar habitats to those of *Rubrivivax gelatinosus*. As depicted in its name, *Rhodoferax antarcticus* was isolated from a microbial mat of an Antarctic habitat (Madigan et al., 2000). Accordingly, with an optimum temperature of 12–20°C and a range from 0–25°C, it appears to be better adapted to the lower range of environmental temperatures than are the other phototrophic purple bacteria.

Isolation

Selective Enrichment

Growth conditions employed for purple nonsulfur bacteria in general (Imhoff, 1988; Imhoff and Trüper, 1992) are also suitable for the enrichment of phototrophic β-Proteobacteria. Because

species of these bacteria cannot use sulfide or other reduced sulfur sources as electron donors for photosynthesis, but are strongly inhibited by low concentrations of them, addition of reduced sulfur compounds in enrichment media should be avoided.

The vitamin B_{12} requirement and its unusual carbon nutrition provide properties for selective enrichment and isolation of *Rhodocyclus purpureus*. From a suitable habitat, it should be possible to selectively enrich this species with benzoic acid as a carbon source and in the presence of vitamin B_{12}; anoxic conditions in the absence of reduced sulfur compounds, in particular hydrogen sulfide, should be established.

Suitable conditions for selective enrichment of *Rhodocyclus tenuis* are not available. This species will develop in suitable media for purple nonsulfur bacteria (Imhoff, 1988; Imhoff and Trüper, 1992) under exclusion of oxygen by sodium ascorbate and in the absence of hydrogen sulfide, vitamins and complex nutrients.

Utilization of citrate has proven to be useful for the selective enrichment of *Rubrivivax gelatinosus*, though this substrate also can be used by several other species.

Although it is not easy to perform selective enrichment of *Rhodoferax fermentans* from environmental samples, the addition of 0.5 mM EDTA to the enrichment medium may be effective for suppressing the overgrowth of possibly co-existing, fast-growing phototrophic species, such as *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. For enrichment, a basal mineral medium with one or more simple organic compounds (e.g., 0.1% acetate or 0.2% glucose) and a vitamin mixture or 0.01% yeast extract are suitable. Favorable incubation conditions are anaerobic in the light (incandescent illumination at 1,000–2,000 lux) at 28°C.

Although the upper temperature limit of *Rhodoferax antarcticus* prevents growth of this species at temperatures above 25°C, the application of low temperatures must not necessarily be of selective advantage because many bacteria with growth optima between 20–30°C are able to grow at 4°C. It should be noted that growth responses of most of the purple nonsulfur bacteria at temperatures below 15°C have not been determined.

Isolation Procedures

The methods employed for enrichment, isolation and maintenance of phototrophic α-Proteobacteria (Imhoff, 1988; Imhoff and Trüper, 1992) also are suitable for species of *Rubrivivax*, *Rhodocyclus* and *Rhodoferax*. Media and standard techniques for the isolation of anaerobic phototrophic bacteria in agar dilution series and on agar plates also can be applied for these bacteria (Biebl and Pfennig, 1981; Imhoff, 1988; Imhoff and Trüper, 1992).

A simple medium used for *Rhodoferax fermentans* (MYCA medium) contains 0.1% DL-malate, 0.3% yeast extract, 0.2% casamino acids and 0.05% $(NH_4)_2SO_4$ and has a pH of 6.6–6.8 (Hiraishi et al., 1991).

Identification

The phototrophic β-Proteobacteria have thin, slender cells of less than 1 µm diameter, single short tubes as internal photosynthetic membranes (if any at all), bacteriochlorophyll *a* as a reaction center and light-harvesting bacteriochlorophyll, a small type cytochrome c_{551} (Ambler et al., 1979), adenosine-5′-phosphosulfate (APS) as intermediate during assimilatory sulfate reduction (Imhoff, 1982), respiratory quinones with eight isoprenoid units (ubiquinone-8, menaquinone-8 and/or rhodoquinone-8; Imhoff, 1984a; Hiraishi et al., 1991), and characteristic molecular features of the lipid A moiety of the lipopolysaccharides, i.e., the amide linked 3-OH-10:0 fatty acid (not known for *Rhodoferax fermentans*, where 3-OH-8:0 is the major 3-OH-fatty acid) (Weckesser et al., 1979; Weckesser et al., 1995).

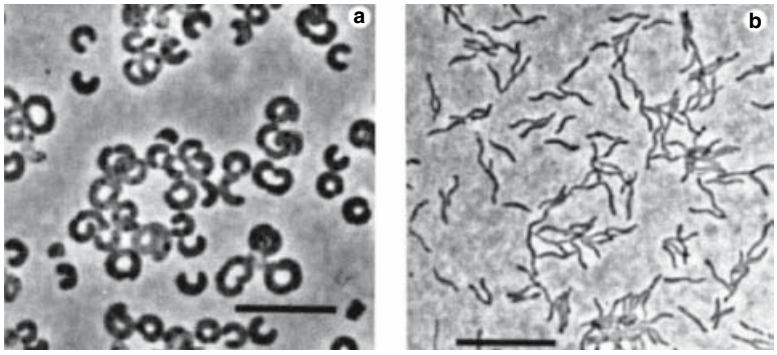

Fig. 2. The morphology of the two *Rhodocyclus* species; phase contrast microscopy. (a) *Rhodocyclus purpureus*; (b) *Rhodocyclus tenuis*. Bar = 10 µm.

Properties that enable the distinction and identification of the species of the genera *Rhodocyclus*, *Rhodoferax* and *Rubrivivax* are summarized in Table 1.

Preservation

For short-term preservation, late log-phase cultures may be kept in closed, air-tight screw-cap bottles at 6–10°C in a refrigerator or at room temperature for several months. Maintenance transfer of liquid cultures should occur at intervals of 2–6 months. For long-term storage, preservation in liquid nitrogen is recommended. Well-grown cultures are supplemented with 50% dimethyl sulfoxide to give a final concentration of 5%, thoroughly mixed, dispensed in plastic ampoules, sealed and frozen in liquid nitrogen.

Physiology

Most phototrophic β-Proteobacteria of the genera *Rhodocyclus*, *Rubrivivax* and *Rhodoferax* are mesophilic and neutrophilic fresh water bacteria with optimal growth temperatures of 25–30°C. *Rhodoferax antarcticus* has been isolated from Antarctic microbial mats and grows best at 12–20°C (Madigan et al., 2000). The species of *Rhodocyclus*, *Rhodoferax* and *Rubrivivax* are facultative anaerobic bacteria which preferably grow under anoxic conditions by anoxygenic photosynthesis. All species grow photoheterotrophically and most species are able to grow photoautotrophically with hydrogen as the photosynthetic electron donor. Under these conditions, the Calvin cycle is fully active. They all are able to grow aerobically to microaerobically in the dark by oxygen-dependent respiration. Under dark anaerobic conditions, most species slowly ferment their storage polysaccharides. Most pronounced fermenting capacities with external substrates were recorded for *Rubrivivax gelatinosus* (Gürgün et al., 1976) and *Rhodoferax fermentans* (Hiraishi et al., 1991).

They are true "nonsulfur" bacteria in being unable to use reduced sulfur compounds as photosynthetic electron donors and consequently cannot form elemental sulfur globules either in or outside their cells. All species are capable of assimilatory sulfate reduction, as far as tested via adenosine-5′-phosphosulfate (Imhoff, 1982).

Rhodocyclus

Cells grow well with simple organic compounds as electron donors and carbon sources and in complex media containing peptone, yeast extract or casamino acids. Photoautotrophic growth with hydrogen and photoheterotrophic growth with a variety of carbon compounds as electron donors are possible. Chemotrophic growth is possible by respiration under microoxic to oxic conditions in the dark and by fermentation.

Rhodocyclus purpureus assimilates only a few carbon compounds, but is capable of anaerobic degradation of aromatic compounds. Benzoate and cyclohexane carboxylate are both used, which may indicate the same pathway for anaerobic benzoate degradation is used by *Rhodocyclus purpureus* and *Rhodopseudomonas palustris* (Dutton and Evans, 1969; Pfennig, 1978; Gibson and Harwood, 1995). *Rhodocyclus tenuis* is more versatile with respect to carbon source utilization, although it is unable to break down aromatic compounds.

A requirement of vitamin B_{12} is not common among the purple nonsulfur bacteria (PNSB). Besides *Rhodocyclus purpureus*, only single strains of *Rhodocyclus tenuis* and *Rhodopseudomonas palustris* (Siefert and Koppenhagen, 1982) and a few more species have been reported to require vitamin B_{12}.

Rhodocyclus purpureus and *Rhodocyclus tenuis* also differ significantly in their nitrogen nutrition. Whereas the former species uses only ammonia and glutamine as nitrogen sources and is unable to fix dinitrogen (a property common to most species of the phototrophic α- and β-Proteobacteria), the latter species utilizes a greater number of amino acids as well as urea, dinitrogen, yeast extract, peptone and casamino acids (Masters and Madigan, 1983). Alanine dehydrogenase was absent from both species. Glutamate dehydrogenase (NADPH-dependent) is found in *Rhodocyclus purpureus* in high activities under all growth conditions, and the glutamine synthetase inhibitor methionine sulfoximine exerts no growth inhibition. This may be taken as an indication that the major route of nitrogen assimilation in *Rhodocyclus purpureus* is via glutamate dehydrogenase (unlike that in all other investigated PNSB). *Rhodocyclus tenuis* employs the glutamine synthetase/glutamate synthase (NADPH-dependent) pathway—as found in most phototrophic PNSB—for the assimilation of ammonia (Masters and Madigan, 1983; Drews and Imhoff, 1991).

Rubrivivax

Cells grow well with simple organic compounds as electron donors and carbon sources and in complex media containing peptone, yeast extract or casamino acids. Photoautotrophic growth occurs with hydrogen and photoheterotrophic growth with a variety of carbon compounds as electron donors. Chemotrophic growth is possi-

ble by respiration under microoxic to oxic conditions in the dark and by fermentation.

A characteristic property of *Rubrivivax gelatinosus*, shared only with a few other phototrophic purple bacteria, is the hydrolysis of gelatin owing to an extracellular protease (Klemme and Pfleiderer, 1977). *Rubrivivax gelatinosus* grows well with citrate as carbon source and thereby excretes large amounts of acetate into the medium, which serves as carbon source after citrate is exhausted (Schaab et al., 1972). Citrate lyase, the key enzyme for growth on citrate, has been characterized in this species (Giffhorn et al., 1972; Beuscher et al., 1974). However, because the other key enzyme, malate synthase, is lacking in acetate-grown cells, the glyoxylate cycle does not function and the serine-pathway has been proposed to be used for acetate assimilation in this species (Albers and Gottschalk, 1976). *Rubrivivax gelatinosus* also can be adapted to grow with CO as sole energy and carbon source under anoxic conditions in the dark (Uffen, 1976). Under these conditions, activities of enzymes of the serine pathway and ribulose bisphosphate carboxylase are enhanced (Uffen, 1983).

Rhodoferax

Growth is possible by photosynthesis, aerobic respiration, and fermentation. Photoheterotrophy with various organic compounds as carbon sources is the preferred mode of growth. Cells grow well with simple organic compounds as electron donors and carbon sources and in complex media containing peptone, yeast extract or casamino acids.

Fermentative growth of *Rhodoferax fermentans* in darkness occurs on pyruvate and sugars, among which fructose is the best substrate (Hiraishi and Kitamura, 1984b). *Rhodoferax fermentans* rapidly produces acid from glucose. The addition of bicarbonate enhances anaerobic growth in the dark significantly (Hiraishi, 1988a). The end products of fructose fermentation are acetate, formate, lactate, succinate and ethanol. Hypophosphite, a potent inhibitor of pyruvate-formate lyase, suppresses the production of formate completely and increases the amount of succinate excreted. These observations suggest that the bicarbonate-dependent fermentative growth may be linked to CO_2 fixation via part of the reductive tricarboxylic acid (TCA) cycle and the subsequent reduction of fumarate to succinate. The guanine diphosphate (GDP)-dependent phosphoenolpyruvate carboxykinase was suggested to function as a key enzyme for CO_2 fixation (Hiraishi, 1988b).

While several species of phototrophic purple nonsulfur bacteria exhibit anaerobic growth in darkness coupled with reduction of trimethylamine-*N*-oxide or dimethylsulfoxide as the terminal electron acceptor, *Rhodoferax fermentans* lacks these properties. Most strains of this species also are devoid of nitrate reductase activity, though one of the strains assigned to this species (strain DSM 10139) is highly active in nitrate reduction (Hougardy and Klemme, 1995).

Rhodoferax antarcticus, like *Rhodoferax fermentans*, can grow well with glucose and fructose, but is unable to ferment these substrates (Madigan et al., 2000).

Applications

The frequent occurrence of *Rhodocyclus*, *Rubrivivax* and *Rhodoferax* species in sewage treatment plants and lagoons (Siefert et al., 1978; Pfennig, 1978) points towards a function and possible application of these bacteria for the treatment of sewage. Sasaki et al. (1981) have developed a successful process for single-cell protein production from soybean wastes using *Rubrivivax gelatinosus*. Processes of single-cell protein production with *Rubrivivax gelatinosus* also have been developed using the agricultural byproduct wheat bran as carbon substrate (Shipman et al., 1975; Shipman et al., 1977).

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Prokaryotes (2006) 5:602–647
DOI: 10.1007/0-387-30745-1_26

CHAPTER 3.2.2

The *Neisseria*

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Introduction

The genus *Neisseria* includes a group of closely related Gram-negative diplococci that are primarily commensal organisms of the mucous membranes of mammals (Knapp, 1988a). Several *Neisseria* spp. are opportunistic pathogens causing disease in immunocompromised hosts. Two species, *N. gonorrhoeae* (the gonococcus) and *N. meningitidis* (the meningococcus), are important human pathogens.

Phylogeny

Species Groups

While the 1994 edition of *Bergey's Manual of Determinative Bacteriology* lists 13 species within the genus, the systematics of *Neisseria* have been problematic, and strains have been frequently reclassified as new techniques have become available. DNA-DNA hybridization techniques, numerical taxonomy procedures (Barrett and Sneath, 1994) and sequence analysis of various genes (Smith et al., 1999) have divided the *Neisseria* into two subgroups: 1) the closely related pathogens *N. gonorrhoeae* and *N. meningitidis*, a related group of commensal *Neisseria* species that have been consolidated into the species *N. subflava* (Reyn, 1974) and *N. lactamica* (Hollis et al., 1969); and 2) a group of commensal organisms isolated from man and other animals (*N. cinerea*, *N. polysaccharea*, *N. canis*, *N. denitrificans*, *N. elongata*, *N. macacae*, *N. animalis*, *N. dentiae* and *N. weaveri* (Andersen et al., 1993; Bovre and Holten, 1970; Dent, 1982; Dewhirst et al., 1993; Sneath and Barrett, 1996). However, recent data have shown that while most *Neisseria* species can be separated into groups of related species, the relationship among and between species is distorted by interspecies recombination (Smith et al., 1999).

Relation to Phenotypic Characteristics

Phylogenetic analyses by rRNA similarities and DNA-DNA hybridizations have placed *N. meningitidis*, *N. gonorrhoeae*, *N. lactamica* and *N. cinerea* in a subgroup with particularly close interspecies relatedness (Guibourdenche et al., 1986; Kingsbury, 1967; Rossau et al., 1989). While these bacteria are closely related, they express very different pathogenicities. *Neisseria lactamica* and *N. cinerea* are typically nonpathogenic; *N. meningitidis* is an opportunistic pathogen and *N. gonorrhoeae* is an obligate pathogen. When *N. meningitidis* colonizes the nasopharynx, it does not cause disease. However, it may spread from there into the bloodstream, causing septicemia before crossing the blood-brain barrier to induce meningitis. *Neisseria gonorrhoeae* colonizes and invades the epithelium of the genitourinary tract, causing a localized inflammatory response.

Classification Errors and Problems

The species currently classified in the genus *Neisseria* are naturally competent for DNA uptake. As such, the genomes of these organisms are continually exposed to exogenous DNA. Using the pattern of nucleotide sequence variation among defined genes as a mechanism of classifying strains within the species has produced different genotypic relationships, depending on the gene used for the classification (Smith et al., 1999). These data indicate that the human commensal *Neisseria* spp. can be separated into discrete groups of related species but that the relationships both within and among these groups, including those reconstructed using 16S rRNA, can be distorted by interspecies recombination events. Proper speciation requires the analysis of multiple genes or the use of multiple classification methods.

Current Species

The genus *Neisseria* currently contains the following species: *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *N. cinerea*, *N. sicca*, *N. subflava*, *N. flavescens*, *N. mucosa*, *N. canis*, *N. denitrificans*, *N. elongata*, *N. polysaccharea*, *N. macacae*, *N. animalis*, *N. dentiae* and *N. weaveri*.

Habitat

In Humans

The *Neisseria* spp. colonize the mucosal surfaces of mammals. The nonpathogenic *Neisseria* spp. are part of the normal flora of the oro- and nasopharynx and are rarely isolated from other sites. Owing to taxonomic and methodologic problems, the prevalence of *Neisseria* spp. cannot be assessed accurately from the literature of early studies. However, when one examines the nature of the species identified, two types of Neisserial colonization were detected: heavy colonization with strains of the saccharolytic species (*N. perflava*, *N. sicca* and *N. mucosa*) and sparse colonization with many different species (Knapp, 1988a; Knapp and Hook, 1988b).

Only *N. gonorrhoeae* is considered to be always pathogenic. *Neisseria gonorrhoeae* causes the sexually transmitted disease gonorrhea. This disease is most frequently manifested as a localized infection of the columnar epithelial cells of mucosal surfaces, i.e., urethra, cervix, rectum, pharynx and eye; squamous epithelial cells are not susceptible to infection by gonococci. Pharyngeal and rectal infections are frequently asymptomatic. The organism is transmitted principally through sexual activities. Ocular infections with *N. gonorrhoeae* are seen most commonly in neonates, whose infections are acquired during passage through an infected birth canal.

Strains of *N. meningitidis* may colonize the oro-and nasopharynx. The organism may be asymptomatically carried by up to 25 percent of a population, where only a few of the infected individuals will go on to develop invasive disease (Hart and Rogers, 1993). The organism is transmitted principally by respiratory droplets from the nose and throat of an infected person. It can cause sudden serious illness and death in a previously healthy person, and as a consequence, meningococcal disease often causes considerable anxiety and even panic in the community. For many years, epidemics were believed to be more likely when the carriage rate rose above 20 percent, but now carriage rates at these levels and the beginning of epidemics do not appear to be associated (Apicella, 1995).

Neisseria lactamica frequently is isolated in children, but infrequently in adults. Correct identification of this species is important because it is able to grow on gonococcal selective media and may be misidentified as *N. gonorrhoeae* if inappropriate differential tests are performed to distinguish between these species. Strains of *N. cinerea* are more prevalent than previously recognized, probably because strains occur in small numbers in the oropharynx and are overgrown in cultures of specimens inoculated on nonselective media such as blood agar (Knapp, 1988a).

Most of the other commensal *Neisseria* spp. have been recognized as opportunistic pathogens. While the significance of the diseases caused by commensal organisms may be life threatening, the incidence of disease is quite low. Septic shock cases have been reported for *N. flavescens* (Quintero Otero et al., 1990). Strains of *N. mucosa* have been isolated from children with pneumonia and adults with endocarditis (Lechowski et al., 1995; Véron et al., 1959). Strains of *N. lactamica* have caused meningitis, septicemia or otis media (Denning and Gill, 1991; Orden and Amerigo, 1991). Strains of *N. cinerea* have been isolated from individuals suffering from proctitis, meningitis, septicemia, pneumonia and conjunctivitis (Dolter et al., 1998; Dossett et al., 1985; Kirchgesner et al., 1995). *Neisseria sicca* has been reported to cause meningitis, peritonitis, endocarditis, pneumonia, Bartholin's gland abscess and osteomyelitis (Gris et al., 1989; Lopez-Velez et al., 1994). *Neisseria subflava* has been associated with abscesses, meningitis, peritonitis and endocarditis (Berger and Muller, 1973; Denis et al., 1970; Scott, 1971; Vermeij et al., 1999).

In Animals

Neisseria also may colonize the mucosal membranes of animals. *Neisseria animalis* has been isolated from guinea pigs (Berger, 1960), *N. canis* from the throats of dogs (Berger, 1962), and *N. macacae* from the throats of rhesus monkeys (Vedros et al., 1983); *N. mucosa* has been isolated from dolphins (Vedros et al., 1973). Dent (1982) characterized 97 isolates of *Neisseria* spp. in dental plaque from 15 species of animals, including a variety of primates (five species), tenrecs, yaks, deer, kangaroos, llamas, black bears, panda bears, sheep, dairy cattle and domestic cats. Isolates were assigned to one of three clusters of organisms differentiated on the basis of acid production from maltose and polysaccharide production. Although nitrate reduction was determined, this characteristic was not used as a differential characteristic to define physiologic groups. While Dent (1982) did not attempt to identify isolates to the species level because the criteria for describing species overlapped, this study indicates that *Neisseria* spp. are inhabitants of a wide variety of animal species.

Isolation

Strains of *N. gonorrhoeae* and *N. meningitidis* may be detected, isolated and identified with similar procedures. Strains of both species are

fastidious and are susceptible to unfavorable environmental factors such as desiccation, heating or chilling, the action of autolytic enzymes, and acid and alkaline conditions. In addition, strains of both species require a CO_2-enriched atmosphere for primary isolation, although the supplemental CO_2 often is not required on subculture.

Neisseria gonorrhoeae

This nutritionally fastidious organism requires an enriched medium in a moist environment of 35–37°C with an atmosphere of 3–5% CO_2 for growth. Growth of *N. gonorrhoeae* from clinical specimens has been enhanced by the use of selective media that inhibit the simultaneous growth of other microorganisms (Thayer and Martin, 1966). Some gonococcal strains are susceptible to vancomycin and, consequently, may not grow on selective media (Windall et al., 1980). The gonococcus does not grow well in the presence of commensal organisms, and small numbers may be present in clinical material (Kraus et al., 1976). Optimum recovery of *N. gonorrhoeae* from endocervical specimens in women is achieved by direct inoculation of a selective gonococcal agar medium with direct incubation at 35°C in CO_2 (Knapp and Rice, 1995). However, the direct plating technique is not always practical, or even feasible, in some clinical facilities. To circumvent this need, specialized transport systems have been developed to allow for the transport of the test sample to an appropriately equipped clinical laboratory (Arbique et al., 2000).

The specimens selected for diagnosing gonorrhea depend on the gender, age and sexual preference of the patient. All specimens should be collected prior to the initiation of antimicrobial therapy. Urethral and cervical specimens routinely are collected from men and women, respectively. Isolation of the gonococcus from specimens obtained from the urethra and Skene's and Bartholin's glands of women also may be attempted. In addition, rectal and oropharyngeal specimens are collected from women and homosexual men; in 5–10% of women, the rectum may be the only site from which the gonococcus may be isolated.

While culture is routinely used for the laboratory diagnosis of gonorrhea in men, a presumptive diagnosis of gonorrhea may be made by performing a Gram stain of purulent materials and observing polymorphonuclear leukocytes and intracellular Gram-negative diplococci. Presumptive diagnosis based on culture results may be made by isolating colonies that contain Gram-negative, oxidase-positive diplococci on selective media inoculated with urethral, cervical or rectal specimens. Blood cultures and cultures of synovial aspirates or skin lesions should be attempted for patients with suspected disseminated gonococcal infection (DGI); blood should be inoculated directly into a blood culture medium.

Neisseria meningitidis

To isolate *N. meningitidis*, samples are taken from the nasopharynx, blood, cerebrospinal fluid and skin lesions and cultured on an enriched, selective medium such as modified Thayer-Martin medium (MTM) or Chocolate agar grown under increased carbon dioxide tension (Creitz et al., 1971). Medium to large, blue-gray, mucoid, convex colonies form in 48 h at 35–37°C.

Confirmation of the diagnosis of invasive meningococcal disease requires bacteriological isolation of *N. meningitidis* from a usually sterile site such as blood or cerebrospinal, synovial, pericardial or pleural fluid or from a petechial or purpuric lesion. A presumptive diagnosis is suggested by finding Gram-negative diplococci in cerebrospinal or synovial fluid or the aspirate from a petechial or purpuric lesion. Because *N. meningitidis* can be part of the normal flora of the nasopharynx and conjunctiva (Gold et al., 1978), isolation of this organism from a throat swab, nasopharyngeal aspirate, or conjunctival swab provides no information about the cause of the invasive disease.

Other *Neisseria* Species

Commensal *Neisseria* spp. have been isolated on blood agar medium (Berger and Wulf, 1961). Because blood agar is not selective, many *Neisseria* isolates may be overgrown by other bacteria that are normal flora in the sites from which the specimens are taken. A selective differential medium (LBVT.SNR) has been developed for the isolation of all *Neisseria* spp. that could grow on a simple nutrient medium. Through the incorporation of sucrose and an indicator in this medium, it is possible to differentiate colonial types of several strains of the same species present in a specimen (Knapp and Hook, 1988b). *Neisseria* spp. associated with animals have not been studied to the same extent as the human species. However, similar to studies of the human species, *Neisseria* spp. from animals have been isolated on blood agar (Berger, 1962).

Identification

Neisseria spp. are not very biochemically active. Most species are asaccharolytic or use relatively few carbohydrates and exhibit few other differ-

ential biochemical reactions. Not many tests are available that differentiate between species, particularly the species associated with animals. Many of the traditional differential biochemical reactions for human *Neisseria* spp. have been incorporated in rapid tests that allow one to determine if the isolate is a gonococcus, a meningococcus or another species. Serological (Menck, 1976) and nucleic acid probe tests have been developed to identify strains of *N. gonorrhoeae* (Black and Morse, 2000). Accurate identification is important because the misidentification of nongonococcal species as *N. gonorrhoeae* may have serious medicolegal implications.

Tests for the identification of *Neisseria* and related species include traditional biochemical tests that must be incubated for 24–48 h before results can be obtained (McDonald and Johnson, 1975) and rapid tests that permit the identification of *Neisseria* spp. after incubation for 2–4 h (Young et al., 1976). However, some isolates may not be identified correctly using rapid tests that lack the specific biochemical test critical for the accurate identification of an isolate. It is important to note, also, that many rapid tests may only be used to identify Gram-negative, oxidase-positive isolates that were isolated on gonococcal selective media. Other rapid tests may be used to identify isolates to the species level regardless of the medium on which they were isolated. Thus, the choice of a specific confirmation test will depend on the clinical or legal significance of an isolate and whether a "presumptive" or confirmed identification is required (Whittington et al., 1988).

Morphology and Colonial Characteristics

The *Neisseria* spp. are Gram-negative diplococci which typically grow in pairs or occasionally grow in tetrads or clusters (Fig. 1). With the exception of *N. elongata*, which are coccobacilli, the *Neisseria* spp. are readily distinguished by their characteristic kidney-bean shaped cells arranged with flattened adjacent sides. Individual cells range in size from 0.6–1.0 μm; strains of some species may form giant cells (Berger, 1963). Flagella and swimming motility are absent, but most species can be piliated and show "twitching motility" (Henrichsen, 1975).

Colony morphology varies among the different species and ranges from small, smooth, transparent, butyrous colonies to wrinkled, dry adherent colonies (Reyn, 1974). Gonococcal and meningococcal strains tend to be nonpigmented; pigmentation has little taxonomic value because pigment expression is highly dependent on growth conditions. Many species have the ability to variably express different surface components, and these changes can be recognized by changes in colony morphology. For example, gonococci grown on clear agar-based medium form colonies that exhibit diversity with respect to size, edge morphology and light refractivity. Figure 2 demonstrates some of the difference in colony morphology when pili are expressed (piliated, T1 or T2) or when pili are absent (non-piliated, T3

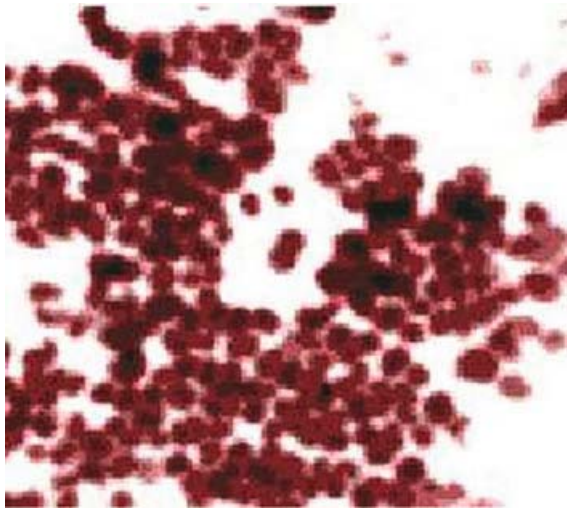

Fig. 1. Gram stain of *Neisseria gonorrhoeae* strain F62.

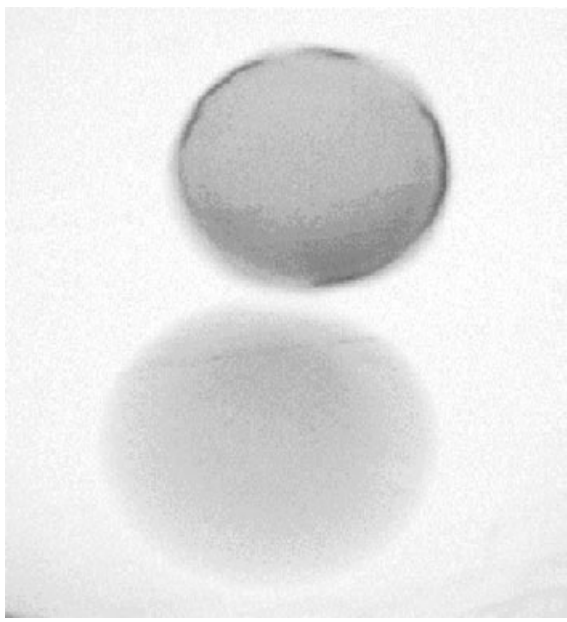

Fig. 2. Colony morphology variation due to the presence or absence of pili. The colonies were visualized under a dissecting microscope using light refracted with a sintered glass filter. The colony on the top represents a piliated colony, while the one on the bottom represents a non-piliated colony.

or T4 organisms). Variation in colony morphology also has been observed in other species and cannot be used as a differential character for the identification of *Neisseria* spp.

Strains of *N. meningitidis*, *N. lactamica*, *N. cinerea* and *N. polysaccharea* grow as translucent, nonpigmented colonies that closely resemble the gonococcus on isolation media (Morello et al., 1985). The colonies observed in young cultures (16–18 h) of most strains are smooth but may become sticky due to lysis of the cells with prolonged incubation. Strains of the *N. subflava*, *N. sicca* and *N. mucosa* are generally colistin-susceptible and do not grow on gonococcal isolation media. The colonies of most commensal strains are pigmented or opaque. The colonies of older cultures (>48 h) of *N. sicca* strains may be distinctly wrinkled.

Biochemical Characterization

Table 1 provides a list of traditional tests used to identify strains of *N. gonorrhoeae*.

Nucleic Acid Probes

The microbiological diagnosis of gonorrhea based on culture on selective medium produces about 80–95% sensitivity, with false-negative results attributed to poor specimen storage, transport problems, and inhibition of growth by the components of selective media (Hook and Handefield, 1990). As an alternative diagnostic test, DNA hybridization techniques have been developed and offer the promise of eliminating transport and specimen collection issues, which are believed to affect test sensitivity in the field setting.

Since 1992, two new nucleic acid-based tests for the diagnosis of gonococcal infection have been approved by the United States Food and Drug Administration (Gen-Probe Pace 2 and Abbott LCR). These nucleic acid hybridization tests are based on the ability of complementary nucleic acid strands to specifically align and associate to form stable double-stranded complexes with their target seqeunces. The GEN-PROBE PACE 2C System uses single-stranded DNA probes with chemiluminescent labels that are complementary to the ribosomal RNA of the target organisms. After the ribosomal RNA is released from the organisms, the labeled DNA probes combine with the ribosomal RNA of the target organisms to form stable DNA:RNA hybrids. The labeled DNA:RNA hybrids are separated from the nonhybridized probes and the amount of signal retained by the test sample is measured in a luminometer. The sensitivity for detecting *N. gonorrhoeae* infections with this system is ~96%, with a specificity of 99% (Iwen et al., 1995).

The ligase chain reaction (LCR) is an in vitro nucleic acid amplification technique that exponentially amplifies targeted DNA sequences. This assay amplifies target sequences within the *N. gonorrhoeae* opacity gene. The sensitivity for detecting *N. gonorrhoeae* infections using the LCR assays is also quite high, ~98% sensitivity, with a specificity of 99.7% (Ching et al., 1995).

Table 1. Differential characteristics of *Neisseria* spp.

Species	Acid from						Nitrate reduction	Polysaccharide from sucrose	DNAse	Colistin resistance
	G	Ma	Su	F	Mn	L				
N. gonorrhoeae	+	–	–	–	–	–	–[a]	–	–	R
N. meningitidis	+	+	–	–	–	–	–[a]	–	–	R
N. lactamica	+	+	–	–	–	+	+	–	–	R
N. cinerea	–	–	–	–	–	–	+	–	–	R
N. sicca	+	+	+	+	–	–	+	+	–	S
N. subflava	+	+	d	d	–	–	+	+/–	–	S
N. flavescens	–	–	–	–	–	–	+	+	–	S
N. mucosa	+	+	+	+	–	–	+	+	–	S
N. canis	–	–	–	–	–	–	–[a]	–	–	
N. denitrificans	+	–	+	+	+	–	+	+	–	
N. elongata	–	–	–	–	–	–	+	–	+	S
N. polysaccharea	+	+	–	–	–	–		+	–	S/R
N. macacae										
N. animalis	–	–	–	–	–	–	–	+		
N. dentiae										
N. weaveri	–	–	–	–	–	–	–			

Symbols: +, present; and –, absent.

Abbreviations: G, glucose; Ma, maltose; Su, sucrose; F, fructose; Mn, mannitol; L, lactose; R, resistance; S, susceptibility; S/R, some strains are resistant and some are sensitive; and d, delayed reaction.

[a]Reduction can occur on low levels of nitrate; higher levels are toxic.

Preservation

Pathogenic species of *Neisseria* are notoriously difficult to maintain in a viable state for long storage periods (Cody, 1978). Storage at 4°C quickly leads to loss of viability. Freeze-drying is a convenient method for long-term storage of these organisms; however, many laboratories are not properly equipped to use this method. Long-term storage of Neisserial strains typically is achieved by suspending cells in trypticase soy broth supplemented with 15% glycerol, with subsequent freezing and storage of cells at –60°C or lower. *Neisseria gonorrhoeae* and *N. meningitidis* remain viable for up to 6 months when resuspended in a gelatin-based media, with storage at –20°C (Harbec and Turcotte, 1996).

Physiology

Metabolism

Oxygen Requirements *Neisseria gonorrhoeae* has been isolated from body sites where anaerobes can be isolated, suggesting that the gonococcus can grow under reduced oxygen tensions. *Neisseria gonorrhoeae* has been shown to grow anaerobically in the laboratory when provided with nitrite as a terminal electron acceptor for anaerobic respiration (Knapp and Clark, 1984a). Because nitrite is present in biological fluids, the ability to grow aerobically or anaerobically by anaerobic nitrite respiration may be one of the factors responsible for the diversity of body sites from which gonococci can be isolated. The gonococcus possesses a copper-containing nitrite reductase (AniA) in its outer membrane (Householder et al., 2000), whose expression is tightly regulated by oxygen (Hoehn and Clark, 1992b). The AniA is essential for growth and survival of gonococci in oxygen-depleted environments (Mellies et al., 1997). Gonococci cannot grow anaerobically by fermentation, and it is likely that the reduction of nitrite must be essential to the maintenance of the redox potential of the cell. Nitrite is reduced by all *Neisseria* spp. isolated from humans with the possible exception of some serogroups of *N. meningitidis* and some strains of *N. lactamica*, *N. cinerea* and *N. polysaccharea* (Morse and Knapp, 1987).

At least three gonococcal outer-membrane proteins (OMPs) are induced and at least five OMPs are repressed by anaerobic growth in gonococcal strain F62. In addition, AniA (formerly Pan1) is the major anaerobically induced OMP and its expression is restricted to anaerobically grown cells (Clark et al., 1987). The presence of antibodies in serum samples from patients with gonorrhea that react with one or more of the anaerobically induced proteins provides further evidence that gonococci are growing anaerobically in vivo (Clark et al., 1988).

Superoxide dismutase is thought to be ubiquitous in aerobes and a requirement for aerobic life (Fridovich, 1975). Gonococci are an apparent exception to this rule. Among aerotolerant cells, the gonococcus is unusual because despite its frequent isolation from purulent exudates containing polymorphonuclear leukocytes vigorously evolving O_2^- and H_2O_2, it contains no superoxide dismutase (SOD). The absence of SOD from *N. gonorrhoeae* strains has been demonstrated under a variety of oxygen-stress conditions. The high tolerance of *N. gonorrhoeae* for extracellular O_2^- and H_2O_2 appears to be due to very high constitutive levels of peroxidase and catalase activity combined with a cell envelope impervious to O_2^- (Archibald and Duong, 1986). Meningococci and the nonpathogenic *Neisseria* spp. differ from the gonococcus in that they possess SOD (Norrod and Morse, 1979).

Among the Neisseriae, *N. gonorrhoeae* is unusual in that the vast majority of strains produce large amounts of catalase (Archibald and Duong, 1986). Gonococci have catalase levels that are 50–5,000 times greater than those of *N. meningitidis* and the other *Neisseria* spp. (Norrod and Morse, 1979). The high catalase levels in gonococci may be partially responsible for its ability to survive aerobically in the absence of SOD. It has been hypothesized that the large amounts of catalase produced by *N. gonorrhoeae* effectively convert H_2O_2 into water and molecular oxygen, affording the organism direct protection against damage by H_2O_2 and indirect protection against damage by hydroxyl radicals formed from H_2O_2 produced by phagocytes (Hassett and Cohen, 1989). The ability to produce high levels of catalase also improves the survival of *N. gonorrhoeae* in the presence of H_2O_2-producing strains of *Lactobacillus acidophilus*, which suggests that catalase may contribute significantly to the ability of *N. gonorrhoeae* to colonize tissues of the female genital tract (Zheng et al., 1994).

Carbon Dioxide Requirement The gonococcus and the meningococcus both require an increased CO_2 tension for isolation from clinical specimens (Griffin and Racker, 1956; Tuttle and Scherp, 1952). *Neisseria gonorrhoeae* strains vary widely in their requirements for CO_2 and/or the HCO_3^- anion. Incubation in the presence of ambient CO_2 tends to maximize the growth response on solid medium of those strains that require it for growth. With a broth culture, $NaHCO_3$ (0.009 M) greatly reduces the lag phase and also increases the total growth (Talley and Baugh, 1975). The presence of ambient CO_2 is

particularly important if growth is to be obtained after the plating of small inocula. Medium containing 0.1% $NaHCO_3$, if incubated in a closed environment, appears to be equivalent to medium without bicarbonate incubated in ambient CO_2 in supporting the growth of some but not all strains of *N. gonorrhoeae* (Jones and Talley, 1977). The requirement for CO_2 is often lost on subculture (Platt, 1976).

The stimulatory effect of exogenous CO_2 or bicarbonate is linked to the initiation of growth and is most apparent during the lag phase. The amount of exogenous CO_2 required varies inversely with the size of the inoculum, suggesting that cellular metabolism can contribute to the CO_2 pool. Carbonic anhydrase is known to be involved in the assimilation of CO_2 by *Neisseria* spp. This enzyme has been cloned and characterized and is homologous to carbonic anhydrases from the animal kingdom (Chirica et al., 1997). Phosphoenolpyruvate (PEP) also can be carboxylated by the gonococcus (Cox and Baugh, 1977; Jyssum and Jyssum, 1962).

Carbohydrate Metabolism Much of our knowledge of intermediary metabolism in the Neisseriaceae has come from studies done in the 1970s and has been summarized in several reviews (Chen et al., 1989; Morse, 1976; Morse et al., 1977). From these studies it has been shown that *Neisseria* spp. do not catabolize many carbohydrates and some species (*N. cinerea*, *N. flavescens* and *N. elongata*) are asaccharolytic (Knapp et al., 1984c). Glucose is the only carbohydrate that can be used as an energy source by the gonococcus. The disaccharides maltose, lactose and sucrose are used by several *Neisseria* spp., and their utilization has been used as an aid in speciating isolates of *Neisseria* (White and Kellogg, 1965).

Amino Acid Metabolism The biosynthesis of amino acids by *Neisseria* spp. occurs by pathways similar to those in other microorganisms. The *Neisseria* spp. vary widely with respect to their amino acid requirements. In general, the nonpathogenic *Neisseria* spp. are able to grow in a defined medium containing one to five amino acids (McDonald and Johnson, 1975). The amino acid requirements of the pathogenic species are more complex. Amino acid requirements have been used to differentiate among isolates (auxotyping) for epidemiologic purposes (Carifo and Catlin, 1973). Auxotyping data have been useful in elucidating some of the pathways of amino acid biosynthesis in the gonococcus by demonstrating that the requirement for certain amino acids is associated with spontaneous mutations in genes encoding enzymes involved in the biosynthesis of amino acids (Lerner et al., 1980; Shinners and Catlin, 1978). While the arginine biosynthetic pathway is probably the best characterized of all of the amino acid biosynthetic pathways (Martin et al., 1990; Martin and Mulks, 1992; Picard and Dillon, 1989), most *Neisseria* biosynthetic pathways are genetically quite similar (Zhou and Spratt, 1992) and seem to share properties seen for most bacteria (Jyssum, 1992).

Most *Neisseria* spp. are able to grow with sulfate as a unique source of sulfur. However, while a few strains of *N. meningitidis* require cysteine, the need for cysteine (which can be satisfied by thiosulfate) is linked to the lack of sulfite-reducing-activity (Le Faou, 1984). The gonococcus exhibits an absolute requirement for cysteine (or cystine; Catlin, 1973).

Amino acids can be used as energy and carbon sources by many *Neisseria* spp. via their oxidation by the tricarboxylic acid (TCA) cycle (Hebeler and Morse, 1976a). Glutamate, proline, and to a lesser extent aspartate are the preferred amino acids (Holten, 1973; Holten, 1976; McDonald and Johnson, 1975). Glutamate dehydrogenase is a key enzyme in the catabolism of glutamate and proline, and all *Neisseria* spp. contain two species of glutamate dehydrogenase (Hebeler and Morse, 1976a; Holten, 1973).

Neisseria spp. posess aminopeptidases that are capable of hydrolyzing L-amino-acid-naphthylamide derivatives of various amino acids (D'Amato et al., 1978). Meningococcocci can be distinguished from gonococci by the presence of *N*-glutamyl aminopeptidase (Delmas et al., 1985). However, some of the nonpathogenic species (*N. mucosa*, *N. sicca*, *N. perflava*, *N. subflava* and *N. flava*) also produce *N*-glutamyl aminopeptidase (Hoke and Vedros, 1982; Riou et al., 1982).

Cellular Structures

Proteins These include the porins, opacity-associated proteins, the Opc protein, reduction-modifiable proteins, the H.8 protein, anaerobically induced proteins, other membrane proteins, iron-binding proteins, IgA protease and RTX homologs.

Porins The porins are the most abundant outer-membrane proteins on the Neisserial surface. Most *Neisseria* spp. express only one type, referred to as "Por"; the meningococcus is an exception, expressing two, PorA and PorB. The gonococcus is the only other *Neisseria* spp. known to have a *porA* gene, but this gene is not expressed owing to frameshift and promoter mutations (Feavers and Maiden, 1998). Although Por is antigenically diverse among strains, its expression is thought to be stable

within a given strain. Thus it exhibits allelic variability. These properties make the gonococcal porin an excellent marker for strain classification and epidemiologic studies. Immunological and biochemical data have determined that there are structural variants of the porin, allowing for their use in classification systems (the serovar typing system is based on reactivity to a panel of Por-specific monoclonal antibodies; Knapp et al., 1984b).

The nomenclature for Por has changed over the years. Initially, all meningococci were described as expressing either a class 2 or a class 3 outer-membrane protein (OMP), and most strains also express a class 1 OMP (P1 protein). The class 1 OMP has been named "PorA," and its gene has been designated "*porA*." Similarly, the class 2 and class 3 OMPs have been named "PorB" and their gene has been designated "*porB*." The amino acid sequences of PorA and PorB do not vary within an isolate, but sequence differences may be used to differentiate strains. The antigenic variety of meningococcal PorB and PorA proteins forms the basis of serotyping and serosubtyping, respectively (Abdillahi and Poolman, 1988; Frasch et al., 1985).

In the gonococcus, Por was originally described as Protein 1 (PI). It is now known that this protein is similar to the *porB* locus of the meningococcus. In the gonococcus, it has been shown to function as an anion-selective porin allowing the passage of small molecules through the outer membrane. The general structure of Por consists of nine internal conserved regions separated by eight surface-exposed regions that are highly variable in both amino acid sequence and length (Carbonetti et al., 1988). Constitutively expressed at high levels in all gonococci, Por is surface-exposed and elicits a strong immune reaction during infection (Ison, 1988).

Alleles of this locus in *N. gonorrhoeae* have been assigned to two homology groups based on close sequence and immunological relationships and are designated as either "PIA" or "PIB" (Carbonetti et al., 1988). These two homology groups differ in molecular weight, susceptibility to proteolysis, and antigenic reactivity. Alleles within each group are much more similar to each other than they are to members of the other group (i.e., PIA and PIB form distinct monophyletic groups which predate the splitting of species within the genus Neisseria), and individual *N. gonorrhoeae* strains express either a PIA or a PIB allele (Smith et al., 1995).

The meningococcal and gonococcal porins are candidates for inclusion in vaccines. They also can induce a T-cell dependent response toward normally T-cell independent antigens (e.g., polysaccharide) in both humans and mice (Donnelly et al., 1990). Many laboratories have characterized variants from many strains, resulting in the availability of DNA sequence for many genes. A three-dimensional structural homology model for *Neisseria* porins has been generated. The data indicate that the protein possesses a 16-strand β-barrel fold characteristic of porins (Derrick et al., 1999). The β-barrel structure is interspersed with more variable regions, forming the putative surface-exposed loops. This model has provided information on the spatial relationships of variable regions of peptide sequences in the PorA and PorB trimers and insights relevant to the use of these proteins in vaccines.

Gonococcal Por has been shown to translocate from the bacterial outer membrane into epithelial cell membranes at the site of contact between the bacteria and the cell membrane, suggesting that Por plays an active role in infection. Also, Por may impair neutrophil function by inhibiting phagocytosis, actin polymerization, the secretion of microbicidal enzymes, and opsonin receptor expression of stimulated neutrophils (Bjerknes et al., 1995; Haines et al., 1988; Haines et al., 1991). Gonococcal Por can inhibit phagosome maturation in human macrophages (Mosleh et al., 1998). Furthermore, translocation of porin to target cells leads to a Ca^{2+} influx, which promotes gonococcal invasion (Müller et al., 1999). The mechanism of Por immunopotentiating ability is unclear; however, in vitro studies have shown that purified Por can act as B-cell mitogens, inducing increased IgM secretion and B-cell proliferation (Wetzler et al., 1996). Mutagenesis of the *N. gonorrhoeae* porin reduces invasion in epithelial cells and enhances phagocyte responsiveness (Bauer et al., 1999).

The nucleotide sequence of Por from a number of *Neisseria* spp. has been examined. Alignment and analysis of all available Por sequences by use of the structurally conserved regions derived from the PorA and PorB structural models have allowed researchers to perform phylogenetic analysis. The phylogenetic relationships of the porin genes from most of the commensal and animal *Neisseria* spp. (*N. animalis*, *N. canis*, *N. cinerea*, *N. dentirificans*, *N. flavescens*, *N. flava* and *N. sicca*) indicate significant sequence variation, with many lineages emerging near the root. Phylogenetic analyses were consistent with an important role for horizontal genetic exchange in the emergence of different porin classes and confirmed the close evolutionary relationships of Por from *N. meningitidis*, *N. gonorrhoeae*, *N. lactamica* and *N. polysaccharea*. Only members of this group contained three conserved lysine residues, which form a potential guanosine triphosphate (GTP)-binding site implicated in pathogenesis. The model placed these residues on the inside of the pore, in close proximity, consistent with their role in regulating pore function

when inserted into host cells (Derrick et al., 1999).

Opacity-associated Proteins Colonies with markedly differing color and opacity characteristics were found when gonococci were propagated on translucent, solid medium. The optical properties were independent of piliation and appeared to be related to the degree of aggregation among the gonococci comprising the colonies. Dark, opaque colonies contained highly aggregated gonococci that were more susceptible to killing and to solubilization of their surface components than were organisms comprising light, transparent colony forms (Swanson, 1978a). Most, but not all, gonococcal Opa proteins are associated with pronounced colony opacity; there is no relationship between colony opacity and expression of Opa proteins in the meningococcus (Hagman and Danielsson, 1989). Gonococci from opaque colonies have cell-wall, outer-membrane proteins, which are lacking from organisms that form transparent colonies. These proteins exhibit heat modification of their apparent subunit molecular sizes, are easily extracted by deoxycholate, have apparent subunit molecular weights varying from 24–29 kDa, and are exposed on the surfaces of gonococci (Swanson, 1980). The opacity-associated proteins are more susceptible to hydrolysis by trypsin than is the major outer membrane protein (OMP), but gonococci possessing the opacity-associated protein(s) also show enhanced susceptibility of their major OMPs to the action of trypsin (Swanson, 1978b). In the older literature, these proteins were referred to as "Opacity proteins," "protein II," or "p.II" in the gonococcus and "Class 5 proteins" in the meningococcus. A standard nomenclature has been adopted and these proteins are now referred to as "opacity proteins" (Opa).

DNA sequence analysis of *opa* genes from a variety of Neisserial strains indicates that Opas have eight transmembrane β strands and four surface-exposed loops (Blake et al., 1981; Malorny et al., 1998). The two-dimensional structural model containing four surface-exposed loops was constructed based on rules derived from porin crystal structure and on conservation of sequence homology within transmembrane strands. In addition, a variety of epitopes recognized by monoclonal antibodies have been mapped to the surface loops (Malorny et al., 1998).

The Opa (opacity) proteins are a family of antigenic and phase-variable OMPs with a monomer molecular mass of approximately 28 kDa found in all *Neisseria* spp. (Stern and Meyer, 1987; Wolff and Stern, 1995). They play a critical role in the colonization, survival, transmission and pathology of Neisserial diseases. Multiple *opa* loci containing different *opa* alleles are scattered around the chromosomes of *N. gonorrhoeae* (11 to 12 *opa* loci; Bhat et al., 1991) and *N. meningitidis* (8 loci; Achtman et al., 1988; Aho et al., 1991).

The importance of Opa proteins in infection is demonstrated by the frequent Opa phase and antigenic variation observed during human infections (Hobbs et al., 1994; Jerse et al., 1994; Schmidt et al., 2000). Figure 3 shows variations in colony morphology due to changes in Opa expression. Intensive research on this area has allowed investigators to define the genetic basis for the variability seen in Opa expression. All *opa* genes sequenced to date contain a repeated pentameric sequence (CTCTT). Changes in the number of pentamers are responsible for the high-frequency phase variable expression seen for these genes (Stern et al., 1986). As a result, a given organism can reversibly express zero, one or multiple different Opa proteins (Blake and Gotschlich, 1984). In addition, among clonally related epidemic meningococcal isolates, there is greater variation of Opa protein expression than can be accounted for by the *opa* gene repertoire of any individual strain. Characterization of *opa* genes by DNA sequence analysis and Southern blot experiments of eight closely related isolates of serogroup A *N. meningitidis* (subgroup IV-1) from a recent meningitis epidemic in West Africa indicated that changes occurred in the *opa* genes of these bacteria as they spread through the human population over a relatively short period of time. The distribution of sequences present in hypervariable (HV) regions of the *opa* genes suggests that duplication of all or part of *opa* genes into other *opa* loci changed the repertoire

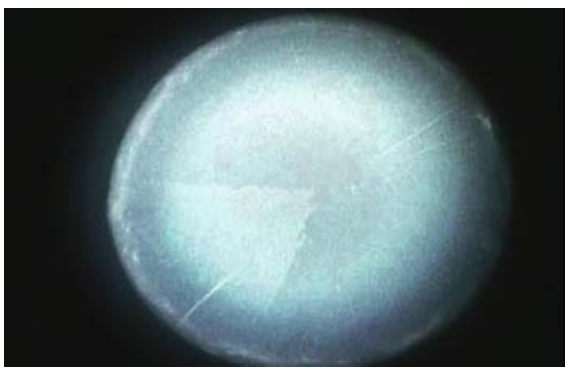

Fig. 3. Variation of Opa expression within a colony. Colonies expressing Opa give rise at high frequency to variants that no longer express Opa. These Opa minus variants appear transparent when the colony is visualized under a dissecting microscope with oblique light. In the colony shown here, the majority portion of the colony is expressing Opa.

of Opa proteins that could be expressed. Additional variability in this gene family appears to have been introduced by horizontal exchange of *opa* sequences from other meningococcal strains and from the gonococcus. These results indicate that processes of recombination and genetic exchange contributed to variability in major surface antigens of this clonal population of pathogenic bacteria (Hobbs et al., 1994).

The large array of Opa proteins provides the Neisserial cell with a diverse surface. Each Opa protein seems to bind to a specific receptor on a eukaryotic cell, with specific Opa proteins mediating adhesion and/or invasion of epithelial cells; different cell lines give different results for the same Opa, indicating that Opa expression is a determinant of cell tropism (Bessen and Gotschlich, 1986; Bos et al., 1997; Grant et al., 1999; Griffiss et al., 1999). The binding of Opa to various eukaryotic cells can initiate different signaling cascades, leading to entry of bacteria via distinct pathways. When Opa mediates binding to heparan sulfate proteoglycan (HSPG) receptors, invasion is mediated through a novel pathway that begins with the localized recruitment of HSPG receptors, F-actin, and tyrosine-phosphorylated proteins at the attachment sites (Grassme et al., 1996; Merz et al., 1996; Merz and So, 1997). This stimulates at least two lipid hydrolysis enzymes, the phosphatidylcholine-specific phospholipase C (PC-PLC) and an acidic sphingomyelinase (Smase; Grassme et al., 1997). This system does not utilize clathrin-coated pits or caveolae (Grassme et al., 1996).

A second uptake pathway directed through HSPG-binding Opa proteins occurs through bacterial binding to vitronectin (Duensing and van Putten, 1997). Infection experiments demonstrated that proteoglycan-deficient Chinese hamster ovary cells efficiently internalized dextran sulfate/vitronectin-coated gonococci, suggesting that soluble sulfated polysaccharides could substitute for cell surface glycosaminoglycans in the internalization process. These results suggest a novel mechanism of vitronectin binding in which sulfated polysaccharides act as molecular bridges, linking the glycosaminoglycan-binding sites of vitronectin and gonococcal Opa (Duensing and van Putten, 1998). Opa-vitronectin interactions promote adhesion and entry into cells, which are nonpermissive for invasion in the absence of vitronectin (van Putten et al., 1998).

A number of Opa proteins function as adhesins through binding to CD66 receptors present on human cells. These CD66 antigens, or carcinoembryonic antigen family members, constitute a family of glycoproteins belonging to the immunoglobulin superfamily. All Opa variants recognize this class of receptors in a differential manner (Bos et al., 1998). A HeLa cell line expressing human CGM1a antigen (HeLa-CGM1a) binds recombinant *Escherichia coli* expressing gonococcal Opa and subsequently engulfs the bacteria (Chen and Gotschlich, 1996a). Some of the carcinoembryonic antigen (CEA) gene family members bind some Opa proteins but not others (Popp et al., 1999). Because single residue changes within Opa, in the so-called "adhesiotopes," can change specificity for Opa binding (Virji et al., 1999), this indicates that (depending on the particular Opa protein expressed) different host cell responses occur, including binding, uptake and the activation of different signal transduction pathways.

Opc Protein The class V outer-membrane protein was cloned in studies of meningococcal antigens and renamed "Opc." The *opc* gene is present in many but not all meningococcal strains and is associated with virulence (Olyhoek et al., 1991; Seiler et al., 1996). Also present in the gonococcal (GC) genome are *opc* homologs of undefined function (Zhu et al., 1999). Expression of *opc* undergoes clonal variation via mutations in a poly-C tract within its promoter region. Spontaneous mutational variation in the number of cytidine residues changes expression levels or eliminates expression altogether (Sarkari et al., 1994). Expression of *opc* in *E. coli* confers a weak adhesive phenotype. Expression of Opc in nonencapsulated meningococci confers on the bacteria the ability to adhere to and invade endothelial cells independently of Opa and pili (Virji et al., 1992; Virji et al., 1995). Pre-exposure of Opc-expressing bacteria to serum increases the number of bacterial interactions at the apical surface, suggesting that Opc binds to serum factor(s) and this in turn increases adherence to tissue culture cells (Virji et al., 1994). The expression of Opc appears to enable bacteria to utilize the normal signal-transduction mechanism of host cells via ligands in sera that adhere to endothelial cell integrins. The Opc protein can bind vitronectin (perhaps indirectly) and it has been proposed that, as with Opa30, vitronectin forms a molecular bridge between the bacterium and integrin receptors on the cell surface (Virji et al., 1994).

Reduction-modifiable Proteins Gonococci and meningococci both contain a protein whose apparent molecular weight in sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) increases upon treatment with a reducing agent such as β-mercaptoethanol (McDade and Johnston, 1980). In older literature, the gonococcal reduction-modifiable protein was termed "protein III," whereas the meningococcal one was designated "the class 4 protein." Under

the new nomenclature for the Neisseria, these proteins are now referred to as "Rmp."

Reduction-modifiable proteins (Rmps) are constitutively expressed and antigenically invariable (Lytton and Blake, 1986). Cross-linking studies with bifunctional reagents suggested that gonococcal Rmp is closely associated with the Por (Leith and Morse, 1980; Newhall et al., 1980). Structurally and immunologically conserved among gonococci (Judd, 1982), Rmp possesses surface-exposed domains (Swanson et al., 1982). The structural genes for Rmp have been cloned from both gonococci and meningococci, and there is 96% homology at the DNA level for these genes (Gotschlich and Seiff, 1987; Klugman et al., 1989). According to its predicted amino acid sequence, the molecular mass of Rmp should be about 24 kDa (Gotschlich et al., 1987). However, this protein contains two disulfide loops and migrates in SDS-PAGE gels at about 32 kDa under reducing conditions. No free C-terminal amino acids are released by carboxypeptidase digestion of Rmp, suggesting that the carboxy terminus is blocked or unavailable for cleavage (Blake et al., 1989). The amino acid sequence of Rmp is homologous to that of the C-terminal part of OmpA from *E. coli* and to that of OprF from *Pseudomonas aeruginosa* (Sugawara et al., 1996).

The function of the Rmp, both in the pathogenesis and in the physiology of the organism, remains unknown. The *rmp* gene is found exclusively in chromosomal DNA of pathogenic Neisseriae, indicating that this protein contributes to the virulence of *N. gonorrhoeae* and *N. meningitidis* (Wolff and Stern, 1995). In the meningococcus, this protein forms complexes with the lactoferrin receptor LbpA, the transferrin receptor TbpA and the siderophore receptor FrpB as well. This complexation apparently resulted in a stabilization of oligomeric forms of these iron-regulated proteins. In vitro experiments further revealed a reduced ability to acquire iron from human lactoferrin in a *rmp* mutant (Prinz and Tommassen, 2000). In the gonococcus, antibody to Rmp increases susceptibility to gonococcal infection (Plummer et al., 1993).

Rmp may have an important role in serum resistance; it is highly immunogenic. Because some murine monoclonal antibodies (MAbs) against Rmp block the serum bactericidal activity of other antibodies directed against gonococci and meningococci (Virji and Heckels, 1988), the blocking action is ascribed to anti-Rmp antibodies competing for binding with other antibody complexes on the gonococcal surface, resulting in the deposition of C5b-9 in a nonbactericidal form, preventing killing of the bacterium (Joiner et al., 1985). Human complement-fixing immunoglobulin G (IgG) antibodies to Rmp block the bactericidal activity of IgG directed to cell-surface antigens such as lipooligosaccharide (LOS; Rice, 1989). Anti-meningococcal bactericidal activity of normal human sera is inhibited by the presence of antibodies directed against Rmp. The ability of Rmp to induce blocking antibodies suggests that its presence in experimental vaccines, based on meningococcal outer membranes, may be antagonistic to the development of effective bactericidal immunity (Munkley et al., 1991). However, antibodies against Rmp have been purified from sera from vaccinees immunized with the Norwegian meningococcal group B outer-membrane vesicle vaccine. While the human sera and purified antibodies reacted strongly with Rmp in immunoblots, experiments with whole bacteria showed only weak reactions, indicating that the antibodies mainly reacted with parts of Rmp that were not surface-exposed. The purified human anti-Rmp antibodies were neither bactericidal nor opsonic against live meningococci. However, these antibodies were not vaccine induced, as they were present also before vaccination. The data indicate that vaccination with meningococcal outer-membrane vesicle vaccines containing the Rmp does not induce blocking antibodies (Rosenqvist et al., 1999).

H.8 Protein The pathogenic *Neisseria* possess an OMP designated "H.8," which possesses a conserved monoclonal antibody (MAb)-binding epitope. The H.8 DNA sequence predicted a 6.9-kD peptide comprising 14 tandemly repeated pentameric sequences. Also predicted was a lipoprotein leader consensus sequence, which probably specified acylation because the *E. coli*-expressed protein was tightly associated with lipid. Lipid appeared to contribute significantly to H.8 antigen's unusual electrophoretic mobility. When outer-membrane preparations from some *Neisseria* spp. are subjected to SDS-PAGE and Western blots are probed with an H.8-specific monoclonal antibody, a cone-shaped monoclonal antibody-binding band with an apparent molecular mass ranging from 18–30 kDa is observed. The protein responsible for this band, designated "the H.8 protein," does not stain with Coomassie blue but can be visualized by staining with silver. The apparent molecular mass of this protein varies in different strains but appears to be constant within a single gonococcal or meningococcal strain (Cannon et al., 1984).

A gonococcal mutant lacking the H.8 protein has been obtained by insertional inactivation of the gene, indicating that this protein is not essential for the growth and survival of gonococci in a complex medium (Woods et al., 1989). The association of the H.8 protein with the pathogenic *Neisseria* spp. has led to the speculation that it

may be important in pathogenesis or as a candidate vaccine. The lipid-modified azurin (Laz), one of two distinct surface proteins recognized by the H.8 monoclonal antibody, is present in all pathogenic Neisseria. The mature protein has two domains; one contains an H.8 epitope and the other has extensive homology to azurins, a class of bacterial copper-binding proteins. The cellular location of Laz and the serum immune response to Laz have been examined in patients with disseminated Neisserial infections and show that Laz is probably contained in the Neisserial outer membrane, although unlike most OMPs, it is Sarkosyl soluble. By probing recombinant bacteriophages encoding the H.8 and azurin domains of Laz, Trees and Spinola (1990) showed that (whereas the H.8 epitope is immunogenic in patients with disseminated Neisserial infections) the azurin domain of Laz plays little role in eliciting an antibody response in these patients.

Anaerobically Induced Proteins When the gonococcus is grown under anaerobic conditions with nitrite as a terminal electron acceptor, at least three gonococcal OMPs are induced and at least five OMPs are repressed by anaerobic growth in gonococcal strain F62. The major anaerobically induced OMP, AniA (formerly Pan1), is tightly regulated, and its expression is restricted to anaerobically grown cells (Clark et al., 1987). Western blot analyses with sera from patients with gonococcal disease indicated that infected individuals responded to AniA, suggesting that AniA is expressed in the host and that the gonococcus encounters an anaerobic environment during infection (Clark et al., 1988). An antigenically related anaerobically induced OMP was detected in all strains of gonococci tested and in a number of commensal *Neisseria* strains but was poorly expressed in *N. meningitidis* strains (Hoehn and Clark, 1990).

Northern blot analysis demonstrated the lack of *aniA* message in aerobically grown cells. Primer extension data from anaerobically grown cells suggested the presence of two RNA transcripts differing in length by only 9 bps representing two overlapping promoters, one with homology to *E. coli* σ70 promoters and the second sharing homology with *E. coli* gearbox promoters (Hoehn and Clark, 1992a). (Gearbox promoters were named for their characteristic of producing a gene product at a rate inversely proportional to the growth rate of the cell.) These promoters are induced during the stationary phase in *E. coli* (Vicente et al., 1991). Also, AniA shares significant identity with copper-containing nitrite reductases (Mellies et al., 1997). Additional structural studies have shown that AniA is a lipoprotein (Hoehn and Clark, 1992b). Immunological data suggest that *N. lactamica* and *N. cinerea* possess homologs to AniA, whereas *N. sicca*, *N. flava* and *N. mucosa* did not. The other commensals tested, *N. subflava* and *N. perflava*, exhibited only a minor reaction (Hoehn and Clark, 1990).

Iron-binding Proteins Iron is an essential component and serves as a cofactor of the membrane-bound electron transport chain and of certain soluble enzymes of most bacteria. Free iron is rare in the human body, generally being complexed by a number of high-affinity binding proteins, including transferrin (Tf) in plasma and lactoferrin in secretions and macrophages (Bullen et al., 1978). Because the free iron concentration in the human host is extremely low, the *Neisseria* spp. must have mechanisms for obtaining iron from its host. The sources of iron on human mucosal surfaces that are available to the gonococcus are not well understood; however, the observation that gonococcal strains deficient in the ability to utilize iron from mucosal surfaces are avirulent (Cornelissen et al., 1998) indicates that there is sufficient utilizable iron on the mucosal surface to support infection. Gonococci and meningococci express many novel proteins when grown in vitro under conditions of iron restriction (Dyer et al., 1988; Mietzner et al., 1984; Norqvist et al., 1978). These proteins are synthesized also in vivo inasmuch as antibodies to these proteins can be detected in serum specimens from patients with meningococcal and gonococcal disease (Black et al., 1986; Fohn et al., 1987).

Many microorganisms synthesize low-molecular-weight, high-affinity, iron-binding compounds called "siderophores," which overcome the insolubility of iron and effectively compete with the host iron-binding proteins transferrin and lactoferrin. While the pathogenic *Neisseria* do not produce siderophores (West and Sparling, 1985), in the milieu of the mucosal surface, neighboring microbes do. Neisserial strains scavenge siderophores made by other bacteria, including the *E. coli* hydroxamate siderophore aerobactin (Beucher and Sparling, 1995; West and Sparling, 1987). Neisserial species also transport the *E. coli* phenolate siderophore ferric enterobactin. The process of iron utilization via a siderophore intermediate requires specific binding of the ferric siderophore to a surface-exposed receptor. Both the meningococcus and gonococcus possess genes that share homology with siderophore receptors. Because exogenous siderophores support the growth of these organisms, it indicates that the gonococcus and meningococcus possess pathways for the uptake of iron-siderophore complexes released by neighboring bacteria (West and Sparling, 1985).

Energy is provided to these TonB-dependent receptors in the form of the proton motive force through the function of an energy-transducing complex of proteins, TonB, ExbB and ExbD (Klebba et al., 1993).

The gonococcus can bind and utilize iron from human transferrin (Blanton et al., 1990; Lee and Schryvers, 1988). Gonococci also can use iron bound to the dihydroxamate siderophores aerobactin, arthrobactin and schizokinen (West and Sparling, 1987; Yancy and Finkelstein, 1981), but they are unable to use the iron bound to the trihydroxamate siderophores desferrioximine B mesylate (Desferal), ferrichrome, ferrichrome A, ferrichrysin, ferricrocin, ferrirubin, and coprogen and the phenolate siderophores enterochelin and vibriochelin (Yancy and Finkelstein, 1981). The gonococcus and meningococcus also utilize iron bound to human lactoferrin, hemoglobin and heme (Mickelsen et al., 1982). The majority of nonpathogenic *Neisseria* spp. cannot use either transferrin or lactoferrin as an iron source (Mickelsen et al., 1982; Mickelsen and Sparling, 1981).

Transferrin Binding In the transferrin-iron internalization system, two proteins (TbpA and TbpB) are essential for the utilization of host-derived iron sources. Mutants defective in the synthesis of either TbpA or TbpB, but not defective in both proteins, can bind transferrin, suggesting that both proteins are surface exposed and function in transferrin binding (Pintor et al., 1998). Because TbpA is similar to other TonB-dependent receptors, it is thought that it forms the pore through which transferrin-bound iron enters the periplasm (Cornelissen et al., 1992). The genes encoding these proteins have been cloned and sequenced from a number of strains.

The TbpA protein is likely to form a TonB-regulated transmembrane β-barrel, through which iron can enter the periplasm. When integrated into the outer membrane, TbpA may form a β-barrel composed of 22 transmembrane strands and probably has a periplasmic globular domain equivalent. Sequence diversity among gonococcal TbpAs is largely confined to four small regions that are postulated to be surface exposed in the gonococcus (Cornelissen et al., 2000). Mutagenesis studies have identified discrete domains within TbpA that are necessary for ligand binding and iron uptake (Boulton et al., 2000). Because expression of the Tf receptor is required to initiate infection in a human challenge model of gonococcal infection (Cornelissen et al., 1998), identification of domains of TbpA that are necessary for the optimal function of this receptor could lead to therapies or prevention strategies aimed at abrogating its function.While gonococcal TbpBs are more diverse than gonococcal TbpAs (Cornelissen et al., 1997), they do not share the great diversity found among meningococcal TbpBs (Rokbi et al., 1993). Meningococcal TbpBs fall into two distinct classes based on molecular mass and sequence characteristics. The low-molecular-weight family, consisting of approximately 26% of tested strains, expresses a TbpB protein of 68 kDa, whereas the high-molecular-weight family expresses a TbpB of 85 kDa (Rokbi et al., 1993). The TbpB protein increases the efficiency of iron uptake from transferrin by virtue of its specificity for the ferrated ligand (Anderson et al., 1994). Also, TbpB is lipidated and exposed on the outer leaflet of the outer membrane (Anderson et al., 1994). Although both Tbps specifically and independently bind human transferrin, TbpB selectively binds the ferrated form of this protein, whereas TbpA binds both ferrated and apo transferrin (Boulton et al., 1998). The study of meningococcal and gonococcal mutants expressing either TbpA or TbpB has shown that both proteins are required for the optimal uptake of human transferrin iron (Cornelissen and Sparling, 1996; Legrain et al., 1993). The conservation of two structurally distinct proteins, both capable of independently binding human transferrin, implies that they play different but related roles. The requirement for both Tbps further suggests that these proteins may interact, forming the functional human transferrin receptor. The meningococcal human transferrin receptor is composed of a TbpA dimer in association with a single molecule of TbpB. This complex binds 1–2 molecules of human transferrin, with TbpB preferentially binding the diferric form of this protein. The TbpA and TbpB proteins bind to distinct separate sites on the human transferrin molecule (Boulton et al., 1998).

Lactoferrin Binding Lactoferrin is the major source of iron on mucosal surfaces. All meningococcal strains possess an iron-regulated cell surface receptor that is specific for human lactoferrin-bound iron as the sole source of iron; many gonococcal strains lack this receptor and cannot grow when iron is complexed with lactoferrin (Mickelsen et al., 1982). Iron uptake from lactoferrin is energy dependent (McKenna et al., 1988). The meningococcal lactoferrin receptor is composed of the integral outer-membrane protein LbpA and the peripheral lipoprotein LbpB. The *lbpA* gene encodes an outer-membrane, lactoferrin-binding protein with homology to the transferrin-binding protein, TbpA. The lactoferrin receptor consists of a macromolecular complex of LbpA and LbpB, where LbpA is present in this complex as a dimer (Prinz et al., 1999). Like TbpA/TbpB, this uptake system is TonB dependent.

The DNA sequence of *lbpA* is highly conserved in the gonococcus and the meningococcus and encodes for a 103-kDa protein. A topology model for LbpA suggests that the protein traverses the outer membrane 26 times in a β-sheet conformation, exposing 13 loops to the bacterial surface (Pettersson et al., 1998). The striking 94% identity between gonococcal and meningococcal genes suggests that the two proteins have the same function. This conservation also suggests that LbpA does not undergo much antigenic variation in vivo, perhaps indicating that it is masked or not highly surface exposed. Primer extension and reverse transcription-polmerase chain reaction (PCR) analysis indicate that *lbpB* and *lbpA* are cotranscribed on a polycistronic iron-repressible mRNA (Lewis et al., 1998). These genes contain a relatively well-conserved ferric uptake regulator (Fur) box upstream of their coding sequence, further supporting the notion that their expression is iron regulated.

The complete nucleotide sequence of *lbpB* has been determined and the gene encodes a 77.5-kDa protein, probably a lipoprotein, with significant homology to the TbpB of *N. meningitidis*. A unique feature of LbpB is the presence of two stretches of negatively charged residues, which are postulated to be involved in lactoferrin binding. Isogenic mutants lacking either LbpA or LbpB exhibit a reduced ability to bind lactoferrin, with *lbpA* mutants being unable to use lactoferrin as a sole source of iron (Pettersson et al., 1998). The genes encoding LbpB have been analyzed from several gonococcal and meningococcal strains and are generally 70–80% identical at the amino acid level, with most of the variability found in two stretches with a high content of negatively charged amino acids. The high degree of variability is disadvantageous for vaccine development, but may be useful for epidemiological studies (Pettersson et al., 1999).

Heme and Hemoglobin Binding Heme compounds are an important source of iron for the pathogenic Neisseriae (Archibald and DeVoe, 1980). They are able to obtain iron from hemoglobin (Hb), haptoglobin-hemoglobin (Hp-Hb) and apo-haptoglobin (apo-Hp; Dyer et al., 1987). Two distinct surface receptors are involved in heme-iron acquisition, HmbR (Stojiljkovic et al., 1996) and HpuA/HpuB (Lewis et al., 1997). The expression of *hmbR* and *hpuAB* undergoes phase variation, with expression controlled by frameshifting involving a polyguanine tract located within the *hmbR* and hpuA loci (Lewis et al., 1999).

The gene for HpuB has been cloned from the meningococcus and the predicted amino acid sequence indicates that HpuB is an outer-membrane receptor belonging to the TonB family of high-affinity transport proteins. Adjacent to and cotranscribed with hpuB is a second open-reading frame (Orf), predicted to encode a 34.8-kDa lipoprotein, HpuA. The 3.5-kb polycistronic *hpuAB* mRNA is transcriptionally repressed by iron, with the transcriptional start site appropriately positioned around consensus promoter and Fur-box sequences. The structure of this operon suggests that HpuA-HpuB is a two-component receptor analogous to the bipartite transferrin receptor TbpB-TbpA (Lewis et al., 1997).

The HmbR receptor is an iron-regulated, 89.5-kDa protein that binds Hb, extracts the heme from it, and transports the heme into the periplasm (Stojiljkovic et al., 1995; Stojiljkovic et al., 1996). An *hmbR* mutant of *N. meningitidis* is attenuated in an infant rat infection model, confirming the importance of Hb acquisition in meningococcal virulence (Stojiljkovic et al., 1995). The HpuAB bipartite receptor is most likely the main Hb receptor of gonococci, inasmuch as the *hmbR* gene contains a premature stop codon in all gonococcal strains tested (Chen et al., 1996b). The lack of *hmbR* and the high prevalence of hpuB in commensals suggest more recent acquisition of *hmbR* by meningococci (Richardson and Stojiljkovic, 1999).

The HemO protein is essential for heme, hemoglobin (Hb), and haptoglobin-Hb utilization. The *hemO* gene is located upstream of the *hmbR* and encodes a protein that is homologous to enzymes that degrade heme. This gene is ubiquitous in commensal and pathogenic Neisseriae. Also, HemO genetic knockout strains of *N. gonorrhoeae* and *N. meningitidis* are unable to use any heme source, whereas the assimilation of transferrin-iron and iron-citrate complexes is unaffected. Furthermore, *hemO* mutants can transport heme into the cell because both heme and Hb were shown to complement an *N. meningitidis* hemA *hemO* double mutant. The expression of the HmbR receptor is reduced significantly by the inactivation of the *hemO*, suggesting that *hemO* and *hmbR* are transcriptionally linked. Comparison of the polypeptide patterns of the wild-type and the *hemO* mutants indicates a general role of HemO in the regulation of gene expression in Neisseriae (Zhu et al., 2000).

The gonococcus and meningococcus can use iron (Fe) from a variety of sources. Insertional mutation of the gonococcal *tonB* homologue results in the failure of gonococci to grow with transferrin, lactoferrin or human hemoglobin as a sole iron source. The *tonB* mutation does not prevent the utilization of Fe from citrate or hemin. This indicates that the pathways for utilization of Fe bound to transferrin, lactoferrin or human hemoglobin are dependent on the TonB

system (Biswas et al., 1997). An alternative TonB-independent system also exists for the utilization of iron from hemoglobin, transferrin or lactoferrin. This system functions with the TonB-dependent receptors HmbR in *N. meningitidis* and HpuB in *N. gonorrhoeae* for growth with hemoglobin (Desai et al., 2000).

A 76-kDa iron-regulated OMP common among Neisserial species, FetA, has homology to the TonB-dependent class of OMPs of Gram-negative bacteria. It is expressed by most Neisserial strains and is a potential vaccine candidate for both gonococcal and meningococcal disease prevention. In addition, FetA functions as an enterobactin receptor. A linked gene, *fetB*, is predicted to encode a protein with sequence similarity to periplasmic binding proteins necessary for transporting siderophores through the periplasmic space of Gram-negative bacteria. Insertional inactivation of *fetB* abolishes ferric enterobactin utilization without causing a loss of ferric enterobactin binding. These data show that FetA binds ferric enterobactin and that FetA/FetB may be part of a system responsible for transporting enterobactin into the cell (Carson et al., 1999).

IgA Protease The pathogenic *Neisseria* secrete immunoglobulin A1 (IgA1) protease, an enzyme that cleaves the hinge of human IgA1 (hIgA1; Plaut et al., 1975). All gonococcal isolates produce one of two similar types of the enzyme (type 1 or 2), which cleave different bonds (Pro-Ser and Pro-Thr, respectively) in the 18-amino-acid hinge region of hIgA1. Production of gonococcal IgA1 protease involves self-directed secretion and autocatalytic processing of a larger precursor protein encoded by the *iga* gene (Klauser et al., 1993). Both proteases are secreted into the culture medium throughout exponential growth. Interestingly, this protein only seems to be found in pathogenic species (Wolff and Stern, 1995).

Numerous functions have been ascribed to it, but its role in pathogenesis remains enigmatic. Alternative roles in gonococcal pathogenesis, other than cleavage of IgA1 at mucosal surfaces, have been proposed for IgA1 protease as it can cleave synaptobrevin II in vitro and, when introduced into the cytosol of bovine chromaffin cells, blocks exocytosis (Binscheck et al., 1995). The type 2 IgA1 protease cleaves LAMP1 (lysosome/late endosome-associated membrane protein; Lin et al., 1997), a major integral membrane glycoprotein of lysosomes. The protease has been proposed to promote bacterial colonization through cleavage of hIgA1 found on the mucosal surfaces. Proteolysis accelerates the LAMP1 degradation rate and results in multiple alterations in the lysosomes of infected cells (Ayala et al., 1998). An *iga* mutant is defective in intracellular growth, compared to the wild-type (WT) parent strain, and this phenotype is likely to be due to the inability of the mutant to cleave LAMP1 and alter lysosomes. In in vitro invasion studies, *iga* mutants are defective in their ability to traverse T84 monolayers (Hopper et al., 2000). A recent human challenge study showed that an *iga* (IgA1 protease gene) mutant is not impaired in its ability to initiate an infection in the human male urethra (Johannsen et al., 1999). These findings suggest that IgA1 protease and other *iga* gene products may contribute to Neisserial pathogenesis at intracellular stages of the infection process. However, a gonococcal *iga* mutant is not impaired in its ability to invade human fallopian tube organ cultures (Cooper et al., 1984).

The IgA1 protease is able to induce proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin, (IL)-1b, IL-6 and IL-8 from peripheral blood mononuclear cells. The capacity of IgA1 protease to elicit such cytokine responses in monocytes is enhanced in the presence of T lymphocytes. Also, IgA1 protease does not induce the regulatory cytokine IL 10. The immunomodulatory effects caused by IgA1 protease require a native form of the enzyme, but the proteolytic activity is not required for the cytokine induction (Lorenzen et al., 1999). The importance of IgA1 protease expression in natural infections is further supported by the observation that invasive isolates of *N. meningitidis* possess protease activity compared to colonizing strains (Vitovski et al., 1999). However, when IgA1 in cervical mucus is examined by Western blotting, no evidence of cleavage fragments characteristic of IgA1 protease activity is seen in gonococcus-infected or control patients. These results suggest that cleavage of IgA1 by gonococcal IgA1 protease within the lumen of the female lower genital tract is unlikely to be a significant factor in the pathogenesis of infections by *N. gonorrhoeae* (Hedges et al., 1998). Taken in total, all of these results suggest that the importance of this protease is in functions other than IgA1 cleavage.

RTX homologs Two iron-regulated proteins (FrpA and FrpC) have been shown to be related to the RTX family of toxins (Thompson and Sparling, 1993a; Thompson et al., 1993b; Thompson et al., 1993c). These genes are predominantly found in some meningococcal isolates. While no hemolytic or leukotoxic activity has been detected for these proteins, their presence in meningococcal strains strongly suggests that they contribute to some aspect of meningococcal pathogenesis.

Other Membrane Proteins. Martin et al. (1997) have reported the identification in the

outer membrane of *N. meningitidis* of a low-molecular-weight protein, Neisserial surface protein A (NspA), that was antigenically conserved and accessible at the surface of intact bacterial cells of all *N. meningitidis* isolates tested. Neisserial surface protein A-specific MAbs were shown to be bactericidal in vitro against several meningococcal isolates. Intraperitoneal injection of these bactericidal MAbs passively protected mice against a lethal meningococcal challenge. They also demonstrated that the injection of recombinant NspA (rNspA) protein produced by *E. coli* protected mice against experimental meningococcal infection. A homolog of this protein also is found in the gonococcus, suggesting that the NspA protein is highly conserved among pathogenic *Neisseria* strains (Plante et al., 1999).

The genes encoding a homologous 85-kDa OMP of *N. gonorrhoeae* and *N. meningitidis* have been cloned and sequenced. The gonococcal gene encodes a 792-amino-acid protein having a typical signal peptide and a carboxyl-terminal phenylalanine characteristic of OMPs. Southern analysis demonstrated that *omp85* is present as a single copy in *N. gonorrhoeae* and *N. meningitidis* (Manning et al., 1998).

A 44-kDa protein has been shown to be structurally invariant among 14 strains of *N. gonorrhoeae*. This OMP fractionated with other Sarkosyl-insoluble OMPs and is susceptible to cleavage in situ by cathepsin. It also covalently binds radiolabelled benzylpenicillin in vitro. Thus, the data suggest that the 44-kDa peptidoglycan-binding OMP and PBP3 are the same OMP (Judd et al., 1991).

Several other Neisserial OMPs have been described. Some of these proteins appear to be both highly conserved and surface exposed. The outer-membrane protein-macromolecular complex (OMP-MC) is a major protein component of the outer membrane, accounting for about 10% of its protein mass (Hansen and Wilde, 1984; Newhall et al., 1980). The OMP-MC has a molecular mass of 800 kDa and is composed of 10–12 identical subunits of 76 kDa. The *omc* gene, which encodes the OMP-MC, has been cloned and sequenced (Tsai et al., 1989). Antibodies against OMP-MC are produced during a natural infection, and anti-OMP-MC antibodies are bactericidal for both homologous and heterologous gonococcal strains in a complement-dependent assay system (Corbett et al., 1988). The function of this protein has yet to be elucidated.

Pathogenic and nonpathogenic *Neisseria* spp. appear to have a common, surface-exposed 70-kDa antigen (Martin et al., 1986). There is some evidence suggesting that anti-70-kDa antigen antibodies have a role in natural immunity to gonorrhea (Aoun et al., 1988a). Nonpathogenic *Neisseria* spp. possess the 70-kDa antigen structure, but elicited less frequently an antibody response in children (Aoun et al., 1988b).

Yang et al. (1995) identified an open-reading frame (Orf) coding for a protein with a predicted size of 19.2 kDa with a typical lipoprotein signal sequence of 21 amino acids. Antisera raised against this protein reacted with meningococcal membrane fractions on a Western blot but did not elicit complement-dependent bactericidal activity. The DNA sequences of the gene from several strains of *N. gonorrhoeae* and *N. meningitidis* were compared and found to be almost identical, except that the coding sequences from all of the gonococcal strains were terminated prematurely as a result of a frameshift mutation.

Lipooligosaccharide Neisserial lipooligosaccharide (LOS) has been examined by chemical, biological and immunological techniques, as well as through visualization after SDS-PAGE (Apicella et al., 1981; Connelly and Allen, 1983; Frasch et al., 1976; Guymon et al., 1982; Jennings et al., 1980; McDonald and Adams, 1971). Gonococcal LOS mediates most of the toxic damage in the fallopian tube model (Gregg et al., 1981), is a key target on the cell surface for bactericidal antibody (Ward et al., 1978), and regulates complement activation on the surface of the organism (Joiner et al., 1985). It also is implicated in the attachment of gonococci to host cells by piliated and nonpiliated organisms (Watt et al., 1978), in the presence or absence of opacity (Opa) proteins (Porat et al., 1995; van Putten, 1993).

Lipooligosaccharides are heterogeneous glycolipids without repeating oligosaccharides (Griffiss et al., 1987) that show wide antigenic diversity among different strains (Apicella et al., 1981; Mandrell et al., 1986). In studies using molecular sieve chromatography, fluorescent-activated cell sorting, and SDS-PAGE analysis, it has been shown that within a strain, LOS components differ in their relative concentrations and in the antigens they express (Apicella et al., 1987; Schneider et al., 1985; Schneider et al., 1988). Antigenic differences between strains are the result of substitutions of various glycose units, alterations in the types of linkages that connect the sugars, or additional decorations of the sugar backbone. *Neisseria* LOSs structurally resemble human glycosphingolipids (Griffiss et al., 1988).

Each gonococcal strain has the genetic potential to make a series of structurally related molecules (Gibson et al., 1989). The oligosaccharides (OSs) of LOS are multiantennary and can branch at two basal heptose residues and at an internal galactose residue (Gibson et al., 1989;

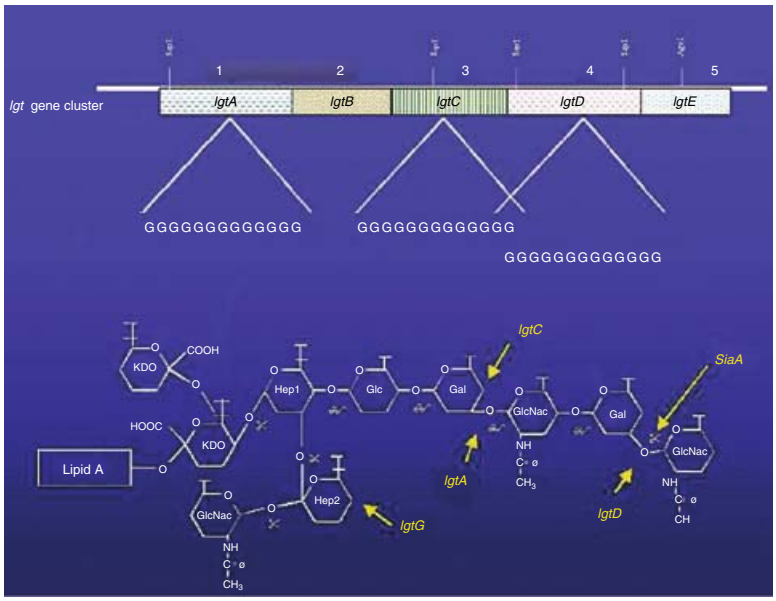

Fig. 4. Schematic depiction of genes and structures of gonococcal LOS. The top part of the figure is a depiction of a gene cluster that is responsible for the synthesis of the major carbohydrate portion of gonococcal LOS (See Gotschlich, 1994). The poly G region indicates the presence of strings of guanines that are responsible for the variable expression of the protein. The bottom part of the figure is a schematic representing the chemical structure of gonococcal LOS. The gene designations indicate those genes whose expression is regulated by changes in the polyguanine tract. When the gene contains enough guanines so that the reading frame for the entire gene is out of frame, the LOS molecule's structure would terminate at this point.

Schneider et al., 1984; see Fig. 4). Besides lipid A and ketodeoxyoctonate (KDO), this molecule contains heptose, glucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine. A culture of gonococcal cells consists of different variants or clonotypes, each of which makes one or more LOS and each of which can interconvert at high frequency (10^{-3}/cell/generation; Apicella et al., 1987; Gibson et al., 1989; Schneider et al., 1984; see Fig. 5 for an example of LOS variation within a colony). The LOS repertoire of the population as a whole represents a summation of the proportional contributions of each clonotype.

In addition to possessing the ability to produce multiple LOS components with different antigenic determinants (Fig. 6), the gonococcus can modify its LOS by "borrowing" host factors and adding them to the OS. Gonococci examined directly from urethral exudates are resistant to the killing action of normal human serum (NHS), but become susceptible upon culture (Apicella et al., 1990; Parsons et al., 1990; Ward et al., 1970). This resistance is due to the fact that the gonococcus sialylates its LOS, adding *N*-acetylneuraminic acid (*NANA*) from cytidine monophosphate-*N*-acetylneuraminic acid to its LOS. The *NANA* can be added to the LOS structure containing the lacto-*N*-neotetrose LOS structure (Mandrell et al., 1990), or the LOS that mimics the human P^k globosyl LOS (Griffiss et al., 2000). Sialylation of Neisserial LOS can contribute to pathogenicity in several ways, including the reduction of phagocytosis by human neutrophils (Kim et al., 1992; Rest and Frangipane, 1992; Wetzler et al., 1992), the binding of IgM and IgA (Vogel et al., 1997), and the oxidative burst in neutrophils (Rest and Frangipane, 1992). In addition, LOS mediates an increased binding of Factor H (a critical downregulator of complement activation; Ram et al., 1998a; Ram

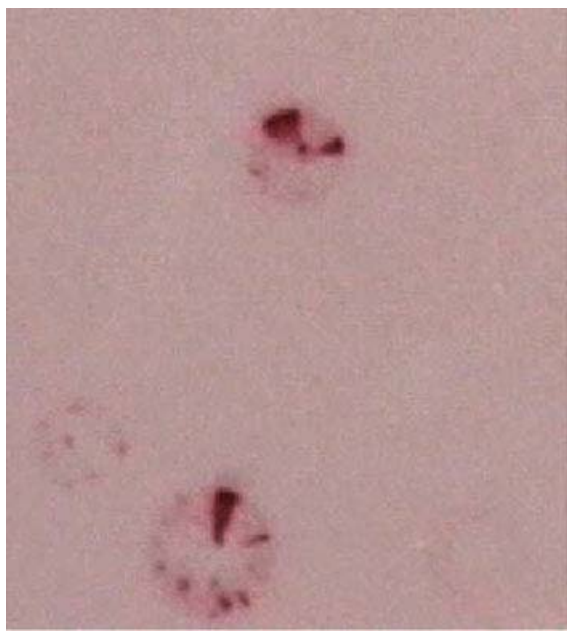

Fig. 5. Demonstration of high-frequency LOS antigenic variation. Colonies of *N. gonorrhoeae* strain F62 were transferred onto Nitrocellulose and analyzed for their ability to bind an LOS specific MAb, 2-1-L8. While the bulk of the colonies transferred were incapable of reacting with the MAb, occasional colonies arose that contained sectors of reactive cells. These sectors are visualized in red.

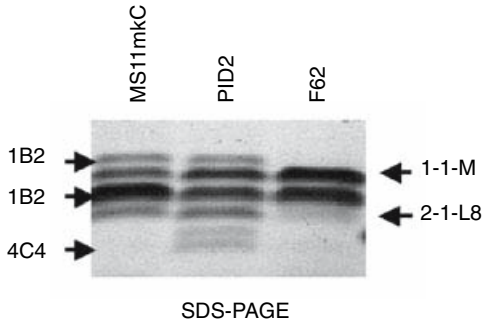

Fig. 6. Complexity of LOS expression. *Neisseria* strains are capable of simultaneously expressing multiple LOS components that each possess unique antigenic properties. The figure represents typical SDS-PAGE profiles that are seen for gonococcal strains. Reactivities with various MAbs have been used as markers for the presence of specific components. The MAb used and its component bound are identified with the arrows.

et al., 1998b). Sialylation also interferes with adherence of bacterial cells to neutrophils in the absence of complement and antibody (Rest and Frangipane, 1992).

The mechanisms by which serum-sensitive gonococci resist the killing action of normal human serum are not fully understood. While gonococci initially recovered from urethral exudates are resistant to the killing actions of nonimmune sera because their LOSs are sialylated (Parsons et al., 1992; Ward et al., 1970), some gonococci are serum resistant even though they lack the LOS form that can be sialylated (Schneider et al., 1985). While the interaction of antibody and complement on the bacterial cell surface may result in the killing of the organism (Rice and Kasper, 1977), some antibodies may block killing by combining with antigens that are identical or adjacent to sites that recognize bactericidal antibody (Joiner et al., 1985). Apicella et al. (1986) demonstrated that the bactericidal activity of lytic IgG antibody can be blocked by IgA antibodies directed against the same LOS or a molecule in close proximity. The identification of loci important for serum resistance has been compounded by the use of strains with different OMP profiles (Cannon et al., 1981; Shafer et al., 1982), different assays for serum resistance (Cannon et al., 1981; Hildebrandt et al., 1978), and the study of LOS targets whose expression is variably expressed. Genetic studies indicate that multiple genes influence the degree of gonococcal resistance to normal human serum (Cannon et al., 1981; Shafer et al., 1982). Our understanding of the role of innate immunity in preventing gonococcal infection is hampered by the lack of information on the structure of the relevant antigens.

The allocation of genetic information and metabolic energy to the task of rapidly changing its surface glycan structures implies that LOS is important to the organisms' survival. *Neisseria gonorrhoeae* is strictly adapted to the molecular environment of the mucous membranes of man. In vivo, LOS phase shifts occur (Apicella et al., 1990) and persistence and induction of symptomatic urethral gonorrhea require that the infecting strain shift to clonotypes that make higher molecular weight OS that share surface epitopes with human polymorphonuclear leukocytes. Also, LOS provides a library of potential surface structures that can be rapidly shifted and that are necessary for the organism's adaptation to the molecular environments of different epithelial surfaces.

A variety of MAbs has been produced that have been used to distinguish between the various LOS components (Kim et al., 1988; Schneider et al., 1985). The MAb 2-1-L8 finds its epitope on an internal α-chain lactose (Lac) residue attached to Hep1 of the basal oligosaccharide (Schneider et al., 1984) forming a highly conserved 3.6-kDa LOS (Kim et al., 1988; see Fig. 4 for structures). Additions of a *N*-acetyl lactosamine (LacNac) residue to the third carbon of the terminal chain lactose can give rise to a component with an apparent molecular mass of 4.5 kDa, capable of binding MAb 1B2 (Kim et al., 1988; Schneider et al., 1985). Further extensions or modifications of this chain are also possible, adding Gal-NAc (binds MAb 1-1M), adding an additional lactosamine (binds 1B2; John et al., 1999) or adding host-derived cytosine monophosphate (CMP)-*NANA*. The α-chain lactose can alternately be extended by adding a galactose to the fourth carbon, producing a structure that reacts with MAb L1-17-1. Although MAb 3G9 and 2C7 reactivity has been used as a marker for elongation of the β-chain, these MAbs are capable of binding to several structurally distinct LOS clonotypes.

The data from many laboratories indicate that removal or addition of a single sugar from the structures shown in Fig. 4 may result in the exposure of new immunogenic epitopes. Specific MAbs may recognize each of these epitopes. However, the presence of LOS structures with the same apparent molecular weight that fail to bind a structure-specific MAb indicates that there are other heretofore unknown structures or structural modifications. Likewise, the ability of LOSs with different migration distances on SDS-PAGE to bind the same MAb indicates that predicting the structure of an LOS molecule based on its MAb binding can be misleading.

The structure of a sufficient number of LOS molecules has been determined (Gibson et al., 1989; John et al., 1991; Yamasaki et al., 1991a; Yamasaki et al., 1991b; Yamasaki et al., 1994) to form a coherent yet incomplete picture as to how the organism assembles its LOS. *Neisseria* spp. synthesize LOS by sequentially adding monosaccharides onto a basal unit. However, the composition of this basal unit and its role in the biosynthetic process are unknown. Although there appears to be some similarity between LOS biosynthesis and LPS biosynthesis, enough differences have been detected to suggest that our reliance on knowledge of LPS biosynthesis in the enterics, although serving as a useful starting point for studies on LOS biosynthesis, has hampered the development of appropriate models for LOS biosynthesis. The multiantennary nature of LOS molecules permits increased molecular diversity, as oligosaccharide chains can be extended from more than one antenna, i.e., to either or both heptose residues and to either internal (Lac) or terminal (LacNac) galactose (Gal) residues. It is this same biosynthetic flexibility that provides diversity among mammalian cell surface glycolipids. This ability to vary both composition and configuration in LOS is also another sharp reminder of the differences between LOS and LPS.

Most work on the genetic basis of Neisserial LOS biosynthesis has been performed on isogenic sets of gonococcal strains: FA19 and its LOS defective counterparts FA5100 and JWS1; 1291 and its LOS defective counterparts 1291a-1291e and F62. The LOS-defective mutants were originally selected as spontaneous pyocin-resistant mutants and have been described previously (Dudas and Apicella, 1988; Shafer et al., 1982). Strains resistant to the killing action of pyocin have alterations in their LOS (Connelly and Allen, 1983; Dudas and Apicella, 1988; Morse and Apicella, 1982). In fact, SDS-PAGE analysis of FA19 reveals several distinct LOS components ranging in apparent molecular weight from 3.6–5.0 kDa. Strain FA5100 produces a single small molecular weight component consisting of a single heptose linked to two KDO molecules (Gibson et al., 1993): JWS1 produces a single LOS that lacks heptose (D. C. Stein, unpublished observations). The structure of the LOS isolated from 1291 and its LOS-defective variants has also been determined (John et al., 1991). The 1291 mutants differ from each other in the sequential loss of individual sugars on the α-OS chain. The structure of LOS isolated from strain 15253 also has been determined and the data indicate that this strain elongates the β-chain (Erwin et al., 1996; Yamasaki et al., 1994). The structure of LOS isolated from strain MS11mkC possesses an *N*-acetyl-lactosamine repeat added onto the α-chain lacto-*N*-neotetraose structure (John et al., 1999). Comparing the structure of LOS from F62 (Yamasaki et al., 1991b) to that seen in 1291 provides further evidence for additional structural variations. While both strains possess the same basic sugar backbone in their OS, they differ in the composition of their γ-chain (in F62, this is composed of an *N*-acetylglucosamine, whereas in 1291, it is composed of 1, 4 diacylglucosamine).

Glycolipid biosynthesis is extremely complex (Robbins et al., 1967). For its biosynthesis, it requires genes encoding for glycosyl transferases, other lipid biosynthetic enzymes, the synthesis of biosynthetic precursors, and housekeeping molecules involved in transport or extracytoplasmic assembly. By comparing the structures of various pyocin mutants, it is possible to infer the types of mutations that could produce the altered LOS phenotype, but it is not possible to infer the exact function of individual genes.

Most of the genes involved in LOS biosynthesis in the gonococcus have been cloned. The gene *lsi-1* encodes a homologue to the *E. coli* gene, *rfaF*, and can complement the LOS defect in FA5100 and restore it to FA19-like LOS expression and reactivity with LOS-specific MAbs (Petricoin and Stein, 1989; Sandlin et al., 1994). The LOS antigenic variation can be regulated by *lgtA*. This gene encodes a glycosyltransferase involved in the addition of *N*-acetylglucosamine-β(1–3) to galactose (Burch et al., 1997). This gene is the first gene in a cluster of genes needed to synthesize the α-oligosaccharide chain (that chain that is the one that is known to be sialylated, as shown in Fig. 4). This cluster also has been cloned and characterized by several research groups in both the gonococcus and the meningococcus (Gotschlich, 1994a; Jennings et al., 1995; Wakarchuk et al., 1996). Several additional genes involved in LOS biosynthesis also have been identified. By altering the anomeric configuration of heptose, subsequent sugar additions were blocked or, when added, failed to form native conformations (Drazek et al., 1995). This indicates that there is a real need to identify the nature of the genetic defect to understand how the phenotype arose. An additional gene, *lgtG*, is needed for β-chain extension. This gene contains a polycytosine tract. When this gene contains cytosines that would produce a full-length protein, strains extend the β-chain and acquire the ability to bind the MAb 3G9 (Banerjee et al., 1998).

The regulation of LOS antigenic variation operates by a mechanism that is different from that described for Opa and pilin. The activity of the protein expressed by *lgtA* is modulated at the DNA level, depending on the number of gua-

nines found in the middle of its coding sequence. Changes in the number of guanine residues change the reading frame, resulting in the production of a functional protein (makes an LOS that reacts with MAb 1B2) or a nonfunctional protein resulting in the production of a truncated LOS (reacts with MAb 2-1-L8). This same gene also is involved in determining if a strain surface expresses multiple LOS components (Burch et al., 1997). While gonococci expressing the lacto-*N*-neotetraose (binds MAb 1B2) moiety can invade human genitourinary epithelial cell lines Hec1b or PC3 (J. M. Griffiss, presented at the 10th International Pathogenic *Neisseria* conference) or ME180 cells (Song et al., 2000), it is not known if this molecule is required for invasion of all epithelial cells, nor if other LOS components also can mediate this process. It is believed that this type of invasion is mediated by LOS binding to the asialoglycoprotein receptor (Apicella et al., 1996; Harvey et al., 1997; Porat et al., 1995). These data indicate the importance of the lacto-*N*-neotetraose structure in gonococcal pathogenesis. However, because attachment and invasion can occur in the absence of this LOS clonotype (Song et al., 2000; van Putten et al., 1995), it further demonstrates the complexity of what is essential for gonococcal pathogenesis.

Although structural studies on gonococcal LOS have been extremely useful in helping understand the genetic pathways utilized for their synthesis, the focus of these studies on a few strains that express highly similar LOS structures has led to the incorrect conclusion that only a small number of LOS structures are present in the gonococcus. Furthermore, studying the role of this molecule in pathogenesis by utilizing strains that can vary the expression of this molecule, without determining if variation of the molecule occurred during the study, has led to incorrect conclusions for the role of this molecule in pathogenesis.

Meningococcal LOS is a critical virulence factor in *N. meningitidis* infections and is involved in many aspects of pathogenesis, including the colonization of the human nasopharynx, survival after bloodstream invasion, and the inflammation associated with the morbidity and mortality of meningococcemia and meningitis. The meningococcus also can express a diverse range of LOS structures. This diversity has been divided into 12 immunotypes, each recognized by a distinct MAb (Scholten et al., 1994). The genetic loci responsible for LOS synthesis in the meningococcus are highly similar to those identified in the gonococcus (Jennings et al., 1999). However, the genetic organization of the *lgt* gene cluster appears to be more variable, with deletions of genes needed to add the terminal Gal*N*ac onto the lacto-*N*-neotetraose precursor commonly occurring. These data indicate that LOS variability in the meningococcus has fewer options owing to the loss of genetic material.

However, the type of LOS structure expressed by the meningococcus is a key factor in meningococcal-host cell interactions. Strains expressing Opc and a sialyated LOS are noninvasive relative to bacteria expressing a nonsialyated LOS (Virji et al., 1995). Studies in the mouse model of meningococcal infection indicate that the L8 immunotype (the α-chain consisting of a lactose) predominates in the nasopharynx, though the L3, 7 and 9 immunotypes are found most commonly in the blood of infected mice (Mackinnon et al., 1993). These studies strongly support the role of terminal LOS structures and their phase variation in pathogenesis of meningococcal disease.

Recent research has shown that meningococcal LOS possesses both quantitative and qualitative differences in immunotype expression. The LOSs are more heterogeneous than what would be predicted using current LOS serotyping (Griffiss et al., 2000). Meningococcal strains, like gonococcal strains, use different kinases and glycosyl transferases to substitute LOS in a variety of ways. The structure of the surface-expressed molecule can vary owing to changes in growth conditions and changes in levels of expression of glycosyl transferases. When a strain has the ability to express multiple LOSs, one may predominate. However, the role that these less well-expressed LOSs play in pathogenesis cannot be understated. Whether these organisms express LOS in vivo and whether certain LOS molecules that are poorly expressed in vitro are necessary for pathogenesis remain to be determined.

Most studies on LOS biosynthesis in the Neisseriaceae have focused on pathogenic strains. However, several commensal *Neisseria* spp. make LOS that share epitopes with the gonococcus and the meningococcus (Kim et al., 1989). However, studies of LOS expressed by a variety of commensal *Neisseria* spp. indicate that considerable heterogeneity exists with respect to size of the molecules made and their chemical composition (Johnson et al., 1976; Sandlin and Stein, 1991). The SDS-PAGE analysis of LOS expressed by several commensal species indicates that some are capable of expressing LPS. This clearly demonstrates another biosynthetic potential found within the genus.

Recently, Virji and coworkers demonstrated the presence of the phosphorylcholine in the LOS of several species of commensal Neisseriae and this property could be used to differentiate commensal from the pathogenic strains. Furthermore, the expression of this epitope is encoded by genes that could undergo antigen variation. They further postulated that the incorporation of

this epitope in LOS has the potential to confer the property of immune avoidance and thus of persistence on mucosa (Serino and Virji, 2000).

Pili Neisseria spp. elaborate type IV pili. Pili are filamentous cell surface structures composed of pilin monomers and a small number of accessory proteins. They mediate the initial attachment of the gonococcus to host mucosal epithelial cells (Swanson, 1973) and are essential in the establishment of infection. Gonococcal pilus phase variation is characterized by a rapid on/off switch in which piliated cells give rise to nonpiliated variants and vice versa. In strain MS11, two regions of the gonococcal chromosome act as pili expression loci (*pilE1* and *pilE2*), whereas several other chromosomal regions contain silent (nonexpressing) pilin sequences (Hagblom et al., 1985).

Pilin is encoded by *pilE* (Meyer et al., 1982). Pilin variation depends on a family of variant genes that undergo homologous, intragenic recombination. When the structural gene is located on the chromosome where it will be expressed, the pilin gene is said to be in the *pilE* site. Additional copies of the gene are located in silent sites (*pilS*). Strain MS11 contains a total of 17 silent copies, which are to varying degrees truncated at their 5′ end. These silent loci can be grouped in seven distinct *pilS* loci (Haas et al., 1992).

Antigenic variation of the gonococcal pilus results from the nonreciprocal transfer of partial pilin sequence information from *pilS* into *pilE* (Haas and Meyer, 1986; Hagblom et al., 1985). The resultant, altered *pilE* gene sequence may encode either an immunologically distinct pilin monomer, which can be assembled into functional pili (antigenic variation), or a pilin monomer which is not produced or is inefficiently assembled. This will result in a switch from a piliated (P^+) to a nonpiliated (P^-) colony phenotype (Hagblom et al., 1985).

PCR-based screening has detected homology to a conserved *N*-terminal region of *pilE* in most *Neisseria* spp. examined, including all human commensal isolates. (The three species failing to display homology were isolated from nonhuman sources.) The predicted protein sequences from these species display features typical of all type IV pilins, possessing the two highly conserved regions, SV2 and CYS2. However, a comparative analysis of *pilE* loci from pathogenic and nonpathogenic species reveals two distinct structural groups: one composed of the pilin genes from *N. lactamica*, *N. cinerea*, and the class II pili-producing subset of *N. meningitidis* isolates; the other of gonococcal and meningococcal class I pilin-encoding genes. Because both class I and class II pilin-producing meningococci can act as pathogens, structural relationships among Neisserial pilin genes do not obviously reflect either species membership or ability to cause human disease (Aho et al., 2000).

Pili participate in a surprising number of functions, including bacterial aggregation (Swanson et al., 1971), adhesion to host cells (Nassif et al., 1993; Swanson, 1973), twitching motility (Brossay et al., 1994; Swanson, 1978a), pilus retraction (Merz et al., 2000), dispersal from aggregates and loss of pili (Pujol et al., 1999). These molecules also interact with host cells in a variety of ways, inducing host responses such as cytosolic Ca^{2+} fluxes (Kallstrom et al., 1998), cortical plaque formation (Merz et al., 1999), and cytotoxicity (Dunn et al., 1995; McGee et al., 1981).

The pilus fiber is composed primarily of pilin, an 18-22-kDa polypeptide. Pilin is synthesized as a precursor protein that is processed by the PilD prepilin peptidase/transmethylase (Freitag et al., 1995). This results in a mature pilin subunit with an α-methylated phenylalanine residue at its N terminus (*N*-met-Phe). Neisserial pilins are further posttranslationally modified by *O*-glycosylation and phosphorylation at sites mapping to the exposed surface of the fiber (Forest et al., 1999; Jennings et al., 1998).

The structure of mature GC pilin has been solved to 2.6 Å resolution (Parge et al., 1995). Fiber diffraction, cryoelectron microscopy, antigenic mapping, and molecular modeling indicate that pilin subunits polymerize into a right-handed helical cylinder with fivefold symmetry about the helix axis (Forest et al., 1996; Parge et al., 1995). The pilin tails form a helical coiled-coil bundle; the pilin heads face outward with the globular domain, forming a 30-residue surface-exposed loop. This loop is the hypervariable region of pilin that exhibits the greatest primary sequence and antigenic diversity (Seifert, 1996).

Neisserial pilins undergo primary sequence variation in nature, as well as in the laboratory (Seifert et al., 1994). Variation occurs through a variety of genetic mechanisms at extraordinary rates of 10^{-4} per cell division and is especially prevalent in regions that map to the fiber surface (Seifert, 1996). Small alterations in the primary structures of Neisserial pilins cause changes in immunoreactivity, posttranslational modification, and adhesive function. While mutations that abolish *O*-glycosylation of the meningococcal pilus or phosphorylation of the gonococcal pilus cause only minor effects on adhesion to host cells and other pilus-related functions, other point mutations cause dramatic changes in host cell binding and tropism (Jonsson et al., 1994; Marceau et al., 1998; Nassif et al., 1993; Virji et al., 1991).

Pilus assembly is hypothesized to occur within the cytoplasmic membrane or periplasm (Fuss-

enegger et al., 1997). Multiple gene products are implicated in pilus assembly. Transposon insertion mutants indicate that the *pilF* and *pilD* gene products are required for gonoccocal pilus biogenesis. Mutants lacking the *pilD* gene product, a pre-pilin peptidase, are unable to process the pre-pilin subunit into pilin and were nonpiliated. The *pilF* mutants processed pilin but did not assemble the mature subunit. Both classes of mutants released S-pilin, a soluble, truncated form of the pilin subunit previously correlated with defects in pilus assembly (Freitag et al., 1995), and are present in the bacterial cytoplasm or associated with the bacterial inner membrane.

The formation of surface expressed pili requires the expression of many proteins: 1) a prepilin peptidase that cleaves a short leader peptide from the subunits; 2) a set of integral membrane proteins located in the inner cytoplasmic membrane that may serve as a platform for fimbrial assembly; 3) a hydrophilic nucleotide-binding protein located in the cytoplasm or associated with the cytoplasmic face of the inner membrane that may energize secretion by ATP hydrolysis; and 4) an OM component that forms a channel allowing the translocation of assembled pili through the OM. Pili biogenesis entails three genetically dissociable steps: 1) fiber formation; 2) fiber stabilization; and 3) surface localization of the intact organelle.

In the gonococcus, translocation of pili occurs by the general secretion apparatus where the pre-PilE precursor is processed during secretion into the periplasmic compartment. These molecules are retained in the inner membrane by their hydrophobic *N*-terminal segments, with their hydrophilic C-terminal domains oriented towards the periplasm (Fussenegger et al., 1997). The PilD signal peptidase removes the leader sequence from the cytoplasmic side of the prepilin to generate mature PilE, which can then undergo assembly as subunits associated with their hydrophobic stems. Although their functions are not well understood, PilF, PilG and PilT are among the factors required for this assembly. It has been suggested based on studies of its homologues that PilF may function as an ATPase or kinase (Freitag et al., 1995). The PilG molecule has been proposed to play a role in the optimal localization or stabilization of PilD and PilF (Tønjum and Koomey, 1997). The assembled pili are thought to be translocated across the OM by a gated pore formed by a multimeric form of PilQ (Drake and Koomey, 1995). The PilP molecule appears to function in stabilizing the expression of PilQ as a multimer (Drake et al., 1997). Gonococcal PilT mutants constructed in vitro no longer display twitching motility. In addition, they have lost the ability to undergo natural transformation, despite the expression of structurally and morphologically normal pilus (Wolfgang et al., 1998). The PilC adhesin appears to facilitate passage of the growing organelle through this pore, although the molecular basis for the role of PilC in this process is not well understood (Rudel et al., 1995a; Rudel et al., 1995b).

PilA and PilB are proposed to be members of the two-component family of prokaryotic proteins that transduce environmental signals to cytoplasmic regulators via phosphorylation. The amino-terminal portion of PilA was predicted to contain a DNA-binding motif, and it has been demonstrated that PilA binds DNA in a sequence-specific manner (Arvidson and So, 1995; Taha and Giorgini, 1995). The predicted protein sequence for PilA has significant homology to two GTPases of the mammalian signal recognition particle (SRP), SRP54 and Srα (Taha et al., 1991).

The PilC protein provides gonococcal pili with adhesive properties. The protein is produced in small quantities and is encoded by two variant genes in *N. gonorrhoeae* MS11 (Jonsson et al., 1991). The expression of PilC is controlled by short variable G stretches affecting the translational reading frame and the expression of each *pilC* gene and thus pilus-mediated adherence to epithelial cells (Rudel et al., 1992). The PilC protein has been located at the tip of type 4 pili (Rudel et al., 1995b) as well as on the surface of the gonococcus (Rahman et al., 1997). Surface-bound PilC is involved in DNA uptake (Rudel et al., 1995a) and probably also in pilus transport. Purified PilC, isolated from either gonococci or meningocooci, binds to epithelial cells in vitro (Rudel et al., 1995b; Ryll et al., 1997). Pili mediate adhesion to several human cell types including epithelial cells, endothelial cells and sperm cells (Gomez et al., 1979; Swanson, 1973).

Gonococci and meningococci engage in twitching motility. The *pilT* mutants have more pili than the wild-type strain, adhere avidly (125–200% of wild-type levels) to epithelial cells, and do not twitch (Wolfgang et al., 1998). Furthermore, *pilT* mutants are defective for the DNA uptake step of genetic transformation (Biswas et al., 1989; Wolfgang et al., 1998). The PilT molecule also is involved in pilus retraction. Gonococcal pilus fibers that lack *pilC* do not support epithelial cell adherence, strongly corroborating earlier observations that meningococcal PilC is essential for epithelial adherence.

Peptidoglycan The peptidoglycan composition of the *Neisseria* is typical of other Gram-negative bacteria. The peptidoglycan is composed of muramic acid, glucosamine, alanine, diaminopimelic acid and glutamic acid in an approximate molar ratio of 1 : 1 : 2 : 1 : 1, respectively

(Hebeler and Young, 1976b). The percentage of peptide crosslinking is approximately 41%, which is relatively high for a Gram-negative bacterium (Rosenthal et al., 1980). Gonococcal peptidoglycan is extensively *O*-acetylated, making it more resistant to lysozyme and other human peptidoglycan hydrolases (Blundell et al., 1980; Rosenthal et al., 1983), and this resistance to hydrolases might enable gonococci to persist in vivo or potentiate the biological effects of peptidoglycan in vivo.

Gonococci do not survive long after the cessation of growth. This decrease in viability is often accompanied by cellular lysis (autolysis) that occurs following the depletion of glucose in the medium (Morse and Bartenstein, 1974). Several peptidoglycan-degrading enzymes have been described in the gonococcus by direct biochemical analysis or deduced by the analysis of released peptidoglycan fragments. D-Alanine carboxypeptidase (or endopeptidase; Chapman and Perkins, 1983; Davis and Salton, 1975), *N*-acetylmuramyl-L-alanine amidase (Hebeler and Young, 1976c), transglycosylase (Rosenthal, 1979; Sinha and Rosenthal, 1980), exo-*N*-acetyl-glucosaminidase (Chapman and Perkins, 1983), and endo-*N*-acetylglucosaminidase (Chapman and Perkins, 1983; Gubish et al., 1982) activities have been described in gonococci. It is likely that some of these enzymes normally have a biosynthetic role in cell growth, but that under non-growth conditions (i.e., no cell wall biosynthesis), enzyme activity results in peptidoglycan hydrolysis. Only AtlA, a peptidoglycan transglycosylase that acts as an autolysin in the stationary phase, has been characterized at the molecular level (Dillard and Seifert, 1997).

Other Enzymes And Polysaccharides A variety of *Neisseria* spp. have been shown to synthesize amylosucrase, a glucosyltransferase that synthesizes an insoluble α-glucan from sucrose. The catalytic properties of the highly purified amylosucrase from *N. polysaccharea* have been characterized. This enzyme has been shown to catalyze polymer synthesis, as well as sucrose hydrolysis. Maltose and maltotriose synthesis occur by successive transfer of the glucose moiety of sucrose onto the released glucose. This enzyme also is able to mediate the synthesis of glucosyl transfer onto fructose (Potocki de Montalk et al., 2000). A similar enzyme has been isolated from *N. perflava* (Tao et al., 1988).

An esterase catalyzing the hydrolysis of acetyl ester moieties in cellulose acetate has been purified from *N. sicca* strain SB, a strain that can assimilate cellulose acetate as the sole carbon and energy source. The enzyme is strongly inhibited by phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate, which indicates that the enzyme is a serine esterase (Moriyoshi et al., 1999).

For a discussion of capsules expressed by *N. meningitidis*, please see section below.

Genetics

DNA Content

The genomic DNA sequence of several Neisserial strains has been determined for gonococcal strains FA1090 (http://www.genome.ou.edu/gono.html), for *N. meningitidis* serogroup B strain MC58, and for *N. meningitidis* serogroup A strain Z2491 and *N. meningitidis* serogroup C strain FAM18. Each of these genomes is approximately 2.2 megabases.

Plasmids in *Neisseria* spp. have attracted considerable attention because of their potential role in virulence and association with antibiotic resistance. Many *Neisseria* spp. contain cryptic plasmids, i.e., plasmids with no measurable phenotype. The best-studied plasmid is the gonococcal cryptic plasmid. Almost all gonococcal strains harbor this plasmid (Dillon and Pauze, 1981). The DNA sequence for this plasmid has been determined (Korch et al., 1985b). While early studies suggested that cryptic plasmid sequences could be found in the chromosome of gonococcal strains, subsequent experimentation has shown that this homology is due to the presence of repetitive sequences that can be detected by hybridization under low stringency conditions (Sarandopoulos and Davies, 1993b).

Three promoters have been identified in the cryptic plasmid coding sequence that can direct transcription in *E. coli*. These promoters do not seem to function at a detectable level when the gonococcus is grown in vitro under normal growth conditions. The derived amino acid sequence of a 1.2-kb segment of the plasmid shows a high degree of sequence similarity to the Mob proteins encoded by the colicinogenic plasmids ColA, ColE1 and ColK. An additional Orf has been shown to have significant homology to *cppB* gene, whose product is expressed when the gonococcus is grown under the appropriate growth conditions (Sarandopoulos and Davies, 1993a). Variant cryptic plasmids have been identified that harbored insertion within the *cppB* gene and these insertions would interrupt the expression of this gene. The presence of the insertion suggests a mechanism of modulating the expression of the *cppB*, suggesting an unknown role for this gene in virulence (Roy et al., 1988).

Other *Neisseria* spp. occasionally carry cryptic plasmids. Some strains of *N. meningitidis*, *N. lactamica*, *N. mucosa* and *N. cinerea* carry plas-

mids of various sizes that hybridize with probes made from the gonococcal cryptic plasmid (Ison et al., 1986). Some *Neisseria* spp. also carry cryptic plasmids that do not hybridize to the gonococcal cryptic plasmid probes (Verschueren et al., 1982).

Plasmids encoding β-lactamase have been identified in the gonococcus and meningococcus (Bäckman et al., 1993; Roberts, 1989). These nonconjugative plasmids range in size from 4.1–7.3 Kb in size and share considerable homology with one another (Dillon and Yeung, 1989). These plasmids may have evolved by insertion of the Tn*A* sequence (perhaps introduced from enteric bacteria) into a phenotypically cryptic plasmid of *Haemophilus parainfluenzae*, with subsequent introduction into gonococcal strains (Brunton et al., 1983). Sequence analysis of plasmids of differing molecular weight suggests that these plasmids are all derived from a common progenitor plasmid (Pagotto et al., 2000).

Two types of conjugative plasmids have been described in the *Neisseria* spp. A 24.5-MDa plasmid carries no detectable markers for antibiotic resistance. However, it efficiently mobilizes itself and β-lactamase plasmids between strains of gonococci, meningococci and other commensal *Neisseria* (Roberts and Knapp, 1988b). A 25.2-MDa conjugative plasmid that encodes tetracycline resistance has been identified in gonococci and meningococci. This plasmid was formed by the transposition of the TetM determinant onto the 24.5-MDa conjugative plasmid. Like the β-lactamase plasmids, this plasmid seems to have arisen on several independent occasions (Xia et al., 1995) The 25.2-MDa plasmid will mobilize β-lactamase plasmids and has an extended host range (Roberts and Knapp, 1988a; Roberts and Knapp, 1988b). A variety of Neisserial strains possessing elevated levels of resistance to erythromycin have been identified. Genetic analysis of these strains indicates that they have acquired one or more known rRNA methylase genes, including *ermB*, *ermC* and/or *ermF* (Roberts et al., 1999b). Many of these isolates also are multiresistant and carry a complete copy of the conjugative transposon.

A group of plasmids that are genetically related to the enteric plasmid RSF1010 have been described in *N. meningitidis*, *N. mucosa*, *N. subflava* and *N. sicca* (Facinelli and Varaldo, 1987; Pintado et al., 1985; Rotger et al., 1986). Some of these plasmids specify resistance to sulfonamide alone, whereas others specify resistance to sulfonamide, streptomycin and penicillin.

Genetic Mechanisms

TRANSFORMATION Catlin (1961), who showed that nearly all *Neisseria* spp. were transformable, originally described transformation in the Neisseria. While all gonococcal strains are naturally competent for DNA uptake, successful transformations occur at a much higher frequency when the organism is piliated (Sparling, 1966). Phenotypic expression of competence requires only a utilizable energy source (glucose) and cations, preferentially Mg^{2+} or Ca^{2+}. Piliated gonococci are competent in genetic transformation in all stages of growth in minimal and enriched media, but nonpiliated cells are almost totally incompetent (Biswas et al., 1977).

Experimental data indicate that DNA enters cells in native, double-stranded form (Biswas and Sparling, 1981). More recent experiments suggest that single-stranded DNA intermediates may be part of the transformation process (Chaussee and Hill, 1998). It is known that single-stranded DNA can transform the gonococcus (Stein, 1991). The DNA is taken up into a DNAse resistant state very quickly (Dougherty et al., 1979). In addition, DNA fragments containing the sequence GCCGTCTGAA are preferentially taken up from the environment (Goodman and Scocca, 1988). These sequences are located throughout the genome. They appear to be located more commonly at the end of coding sequences. These sequences are not absolutely required for transformation, as uptake-sequence independent transformation does occur, albeit at a dramatically lower frequency (Boyle-Vavra and Seifert, 1996).

The transformation process can be divided into three distinct steps: 1) sequence-specific uptake of transforming DNA into a DNase-resistant state; 2) transfer of DNA to the cytosol; and 3) processing and recombination of the incoming with the resident DNA. Mutants defective in each of these steps have been identified (Biswas et al., 1989). The process of DNA uptake in gonococci requires the expression of at least three distinct components: pili, PilT and ComP (Wolfgang et al., 1999). Piliated *pilT* and *comP* mutants are deficient in sequence-specific DNA uptake into the cell, the earliest demonstrable step in Neisserial competence (Wolfgang et al., 1998). The ComA, ComL and Tpc factors are not essential for DNA uptake and rather act in a subsequent step. Mutants in *comA* grow normally and are DNA-uptake proficient but blocked in the translocation of DNA into the cytoplasm (Facius and Meyer, 1993). The *comL* gene encodes a peptidoglycan-linked lipoprotein, which is required for efficient transformation; most mutations in *comL* appear to be lethal (Fussenegger et al., 1996a). The *tpc* mutants produce a distinctive rough-colony morphology and bacterial growth in clusters of four (tetrapacs; Fussenegger et al., 1996b). Tetrapacs can be resolved by cocultivation with wild-type gono-

cocci, indicating that Tpc is a diffusible protein. Interestingly, Tpc is absolutely required for the natural transformation competence of piliated gonococci. Each of these mutants lacks the characteristic nucleolytic processing observed with the incoming DNA in both wild-type and non-transformable RecA-deficient gonococci, indicating that they are blocked in the processing and/or the delivery of DNA to the cytoplasm (Facius et al., 1996). Transformation of gonococci with plasmid DNA is markedly more efficient when the transformation recipient contains homologous plasmid DNA (Graves et al., 1982). When the recipient strains lack a homologus sequence, the majority of the transformants identified contain deletions (Sox et al., 1979).

Conjugation A conjugation system was initially discovered in β-lactamase-producing gonococci because they could mobilize small non-self-transmissible R plasmids into other gonococci, *Neisseria* spp. and *E. coli*. This conjugation system is mediated by a self-transmissible plasmid of about 24.2 MDa. This plasmid is found in about 8% of gonococcal strains (Norlander et al., 1979; Sox et al., 1978). A 25.2-MDa tetracycline-resistance plasmid also has been found in gonococci. This plasmid appears to be unrelated to the 24.2-MDa conjugative plasmid (Gascoyne et al., 1990). Restriction endonuclease analysis mapping has shown that there are at least two different conjugative tetracycline-resistance plasmids found in the gonococcus (Gascoyne et al., 1991). Conjugal transfer between various Neisserial and other species occurs, but the rates of transfer can vary significantly. Two highly tetracycline-resistant, β-lactamase-producing *N. gonorrhoeae* strains have been used as donors for conjugation with *N. meningitidis* and commensal *Neisseria* spp. Transfer rates for the 4.4- and 3.2-MDa βlactamase plasmids varied, with frequencies between 10^{-1} and 10^{-9} (Roberts and Knapp, 1988a). These data indicate that once an antibiotic-resistance plasmid finds its way into a Neisserial strain, it is only a matter of time before it will oblate the usefulness of that antibiotic.

Bacteriophage Although many investigators have attempted to isolate bacteriophage capable of infecting various *Neisseria* spp., only a few successes have been reported in the literature (Cary and Hunter, 1967; Phelps, 1967). Phage isolates capable of forming plaques on *N. perflava* were similar in terms of host range, latent period, burst size, antigenic properties, morphology and nucleic acid content. Because neutralization studies with antisera demonstrated that the isolates exhibited a very high degree of serological relatedness, this suggests that the isolates represented a single strain of bacteriophage. This phage exhibited a high degree of host specificity, attacking only one of the several strains of *N. perflava* tested and none of the other species tested (Steinberg et al., 1976). Genomic sequence analysis of FA1090 indicates that this strain possesses many genes that are homologous to phage-encoded genes. However, no evidence indicates that these genes are part of a functional bacteriophage.

Restriction and Modification Systems

Restriction enzymes and their associated methylases have been identified in a wide variety of prokaryotes (Roberts and Macelis, 1993). The presence of modified DNA in the *Neisseria* spp. was first studied by analyzing the ability of various DNA samples to resist cleavage with commercially available restriction endonucleases (Norlander et al., 1981; Prere and Fayet, 1985). These studies suggested that the gonococcus possesses three different DNA methyltransferases (Mtases). Korch and coworkers extended these observations by analyzing DNA sequencing gel profiles generated using chemical sequencing methods. They determined that several palindromic sequences contained methylcytosine and postulated the existence of eight different Mtases (Korch et al., 1985a; Korch et al., 1985b). The molecular basis for each of these enzymes was demonstrated by their cloning (Gunn et al., 1992; Gunn and Stein, 1997; Stein et al., 1992; Sullivan and Saunders, 1988) and biochemical characterization (Clanton et al., 1978; Piekarowicz, 1994; Piekarowicz and Stein, 1995; Piekarowicz et al., 1988a; Piekarowicz et al., 1988b; Piekarowicz et al., 1988c; Piekarowicz et al., 1988d). The data presented in Table 2 summarize what is known about gonococcal R/M systems.

While almost all gonococcal strains examined have been shown to express the Mtases at levels sufficient to give complete modification of the endogenous DNA, the expression of the corresponding endonuclease is highly variable (Gunn and Stein, 1993; Piekarowicz et al., 1988a; Piekarowicz et al., 1988b; Piekarowicz et al., 1988d). Genomic analysis indicates that some of these gene pairs have overlapping coding sequences (Gunn and Stein, 1997; Stein et al., 1995; Stein et al., 1998; Sullivan and Saunders, 1988), suggesting a possible transcriptional regulation mechanism.

The biological implications of expressing multiple R/M systems have been examined. Using strains that differed in their ability to express the S.*Ngo*II R/M system, Stein and coworkers showed the endogenous expression of restriction enzymes is able to efficiently restrict the entry of DNA into the gonococcus via transformation, but not conjugation (Stein et al., 1988). This

Table 2. Mtases present in *N. gonorrhoeae*.

Mtase Specificity[a]	Recognition Sequence[b]	Mtase Detection[c]	Commercial Isoschizomer[d]	Assoc. ENase[e]	ENase[e,f] Detection[c]
S.Ngo	RGCGCY	2, 3	*HaeII*	Yes	1, 3, 4
S.NgoII	GGCC	1, 2, 3	*HaeIII*	Yes	1, 3, 4
S.NgoIII	CCGCGG	1, 2	*SacII*	Yes	1, 3
S.NgoIV	GCCGGC	1, 2, 3	*NaeI*	Yes	1, 3, 4
S.NgoV	GGNNCC	2, 3	*NlaIV*	Yes	1, 3, 4
S.NgoVI	GATC	2	*NdeII*	No	ND
S.NgoVII	GCSGC	3	None	Yes	3, 4
S.NgoVIIIa	GGTGA	2, 3	*HphI*	Yes	1
S.NgoVIIIc	TCACC	2, 3	*HphI*	Yes	1
S.NgoIX	GTANNNNNCTC	1	None	No	ND
S.NgoX	GGCC[g]	1, 2, 3	*HaeIII*	?	ND
S.NgoXI	GGCC[h]	1, 2, 3	*HaeIII*	?	ND
S.NgoXII	GCNGC	3	None	?	ND
S.NgoXIII	Unknown	3	None	?	ND
S.NgoXIV	Unknown	3	None	?	ND
S.NgoXV	Unknown	3	None	?	ND
S.NgoXVI	Unknown[i]	3	None	?	ND

Abbreviations:

[a]Indicates the specificity name given to various gonococcal enzymes, based on their recognition sequence, regardless as to what strain they were isolated from.

[b]Indicates the recognition sequence of the Mtase.

[c]Indicates the method by which the enzyme was detected: 1) purification by column chromatography; 2) protection assays based on the ability of chromosomal DNA to resist cleavage by the named isoschizomer; 3) indicates that the gene has been cloned; and 4) indicates that the presence of the corresponding ENase restricts transforming DNA.

[d]Indicates the ENase used in protection assays.

[e]Indicates that the ENase has been detected, either by column chromatography or via a biological assay.

[f]ND = none detected.

[g]Although this clone encodes an enzyme with the same recognition sequence as *S.Ngo*II, the DNA encoding this sequence shares no detectable homology.

[h]This clone methylates the first cytosine in the recognition sequence at the N4 position. It imparts resistance to cleavage by *Hae*III, but not by *Ngo*PII.

[i]The clone encoding this enzyme has significant homology with the *Eco*K system of *E. coli.*

restriction activity could be prevented with appropriate methylation of the incoming DNA (Butler and Gotschlich, 1991). This host-mediated restriction also could be overcome if the large amounts of transforming DNA are employed, thereby overwhelming the restriction system (Gunn and Stein, 1996).

The presence of multiple restriction systems is not limited to the gonococcus. All *Neisseria* spp. that have been examined have been found to be capable of producing multiple R/M systems (Ritchot and Roy, 1990). Genomic sequence analysis suggests that meningococcal strains possess multiple gene pairs, with each sequenced strain possessing about 16 putative R/M systems (Parkhill et al., 2000; Sullivan et al., 1987). According to the Restriction Enzyme Database (REBASE), which is a collection of information about restriction Enases, Mtases, and the microorganisms from which they have been isolated, thus far eight R-M systems have been solely identified in meningococci (Bart et al., 2000; Bucci et al., 1999; Claus et al., 2000; Roberts and Macelis, 1999a; Sparling and Bhatti, 1984). However, the recognition sites of only a few of the meningococcal R-M systems are known, and only a small number have been cloned and/or defined by their restriction activities in cellular lysates (Claus et al., 2000).

The commensal *Neisseria* spp. also appear to have a wealth of diverse R/M systems (Labbe et al., 1990; Lau et al., 1994; Morgan et al., 1996; Qiang and Schildkraut, 1986; Silber et al., 1988). It has been suggested that because the *Neisseria* spp. are naturally competent for DNA uptake, members of this genus have accumulated these genes from other bacteria over time (Stein et al., 1998). The presence or absence of specific R/M systems in various strains has proven to be useful in studying clonal lineages of epidemic strains (Claus et al., 2000).

DNA Repair

Studies on DNA repair in the gonococcus are limited. The gonococcus is deficient in several DNA repair functions, including photoreactivation (Campbell and Yasbin, 1979) and error-prone repair (Campbell and Yasbin, 1984b). Interestingly, the defect in error-prone repair

may actually reflect the absence of an SOS-like system (an inducible DNA repair system), which is needed to trigger the repair process (Black et al., 1998). Gonococci can repair UV-induced damage using two distinct systems: *uvr*-mediated excision repair (Black et al., 1995; Black et al., 1997; Campbell and Yasbin, 1984a) and RecFOR recombinational repair (Mehr and Seifert, 1998). A broader picture of the DNA repair capacity of the gonococcus has emerged with the availability of the complete genome sequence of *N. gonorrhoeae* strain FA1090. Sequence analysis verifies that the gonococcus lacks the genes associated with error-prone repair and photoreactivation. However, it does possess the genes that encode the necessary proteins for recombinational repair, mismatch repair, very short patch repair, excision repair and oxidative damage repair.

Epidemiology

Strain Typing

NEISSERIA GONORRHOEAE Different serological methods have been used to classify and distinguish between various strains of the gonococcus. The most widely employed method for differentiating between various gonococcal strains is based on a combination of auxotyping and serological characterization. Auxotyping is based on the observations of Catlin and coworkers (Carifo and Catlin, 1973), who determined that most gonococcal strains have defects in various biosynthetic pathways, which prevent the organism from growing on chemically defined media in the absence of specific amino acids. Hence a proline auxotroph will only grow on chemically defined media supplemented with proline.

Knapp and coworkers developed a serological method for differentiating antigenic variants of *N. gonorrhoeae* (Knapp et al., 1984a). This is a Por-based serovar classification system, where serovars are defined by their reactivity with Por-specific MAbs. In combination with auxotyping, serological characterization has proved useful for elucidating the epidemiology of *N. gonorrhoeae* (Van Looveren et al., 1999).

Isoenzyme typing, based on multilocus enzyme electrophoresis, has been applied for the epidemiological studies of gonococcal disease (Selander et al., 1986). Genetic relatedness of gonococcal isolates also has been assessed by using DNA-based typing techniques, including restriction endonuclease analysis using frequently or rarely cutting enzymes (Poh et al., 1992) and random and repetitive-motif-based amplification of polymorphic DNA fragments (Poh et al., 1996). The discriminatory abilities of pulsed-field gel electrophoresis (PFGE) and random and repetitive-motif-based amplification of polymorphic DNA have been shown to be superior to that of traditional auxotype/serovar typing.

The use of various primer sets has been used to develop PCR-based methodologies. For example, O'Rourke et al. (1995) developed a PCR-RFLP method using the *opa* gene as the target for amplification. The 11 *opa* genes are amplified with a single pair of primers and digested with frequently cutting restriction enzymes, and the radioactively labelled fragments are separated on polyacrylamide gels to index the strains to particular *opa* types. This *opa* typing is highly discriminatory and is able to establish the close identity of isolates collected from sexual contacts and of differentiated isolates from a worldwide collection made over the last 30 years.

Although auxotype/serotype classification and DNA amplification/fingerprinting can be used in the epidemiologic characterization of strains, DNA amplification/fingerprinting offers a better discriminatory index than auxotyping or serotyping. It is especially useful for differentiating serologically identical strains and nontypable strains. A combination of serotyping and DNA amplification fingerprinting seems to be the best way to differentiate gonococcal strains in epidemiologic studies, bringing together the simplest techniques and the best discriminatory power among isolates (Camarena et al., 1995).

NEISSERIA MENINGITIDIS This species is classified into serogroups based on the immunological reactivity of the capsular polysaccharide (see Table 3 for the structure of the capsules). Although 12 serogroups have been identified, the three serogroups (A, B and C) account for over 90% of meningococcal disease. The serogroup specificity of *N. meningitidis* is determined by the structure of the expressed capsular polysaccharide. The serogroup A capsule is composed of repeating units of (α1-6)-linked *N*-acetyl-D-mannosamine-1-phosphate (O'Rourke et al., 1995). This structure is chemically distinct from those of the capsules of the other major disease-associated meningococcal serogroups, B, C, Y and W-135, which are composed of or contain sialic acid (Liu et al., 1971).

Many investigators have employed molecular typing methods and shown that meningococcal disease is associated with a variety of different epidemiological patterns. The choice of a typing method is dependent upon the epidemiological questions to be answered and on the population genetics of the organism under investigation. With highly clonal populations comprising independent non-recombining lineages, such as serogroup A meningococci, ribotyping, multilocus

Table 3. Structure of the capsules of *N. meningitidis*.

Serogroup	Repeating unit	Linkage	Modifications	References
A	2-Acetamido-2-deoxy-D-mannopyranosyl phosphate	α1-6	*O*-acetyl	Bundle et al., 1974
B	D-*N*-Acetylneuraminic acid	α2-8	None	Jennings et al., 1985
C	D-*N*-Acetylneuraminic acid	α2-9	*O*-acetyl	Jennings et al., 1985
H	Glycerol-D-gal		*O*-acetyl	van der Kaaden et al., 1984
I	α-Gulp*N*AcA-β-ManpNA	β1-3	*O*-acetyl	Michon et al., 1985
K	β-D-ManpNAc-β-D-ManpNAc	β1-4	*O*-acetyl	Van der Kaaden et al., 1985
L	2-Acetamido-2-deoxy-D-glucosyl		Phosphate	Jennings et al., 1983
W135	4-*O*-α-D-Galactopyranosyl-*N*-acetylneuramic acid	α2-6		Bhattacharjee et al., 1976
X	2-Acetamido-2-deoxy-D-glucopyranosyl phosphate	α1-4		Bundle et al., 1974
Y	4-*O*-α-D-2-Glucopyranosyl-*N*-acetylneuramic acid	α2-6	*O*-acetyl	Bhattacharjee et al., 1976
Z	7-*O*-α-D-Acetoamido-α-deoxygalactopyranosyl-2-keto-3-deoxy-D-octulosonic acid	α2-3	*O*-acetyl	
29E				

Abbreviations: Gulp; NAc, neuraminic acid; and Manp, mannosyl phosphate.

enzyme electrophoresis (MLEE), pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and PCR with arbitrary primers (RAPD) or with other gene-based primers provide a constant measure of the relationship between strains. A more restricted portfolio of molecular methods—PFGE, MLEE and MLST—is appropriate for the investigation of the less clonal serogroup B and C meningococci from localized outbreaks (Yakubu et al., 1999).

Most epidemiological investigations of meningococcal disease utilize classification schemes based on differences among meningococcal cell envelope molecules. Like the gonococcus, variations in PorB form the basis for meningococcal serotyping. *Neisseria meningitidis* serogroups B and C were originally subdivided into serotypes by the use of two different classification systems based upon type-specific bactericidal antibodies and immunoprecipitation in agar gels, respectively. The serotype specificities were later found to be associated with different MOMPs and with the lipopolysaccharide. The four or five MOMPs were physiochemically characterized. A new serotyping nomenclature and scheme based on these four or five proteins and the LPS is now used.

Disease Incidence

GONORRHEA The number of reported cases of gonorrhea increased steadily from 1964 to 1977, fluctuated through the early 1980s, increased until 1987, and since 1987 has decreased annually. The incidence of gonorrhea is highest in high-density urban areas among persons under 24 years of age who have multiple sex partners and engage in unprotected sexual intercourse. In 1997, there were 324,901 cases of gonorrhea reported in the United States. The overall rate of gonorrhea has declined 74% since 1975; however, the rate of infection in 1997 was still 122.5 cases per 100,000 persons. The overall gonorrhea rate is much higher in large cities (229.1 per 100,000 people in cities with populations over 200,000). Owing to underreporting, the actual number of cases in the United States is estimated to be about 800,000 cases annually. The annual cost of gonorrhea and its complications is estimated at close to $1.1 billion per year.

MENINGITIS *Neisseria meningitidis* is the leading cause of bacterial meningitis in children and a major cause of septicemia. The majority of cases of invasive meningococcal disease have been caused by *N. meningitidis* serogroup B (46%) and serogroup C strains (45%; Advisory Committee on Immunization Practices, 2000). Disease is most common in children younger than four years, with an attack rate of about eight cases/100,000 individuals. The case fatality rates range from 10–20%, even with rapid and appropriate care. The mortality rate is higher for fulminant meningococcemia than for meningococcal meningitis.

In North America, disease is most common in the late winter and early spring. Blacks have a higher rate of infection than Caucasians (1.5 versus 1.1 in 100,000), and males account for 55% of the cases. People at increased risk for disease include close contacts of index cases, travellers to countries recognized to have hyperendemic or epidemic rates, and persons with functional or anatomic asplenia, properdin deficiency or a terminal complement deficiency (C5 through C8) (Figueroa and Densen, 1991).

Antecedent viral infection, household crowding, chronic underlying illness, and both active and passive smoking also are associated with increased risk for meningococcal disease. During outbreaks, bar or nightclub patronage and alcohol use also have been associated with higher risk for disease. In the United States, persons of low socioeconomic status have been consistently at higher risk for meningococcal disease. However, race and low socioeconomic status are likely risk markers, rather than risk factors, for this disease (Advisory Committee on Immunization Practices, 2000).

Meningococcal meningitis occurs globally. The disease is endemic in temperate climates, causing a steady number of sporadic cases or small clusters with a seasonal increase in winter and spring. A different pattern, with epidemics flaring for two to three consecutive years, has been observed in countries in sub-Saharan Africa. This area has experienced epidemic cycles every 8–12 years in the past, and the intervals between major epidemics have become shorter and more irregular since the beginning of the 1980s. The largest epidemics of meningococcal meningitis are experienced by sub-Saharan African countries within the "meningitis belt" which extends from Ethiopia in the east to Senegal in the west. Epidemics occur in the dry season in this area. While the highest disease rates are found in young children, during epidemics older children, teenagers and young adults also are affected.

Disease

Gonorrhea

Gonorrhea is one of the most common bacterial venereal diseases. Gonorrhea is considered epidemic in the United States. Complicating the high incidence of disease is the increasing appearance of multiple antibiotic resistance. The disease is generally spread via sexual activity; however, gonococcal eye infections can occur in infants infected during the process of passing through the birth canal.

Disease in men occurs after an incubation period of from 2–14 days. Onset of disease is usually marked by mild discomfort in the urethra, followed a few hours later by dysuria and a purulent yellowish green urethral discharge. In women, symptoms usually begin within 7–21 days after infection. A significant portion of infected women is asymptomatic. However, onset is sometimes severe, with dysuria and vaginal discharge. The cervix and deeper reproductive organs are the sites most frequently infected, followed by the urethra, rectum, Skene's ducts and Bartholin's glands. Salpingitis is a common complication.

In women and homosexual men, rectal gonorrhea is common. Women are usually asymptomatic, but perianal discomfort and a rectal discharge may occur. Severe rectal infection is more common in homosexual men. Patients may note a coating of mucopus on stools and report pain on defecation or rectal intercourse. Gonococcal pharyngitis from orogenital contact is usually asymptomatic, but some patients complain of a sore throat and discomfort on swallowing; the pharynx and tonsillar area may be red, exudative and occasionally edematous.

Diagnosis Even though newer nonculture-based molecular methods such as gene amplification and other techniques such as nucleic acid probes are becoming more widely used in diagnostic laboratories, culture is still the most commonly used method of identifying individuals infected with gonorrhea. A Gram-stained smear of urethral discharge allows rapid identification of the gonococcus in >90% of men. However, the cervical Gram stain is only about 60% sensitive in women. This is probably due to the fact that the concentration of *N. gonorrhoeae* in the endocervix can be as low as 10^2 CFU/ml (Perry, 1997). Culture requires that exudates from the urethra, cervix, rectum and other infected sites be inoculated onto a suitable medium (e.g., modified Thayer-Martin medium) and incubated at 35–36°C for 48 h in an atmosphere containing 3–10% CO_2 (Knapp and Rice, 1995).

Nonculture methods, such as enzyme immunoassays and methods based on current molecular biology techniques (i.e., DNA probes, ligase chain reaction, etc.), have been recently incorporated into many clinical laboratories. The advantages to nonculture methods include rapid turnaround time, batching of tests, and the ability to detect nonviable *N. gonorrhoeae*. The enzyme immunoassay methods produce variable results with specimens isolated from women (Donders et al., 1996). The DNA probe assay appears to be much more effective in identifying organisms, with reported sensitivity ranging from 96.3–100% and a specificity of greater than 99% (Iwen et al., 1995; Stary et al., 1993; Stary et al., 1997). While these techniques are more sensitive and more specific and allow testing of urine and vaginal swab specimens, there is still a need for rapid, less expensive, accurate diagnostic tests for gonococci.

Complications In men, postgonococcal urethritis, a common sequela, actually results from infection with other organisms (e.g., *Chlamydia trachomatis*). These two agents are acquired simultaneously but chlamydia has a longer incubation period and does not respond to penicillins and cephalosporins. Epididymitis is

uncommon and usually unilateral. Infection ascends from the posterior urethra along the vas deferens to the lower pole of the epididymis, which becomes painful, warm, tender and swollen. A secondary hydrocele may follow. Abscesses of Tyson's and Littré's glands; periurethral abscesses; infection of Cowper's glands, the prostate, and the seminal vesicles; and urethral stricture are less common.

In women, salpingitis (pelvic inflammatory disease) is the most important clinical problem. This syndrome has two important consequences: 1) sterility and ectopic pregnancy resulting from scarring of the fallopian tubes; and 2) susceptibility to chronic infections due to alterations of fluid flow in the damaged area. Disseminated gonococcal infection (DGI) with bacteremia is more common among women than men.

Gonococcal arthritis, a more focal form of DGI, may be preceded by symptomatic bacteremia. The onset typically is acute, with fever, severe pain and limitation of movement in one or a few joints, unlike DGI, which involves multiple joints. Joints are swollen and tender, and the overlying skin is warm and red. Synovial fluid is usually purulent (WBCs >25,000/μl) and contains gonococci demonstrable on Gram stain and culture. Patients with DGI rarely have positive blood and synovial fluid cultures simultaneously. After aspiration, treatment is started immediately to limit destruction of the articular surfaces of the joint. Pericarditis, endocarditis, meningitis and perihepatitis occur rarely. Ocular infections occur most frequently in newborns but are prevented by prophylaxis.

Prevention At this time, there is no vaccine to prevent gonorrhea. Several prototype gonococcal vaccines have shown limited or no protection against reinfection with *N. gonorrhoeae* despite the generation of serum antibody responses against the vaccine antigens (Boslego et al., 1991). Although candidate vaccines consisting of pilus protein and Protein I (PI) have been evaluated (Tramont, 1989), the results from vaccine trials parallel observations regarding natural gonococcal infections, where local and systemic antibodies have been detected by immunofluorescence in secretions and serum from infected patients, yet there is a high rate of recidivism of gonococcal infections among patients attending sexually transmitted disease (STD) clinics (Tapchaisri and Sirisinha, 1976). The development of a vaccine also is hampered by the lack of an animal model in which to test candidate vaccines. The estradiol-treated mouse model may serve as a useful tool for the evaluation of potential gonococcal vaccine candidates (Plante et al., 2000). There is still hope for the development of effective vaccines because serovar-specific immunity among sex workers has been reported (Plummer et al., 1989).

Meningitis

Two clinically overlapping syndromes—meningitis and bloodstream infection (meningococcemia)—are caused by infection with *N. meningitidis* (meningococcal disease). Meningitis is an infection of the membranes (meninges) and cerebrospinal fluid (CSF) surrounding the brain and spinal cord and is a major cause of death and disability worldwide. The etiology of bacterial meningitis varies by age group and region of the world. Approximately one million cases of bacterial meningitis occur each year, with as many as 200,000 fatalities associated with this infection. Up to 54% of survivors are left with disability due to bacterial meningitis, including deafness, mental retardation and neurological sequelae.

Transmission of the causative agent is by direct contact, including respiratory droplets from nose and throat of infected persons. Most infections are subclinical and many infected people become symptomless carriers. Approximately 20% of the population is colonized with *N. meningitidis* at any given time.

Meningococcal meningitis is characterized by the sudden onset of an intense headache, fever, nausea, vomiting, photophobia and stiff neck. Neurological signs include lethargy, delirium, coma and/or convulsions. Even when the disease is diagnosed early and adequate therapy instituted, the case fatality rate is 5–10% and may exceed 50% in the absence of treatment. A less common but more severe (often fatal) form of meningococcal disease is meningococcal septicemia, which is characterized by rapid circulatory collapse and a hemorrhagic rash.

Diagnosis The proper collection of clinical specimens is important in the isolation of *N. meningitidis*. Clinical specimens should be obtained before antimicrobial therapy is begun to avoid loss of viability of the etiological agent. Treatment of the patient, however, should not be delayed while awaiting collection of specimens. Presumptive diagnosis can be made by observation of Gram-negative diplococci in CSF. However, CSF and blood should be processed in a bacteriology laboratory as soon as possible to allow for comfirmatory culture. Blood and/or CSF are collected and plated on an enriched agar (trypticase soy agar plus IsoVitaleX), with incubation of the agar plates in a 5% CO_2 at 36°C.

Complications The majority of people who contract bacterial meningitis and meningococcal

septicemia survives and make a full recovery; however, some are left with aftereffects or serious disabilities. Physical disabilities and aftereffects include hearing impairments/deafness/tinnitus (can be temporary), loss of sight/changes in eyesight, brain damage, residual headaches, stiffness in joints, loss of balance, clumsiness or lack of coordination, fits/epilepsy, tissue damage (due to acute septicemia, skin grafts may be needed), amputation of digits or limbs (due to acute septicemia) and arthritis.

Prevention Vaccination has proven effective in reducing the incidence of meningococcal disease. The currently available vaccines protect against some serogroups. A single dose of the vaccine may decrease the risk of disease caused by *N. meningitidis* serogroups A, C, Y and W-135. However, vaccination will not totally eliminate risk of the disease because the vaccines do not protect against serogroup B and because, although they are highly effective, they do not confer 100% protection.

Chemoprophylaxis is used for people in close contact with patients in the endemic situation. High risk individuals who should seek chemoprophylaxis include: household contacts, especially young children; child-care or nursery school contacts during previous 7 days; those directly exposed to index patient's secretions through kissing or sharing toothbrushes or eating utensils; those involved in mouth-to-mouth resuscitation or unprotected contact during endotracheal intubation during 7 days before onset of illness; and individuals who frequently sleep or eat in same dwelling as index patient. However, chemoprophylaxis is not an effective means of interrupting transmission during an epidemic. Potential antimicrobials for chemoprophylaxis are rifampicin, mynocycline, spiramycin, ciprofloxacin and ceftriaxone.

Antibiotic Treatment

Gonorrhea The emergence of drug-resistant gonococci has limited the usefulness of many antibiotics for the treatment of uncomplicated gonorrhea. In addition, coexisting chlamydial infections are sufficiently common to require simultaneous presumptive treatment. The United States Centers for Disease Control and Prevention (CDC) recommends that broad-spectrum cephalosporins and fluoroquinolones be used to treat uncomplicated gonorrhea. The CDC currently recommends regimens of fluoroquinolones (single-dose, oral therapy with ciprofloxacin and ofloxacin) for the primary treatment of uncomplicated gonorrhea. In patients known to harbor penicillin-sensitive gonococci, amoxicillin with probenecid may be used.

Meningococcal Disease The use of antibiotics has resulted in a significant decrease in mortality rates among patients with invasive disease. Meningococcal infections are usually treated with penicillin, ampicillin or a combination of penicillin and chloramphenicol.

Antibiotic Resistance

Gonococcal resistance to antimicrobial agents is an increasing problem in the treatment of gonorrhea. A high prevalence of plasmid-mediated high-level or chromosomally mediated low-level resistance to penicillin or tetracycline has been recognized in Southeast Asia and in African countries (Chalkley et al., 1997). The emergence of gonococcal isolates with reduced susceptibility to fluoroquinolones also has become a significant concern (Ison et al., 1998). Antimicrobial resistance in *N. gonorrhoeae* is defined as organisms having a minimum inhibitory concentration (MIC) greater than ≥2.0 mg/liter for penicillin, ≥2.0 mg/liter for tetracycline, ≥128.0 mg/liter for spectinomycin, ≥1.0 mg/liter for ciprofloxacin and ≥2.0 mg/liter for ofloxacin.

Antimicrobial resistance to a variety of antibiotics has been recently reported for *N. meningitidis*. For example, strains expressing a chloramphenicol acetyl transferase possess an MIC for chloramphenicol of ≥64 mg/liter (Galimand et al., 1998). Isolates of *N. meningitidis* with increased levels of resistance to penicillin have been reported, with resistance generally being due to the development of altered forms of the penicillin-binding protein PBP-2 (Mendelman et al., 1988). The MIC for penicillin has increased about 20-fold, with MICs of 1 μg/ml being common. The production of a β-lactamase as a mechanism of penicillin resistance in meningococci is occasionally seen (Dillon and Yeung, 1989). Although the prevalence of resistance in meningococci is still low, continued surveillance is necessary to monitor trends in their susceptibilities to antimicrobial drugs and to advise clinicians on appropriate empirical therapy and chemoprophylaxis.

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Prokaryotes (2006) 5:648–674
DOI: 10.1007/0-387-30745-1_27

CHAPTER 3.2.3

The Genus *Bordetella*

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Introduction

Description of Genus *Bordetella*

The genus *Bordetella* encompasses a group of Gram-negative, small coccobacilli. They are obligate aerobes and fail to ferment carbohydrates such as glucose. Most members have adapted to live in close association with higher organisms, either as overt primary pathogens or in commensal associations that occasionally result in opportunistic diseases.

Current systematic analysis of 16S rDNA sequences has established *Achromobacter* (Gerlach et al., 2001), not *Alcaligenes* as previously reported, as the bacterial species most closely related to the *Bordetella* (see Fig. 1). *Achromobacter* are free-living organisms that are widespread in soil and water but are also commonly isolated from the hospital environment. They only rarely cause disease, and then in compromised individuals, and appear to lack the constellation of virulence factors associated with *Bordetella*. Interestingly, a missing link uniting the environmental *Achromobacter* and the pathogenic *Bordetella* has been identified recently. A novel microbe, strain Se-1111R (Gerlach et al., 2001), was isolated from river sediment and, unlike the *Bordetella*, is able to grow as a facultative anaerobe under nitrate and selenate-reducing conditions. Comparative analysis of the 16S ribosomal, *rpoB*, *ompA* sequences and other biochemical and genetic factors clearly places this organism in the genus *Bordetella*, and a new species has been proposed, *Bordetella petrii* (Gerlach et al., 2001). Further characterization of this environmental isolate will likely provide important clues to the genetic changes that have occurred, which allow *Bordetella* to evolve from a free-living environmental microorganism to a sometimes pathogenic microorganism within a mammalian host.

Species Within the Genus *Bordetella*

The three main species of *Bordetella*, *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, are respiratory pathogens of mammals and have an important economic impact on both human health and agriculture. Historically, the classification into species was based on host range and severity of clinical disease. However, it is now apparent that by true genetic criteria, they actually comprise a single group of highly related subspecies. *Bordetella pertussis* and *B. parapertussis* appear to be more host-adapted and differentiated and are less representative of the group as a whole than are *B. bronchiseptica* strains. As a result, the designation "*B. bronchiseptica* cluster" has been adopted to more accurately refer to this group of highly related organisms and will be used in this chapter. Distinguishing *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* is useful for clinical purposes and these species designations will likely remain as a part of medical microbiology. *Bordetella pertussis* would be more accurately designated as *B. bronchiseptica* subspecies *pertussis*, for example; however, the terms "*B. pertussis*" and "*B. parapertussis*" will be used to refer to these important human pathogens in this chapter.

Other distinct species of *Bordetella* have been identified. A few characteristics can be used to distinguish between the different species (Table 1). These include the ability to grow on MacConkey agar, motility, the ability to produce a positive oxidase reaction using Kovács' reagent or Gaby-Hadley reagent, urease activity, and production of a brown pigment. *Bordetella avium* is most distantly related to the *B. bronchiseptica* cluster, with a G+C content of only 62%, compared to 66–68% for the *B. bronchiseptica* cluster (Fig. 2). It is a respiratory pathogen of birds, and disease in turkeys is of major economic importance to the poultry industry (Temple et al., 1998). *Bordetella trematum* is a causative agent of ear and wound infections in humans (Vandamme et al., 1996). *Bordetella hinzii* colonizes the respiratory tracts of poultry (Vandamme et al., 1995) and on occasion has caused disease in humans, with one report of a fatal septicemia (Kattar et al., 2000). *Bordetella holmesii* also causes septicemia in humans (Weyant et al., 1995). Comparison of 16S rDNA

Table 1. Properties that distinguish between members of the genus *Bordetella*.

Species	Hosts and diseases	Mol% G+C	Growth on MacConkey	Motility	*Oxidase*[a] *by* K	*Oxidase*[a] *by* G-H	Urease	Pigment
B. bronchiseptica cluster		66–68						
B. pertussis	Human, whooping cough		–	–	+	+	–	–
B. parapertussis	Human, mild whooping cough		+	–	–	?	+	+
B. bronchiseptica	Mammals, respiratory disease Dogs, kennel cough Pigs, atrophic rhinitis		+	+	+	+	+	–
B. holmesii	Human, septicemia, respiratory disease	61.5–62.3	+	–	–	?	–	+
B. hinzii	Birds, respiratory tract infections, human opportunist	65–67	+	+	+	+	+	–
B. avium	Birds, respiratory tract, turkey coryza	62	+	+	+	–	–	–
B. trematum	Humans, wounds, ear infections	64–65	+	+	–	–	–	–

[a]Different oxidase assays can give different results (Vandamme et al., 1996). K assays were performed using Kovács' reagent. G-H assays were performed using Gaby-Hadley reagent.

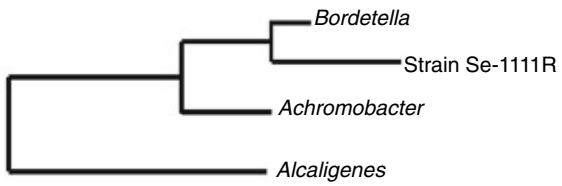

Fig. 1. Phylogenetic relationships of *Bordetella* with other members of the family Alcaligenaceae, based on 16S rDNA sequences. Adapted from Gerlach et al. (2001).

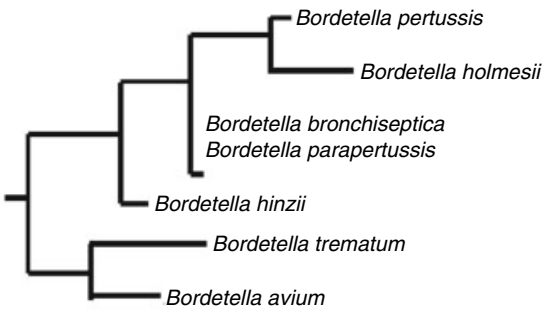

Fig. 2. Phylogenetic relationship of the *Bordetella* based on 16S rDNA sequences. Adapted from Gerlach et al. (2001).

sequences (Gerlach et al., 2001) aligns this species closely with *B. pertussis* (Fig. 2); however, comparison of the 62% G+C content of *B. holmesii* with 67% for *B. pertussis* argues against such a close genetic relationship.

Characterization of the *Bordetella bronchiseptica* Cluster

All members of the *B. bronchiseptica* cluster have been implicated in respiratory disease in mammals. The genomes of a *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* isolate are currently being sequenced (see The *Bordetella pertussis* Genome Project, http://www.sanger.ac.uk/Projects/B_pertussis/ and http://www.medmicro.mds.qmw.ac.uk/bp/). The genomes of *B. bronchiseptica* and *B. parapertussis* are similar in size, approximately 4,400 kilobase pairs (kbp). Interestingly, *B. pertussis* has a smaller genome of approximately 4,000 kbp. It has been hypothesized that in the process of adapting to the human host as its sole pathogenic niche, *B. pertussis* lost genes needed for survival as a free-living organism in the environment. This concept is supported by the fastidious growth requirements of *B. pertussis* and its inability to grow at ambient temperatures much less than 37°C.

Bordetella pertussis is an obligate human pathogen, which causes whooping cough or pertussis. It comprises a distinct and very closely related group of organisms, suggesting recent evolution. The first clinical description of pertussis was recorded in 1640 in France. The unique presentation of whooping cough (the failure of Hippocrates, who accurately described diphtheria and tetanus, to describe pertussis) supports the view that the disease is of recent origin (Cone, 1970). *Bordetella parapertussis* strains also comprise a highly related group of organisms and cause a disease in humans similar to a mild case of whooping cough. Recently, the *B. parapertussis* designation has been applied, inappropriately, to a population of strains that cause

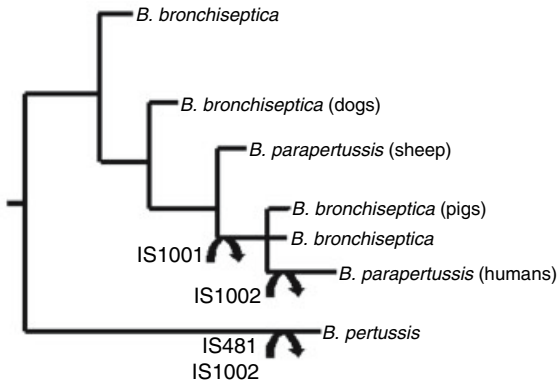

Fig. 3. Genetic relationships between members of the *Bordetella bronchiseptica* cluster based on the presence of genetic elements and multiple enzyme electrophoresis typing. Data from van der Zee et al. (1997) and adapted from Gerlach et al. (2001).

respiratory infections in sheep. These strains have been shown to be more closely related to other *B. bronchiseptica* (van der Zee et al., 1997; Gerlach et al., 2001) than to human *B. parapertussis* isolates (Fig. 3), and this designation should no longer be used for these isolates. *Bordetella parapertussis* will be used only to describe the human cluster in this chapter.

Bordetella parapertussis isolates and human *B. pertussis* appear to have evolved along different evolutionary pathways but interestingly share a unique insertion element, IS1002 (van der Zee et al., 1997), that is present in multiple copies in their chromosomes, which suggests it was shared by horizontal transmission. In addition, *B. pertussis* possesses a unique insertion sequence, IS481. *Bordetella parapertussis* also has a second insertion sequence, IS1001, which is also present in some of the pathogenic *B. bronchiseptica* (van der Zee et al., 1997).

Bordetella bronchiseptica isolates comprise a more diverse group of isolates that vary widely in host range as well as virulence. Members of this group can infect many mammalian species, and the result can vary from persistent colonization without symptoms to serious, debilitating disease. Highly related subgroups have been identified. The strains within a subgroup often have a single preferred host (for example, pigs or dogs) and are more virulent than the broad host-range strains.

Evolution of the *Bordetella* Species

Recent studies suggest that the genus *Bordetella* evolved by a paradigm quite distinct from that put forward by the pathogenicity island theory to explain acquisition of virulence in the enteric species, including *Escherichia*, *Shigella* and *Salmonella* (Ochman et al., 2000). In the pathogenicity island theory, horizontal transmission of blocks of genes (pathogenicity islands) from other species directs evolution into new pathogenic niches. Pathogenicity islands often possess a G+C content quite different from that of the rest of the genome, suggesting they originated from a foreign source, and they are often flanked by tRNA genes, which suggests they were introduced into the chromosome via homologous recombination into these highly conserved, repetitive sites. In contrast to *Escherichia coli*, the genome sequence of *B. pertussis* revealed that the G+C content and codon preferences are uniform for all genes, including those involved in basic bacterial metabolism as well as virulence factors. These observations suggest that the acquisition of virulence traits by *Bordetella* is not recent and the virulence genes evolved in the context of the rest of the genome.

When the genomes of the *B. bronchiseptica* cluster are compared, virulence and adaptation to different hosts appear to involve a selective silencing of genes. The most dramatic example is expression of the pertussis toxin operon. Almost all members of the *Bordetella bronchiseptica* cluster possess the genes for pertussis toxin synthesis and secretion; however, only *B. pertussis* produces pertussis toxin. The inability of *B. bronchiseptica* and *B. parapertussis* to express pertussis toxin appears to be due to the lack of a functional promoter, not to defects in the structural genes. Thus the introduction of a functional promoter allows for expression and secretion of toxin in these strains (Hausman et al., 1996).

A reciprocal relationship is observed with regard to motility and expression of the flagellar genes. *Bordetella bronchiseptica* isolates express flagellar genes and are motile. In contrast, *B. pertussis* isolates possess the genes for flagella, but do not produce flagella (Akerley and Miller, 1993; Leigh et al., 1993) and are not motile. Other examples of selective silencing of genes have been reported for the *Bordetella bronchiseptica* cluster. The interested reader is directed to an excellent review of the evolution of the *Bordetella* (Gerlach et al., 2001).

Significance

Human Diseases

Bordetella pertussis is the causative agent of human whooping cough. While largely controlled by vaccination in industrialized nations, whooping cough remains a major cause of mortality worldwide, resulting in approximately 400,000 deaths a year. Most of the victims are

children. In industrial nations, the disease is rarely fatal, except in infants. *Bordetella pertussis* is spread by coughing and has no environmental reservoir other than infected humans. It has become apparent that milder subclinical disease, which is not recognized as "whooping cough," is rather common in teenagers and adults who were vaccinated as children. This milder form of disease is characterized primarily by a persistent cough that can last four weeks or more (Cherry, 1999b; Jackson et al., 2000). These individuals are thought to serve as the reservoir that allows *B. pertussis* to maintain itself in the human population. All infected individuals are capable of transmitting the organisms, and in a nonimmune individual this can result in a textbook case of whooping cough as described below.

All of the other *Bordetella* species, with the exception of *B. avium*, have been isolated from humans. *Bordetella parapertussis* isolates are primary pathogens and cause a milder form of whooping cough. The other *Bordetella* species cause opportunistic infections and appear to lack the ability to infect a healthy human host.

Agricultural Diseases

Kennel cough in dogs and atrophic rhinitis in pigs (the result of nasal passage deformation due to inappropriate cartilage and bone growth) are the diseases with the biggest economic impact caused by the *B. bronchiseptica* group. *Bordetella avium* is a respiratory pathogen of birds. Turkey coryza is of major economic importance to the poultry industry.

It can be argued that the strains with the greatest agricultural impact are not primary pathogens, but rather are opportunists. While fatal disease is often observed on farms, experimentally induced infections are often asymptomatic and self-limiting. These opportunists take advantage of the crowded, stressful and unhealthy conditions that result from modern large-scale farming conditions. For example, the concentration of ammonia in the air on pig farms can exceed what is allowable for even short-term human exposure. The resulting damage to the respiratory tract enables *B. bronchiseptica* to evade the immune defenses present in the healthy host. In addition, there is evidence for strain-specific susceptibility to disease in agricultural animals, which are extremely inbred. The development of effective vaccines is a goal of both human and agricultural research. Cross protection among different members of the *B. bronchiseptica* cluster has not been demonstrated. For example, the pertussis vaccine can prevent infection by *B. pertussis* but not by *B. parapertussis* (Khelef et al., 1993).

Habitats and Isolation

Pertussis or Whooping Cough (*B. pertussis*)

Whooping cough can be separated into three relatively distinct phases: catarrhal, paroxysmal and convalescent. Disease initiates with the catarrhal phase. The bacteria establish a localized infection in the respiratory tract, resulting in local tissue damage, and produce symptoms similar to the common cold (cough, fever and runny nose). After about a week, the disease progresses to the more serious paroxysmal phase. Patients develop the characteristic cough, where prolonged expulsive coughing ends with a "whoop," in a frantic effort to inhale. Vomiting of thick mucus often follows. Interestingly, following a paroxysm the patient can appear to be quite healthy for several hours until some event triggers another paroxysm. This phase of the disease can be very persistent, and the Chinese call whooping cough the "Cough of One Hundred Days." After prolonged illness, patients enter the convalescent stage and their symptoms gradually improve. Mortality is very rare in children over the age of one with access to good health care, but it can be fatal in infants.

In addition to the characteristic cough, the presence of an elevated white cell count, or lymphocytosis, is an important diagnostic aid. Lymphocytosis is due to the effects of pertussis toxin. Antibiotic treatment is beneficial to the patient only if initiated before whooping has begun. This is consistent with the concept that the early symptoms of the disease result from bacterial damage to the respiratory tract and the later symptoms are due to toxins released by the bacteria. Antibiotics can eradicate the microorganisms but cannot reverse the effects of toxins, which can cause damage far from the site of bacterial growth. Since diagnosis is often delayed until the paroxysmal coughing develops, it is often too late for antibiotics to be helpful.

Isolation and Cultivation of *B. pertussis* and Other Fastidious *Bordetella*

Bordetella pertussis isolates have been isolated only from the human respiratory tract. They are slow growing and will double only once every 4 hours under optimal conditions. It can be difficult to obtain logarithmic growth, suggesting that under such conditions only a subset of the bacteria are replicating, even if the density of the culture is increasing. *Bordetella pertussis* isolates are sensitive to fatty acids, and Dacron swabs, rather than cotton, are used to transfer the bacteria. Bordet-Gengou agar is the most commonly used growth medium for laboratory cultivation. The base medium contains potato infusion, sodium chlo-

ride and glycerol and may be supplemented with casein or peptone digests. After autoclaving, 15% sterile defibrinated blood (usually sheep blood) is added to the cooled agar before plates are poured.

An advantage to using Bordet-Gengou agar is that it allows for visualization of hemolysis, or lysis of the blood cells, which is mediated by the adenylate cyclase toxin, a protein that is only expressed when the bacteria are in the virulent state. All members of the *B. bronchiseptica* cluster undergo a phase variation from a virulent phase, where genes involved in promoting survival in the mammalian host are expressed, to an avirulent state where virulence genes are not expressed. The transition from a virulent to an avirulent state is controlled by a two-component regulatory system encoded in the *bvg* (*Bordetella* virulence genes) locus, which will be described in more detail in the section, Phase Variation and Bvg Control. Spontaneous mutants in the bvg locus can arise at a high frequency when the bacteria are grown in vitro, and it is important to monitor production of hemolysis to ensure the culture has not mutated.

Many clinical laboratories use Regan-Lowe charcoal agar to cultivate *B. pertussis* from an appropriate clinical sample (Tilley et al., 2000). The antibiotic cephalexin can be added at 40 µg/ml to inhibit the growth of other respiratory flora. *Bordetella pertussis* possesses a chromosomally encoded β-lactamase that confers resistance to penicillin and cephalosporin antibiotics. Regan-Lowe medium has a longer shelf life than Bordet-Gengou has (Hill et al., 2000), which is important since pertussis is often sporadic, and it is expensive to maintain a constant supply of medium that is only suitable for cultivation of *B. pertussis*. *Bordetella pertussis* colonies can be identified on either Bordet-Gengou or Regan-Lowe as tiny gray colonies that can take up to 7 days to develop. The colonies have a "mercury droplet" or a pearl-like appearance under a dissecting scope. They are oxidase positive and will not grow on chocolate agar. Agglutination with *B. pertussis* antiserum confirms the diagnosis.

The extra antigens present in complex media are not desirable for vaccine production, and it is preferable to use a defined medium that does not contain animal products such as blood. Stainer-Scholte broth is a simple defined medium that is commonly used in vaccine production (Table 2).

It has been reported that after several days of growth in vitro, *B. pertussis* can induce their own modulation to the Bvg-minus, avirulent state (Bogdan et al., 2001) by a process associated with production of sulfate ions from compounds present in the growth medium. Variability of cultures with respect to the Bvg phenotype could result in considerable variation in experimental results, and investigators wishing to examine the role of bacterial virulence factors need to ensure that the culture being examined is in the Bvg-positive virulent state.

Table 2. Stainer-Scholte Broth

Part 1. Stainer-Scholte salts solution	
Tris hydrochloride	6.36 g
Tris base	1.18 g
Glutamate (monosodium salt)	10.72 g
Proline	0.24 g
NaCl	2.5 g
KH_2PO_4	0.5 g
KCl	0.2 g
$MgCl_2$ (or 0.5 ml of 1 M solution)	0.1 g
$CaCl_2$ (0.135 ml of 1 M solution)	0.02 g
Dissolve in 1 liter of deionized water and sterilize by autoclaving.	
Part 2. Supplements (100 X stock solution)	
L-Cystine	0.4 g
Dissolve in few ml of concentrated HCl, adjust to 100 ml with deionized water. Add	
$FeSO_4 \cdot 7H_2O$	0.1 g
Ascorbic acid	0.1 g
Niacin	0.04 g
Glutathione (reduced)	1.0 g
Filter sterilize, add 10 ml per liter of sterile salts solution (store refrigerated).	

Clinical Diagnosis of Pertussis Widespread use of the pertussis vaccine has greatly reduced (but not eliminated) pertussis in developed nations. Pertussis vaccines appear to confer a sustained level of protection through school age, but it is now apparent that mild disease is common in teenagers and adults (Cherry, 1999a). These individuals likely serve as the source of infection to infants, and infection during infancy is frequently fatal. The disease appears to be on the rise and even today, the source of infection cannot be determined for about a third of all cases in infants. Public health efforts to control the disease could be improved if better diagnostic tests were available.

Diagnosis of pertussis is a challenge, and several methods are commonly used (Hallander, 1999; de Melker et al., 2000; Tilley et al., 2000). Culture from nasopharyngeal swabs or aspirates is the gold standard for diagnosis of disease. Swabs should be immediately transferred to a transport medium of 1% casamino acids in phosphate buffered saline (PBS) and plated within 24 hours if possible. It is difficult to grow these fastidious microorganisms, and not all clinical laboratories can successfully culture *B. pertussis*. Furthermore, sensitivity diminishes with the

duration of illness, leading to the problem that it is difficult to isolate the organisms when pertussis is most likely to be considered in a differential diagnosis. Polymerase chain reaction (PCR)-based tests are available for pertussis, and PCR can be more sensitive than culture, especially in the later stages of disease (Heininger et al., 2000). However PCR suffers from the usual specificity problems, and not all laboratories can reliably perform these tests.

There are also a number of commercially available enzyme-linked immunosorbent assays (ELISAs). Some assays compare initial titers with convalescent titers, looking for an increased response to *Bordetella* antigens. While useful for epidemiological studies, such assays are less useful for diagnostic purposes. Single-point serologic tests are being developed that look for a response above a threshold value thought to be negative based on population studies (de Melker et al., 2000). However, it has become apparent that there may be problems with the existing ELISAs, especially the single-point serologic tests, and the utility of these may be compromised in the future. They all measure titers to antigens present in the acellular vaccines (pertussis toxin, filamentous hemagglutinin (FHA), pertactin, or fimbriae), making it difficult to diagnose pertussis in vaccinated individuals who may already possess high titers to these antigens. Thus, development of new assays that measure responses to antigens not present in the pertussis vaccine is needed.

Treatment of Pertussis The symptoms of clinical pertussis are the result of two different microbial processes. Local damage of the respiratory tract is due to the presence of the bacteria in a normally sterile site of the body. In addition, systemic manifestations are due to the elaboration of toxins, in particular pertussis toxin. Many symptoms of the disease have been replicated in experimental animals treated with purified pertussis toxin in the absence of the microorganisms. These symptoms include lymphocytosis, hyperinsulinemia, hypoglycemia, and sensitivity to treatments that alter metabolic balance, such a serotonin, endotoxin and cold. Antibiotic treatment will eradicate the microorganisms, but has no effect on toxin-mediated symptoms. Therefore, antibiotic treatment is most beneficial to the individual if it is given early in the infection, before significant levels of pertussis toxin have been produced. Antibiotic treatment can prevent transmission and is particularly useful in an epidemic where heightened awareness of the disease improves the chances of early diagnosis. The possible benefits of passive therapy with anti-pertussis immunoglobulin are being evaluated (Granstrom et al., 1991; Bruss and Siber, 1999a; Bruss et al., 1999b).

Bordetella pertussis produces a β-lactamase and is innately resistant to ampicillin and related antibiotics, and these are not useful for treating pertussis. Erythromycin and related antibiotics are used most frequently to treat whooping cough when an early diagnosis is made, and erythromycin is used for prophylaxis of contacts. A few erythromycin-resistant isolates have been recovered from patients that failed to respond to erythromycin treatment, but for the most part it is assumed that *B. pertussis* isolates are universally susceptible to erythromycin. Standardized procedures for antibiotic-resistance testing have been developed (Hill et al., 2000), but routine antibiotic susceptibility testing is not performed.

Secondary infections by other microorganisms can occur and should be treated with antibiotics. Secondary pneumonia was a major cause of mortality from whooping cough in the preantibiotic era and remains a serious complication in individuals without access to good health care.

Isolation and Cultivation of Other *Bordetella*

As discussed above, with the exception of *B. pertussis*, all species of *Bordetella* can be readily cultivated under normal laboratory conditions without the need for special media and are even able to grow on selective medium, such as MacConkey agar (see Table 1). Many grow well at 25°C and 37°C.

Ecophysiology

Infection by members of the *B. bronchiseptica* cluster is characterized by local infection of the trachea. In some instances, especially following infection by *B. pertussis*, systemic symptoms due to toxin production are also observed. The bacteria elaborate numerous virulence factors that allow them to cause disease, which can be broken down into three general categories: adhesins, factors that enable the bacteria to evade the immune defenses, and toxins, as outlined in Table 3. Several putative virulence factors have been proposed based on DNA homology to virulence factors in other bacterial species (Antoine et al., 2000) and are also listed in Table 3. The actual role of these factors awaits experimental evaluation. The pathogenesis of *B. pertussis* is understood in the most detail and will be discussed first. The other species will be discussed primarily with respect to their differences from *B. pertussis*.

Table 3. Virulence factors of *Bordetella bronchiseptica* cluster.

	Known virulence factors			
	Species			
Factor (size)	Expression	Function	Cellular location	In acellular vaccine?
FHA (200kDa)	All	Adhesin	Surface and secreted	Yes
Fimbriae (polymerized)	All	Adhesin	OM	Some
Pertactin (69kDa)	All	Adhesin	OM	Some
BrkA (74kDa)	Variable	Complement resistance/adhesin	OM	No
TCF (64kDa)	*B. pertussis*	Adhesin (?)	OM	No
Vag8 (95kDa)	Variable	Unknown	OM	No
Pertussis toxin	*B. pertussis*	Immunosuppression	Secreted	Yes
Adenylate cyclase toxin	All	Immunosuppression	OM	No
Dermonecrotic toxin	All	Variable	Cytoplasm	No

Putative virulence factors from genome sequence[a]

Adhesins

Proposed gene	Bvg regulation	Homologues
fhaL	Vag	FHA
fhaS	Vag	FHA
adhS	None	Adhesin B, *Streptoccus parasanguinis*
bilA	Vrg	Intimin, *E. coli*
aidB	None	AidA adhesin, *E. coli*

Others

Proposed gene	Bvg Regulation	Homologues
bexB	Vrg(?)	Capsule export
drnB	None	DNAse
phg	None	Autotransporter
sphB1	Vag	Serine protease
sphB2	None	Serine protease
sphB3	None	Serine protease
bfrD	Vag	Ferrisiderophore receptor
bfrE	None	Ferrisiderophore receptor

Abbreviations: FHA, filamentous hemagglutinin; OM, outer membrane; BrkA, "*Bordetella* resistance to killing" protein; TCF, tracheal colonization factor; Vag, Vir-activated gene; and Vrg, Vir-repressed gene.
[a]From Antoine et al. (2000).

Phase Variation and Bvg Control

A hallmark of the *Bordetella* is the use of a two-component global regulatory system, encoded by the bvg (*Bordetella* virulence genes) locus, which regulates expression of the virulence factors. The BvgS protein is an integral inner-membrane protein. The periplasmic N-terminal domain senses environmental conditions, such as temperature and ion concentrations, and transmits this information to the cytoplasmic C-terminal domain (Martinez de Tejada et al., 1996). The cytoplasmic C-terminal domain interacts with a transcriptional activator, the BvgA protein, via a phosphorelay system (Uhl and Miller, 1994). In its activated (phosphorylated) form, BvgA binds to the promoters of target genes and activates transcription (Boucher and Stibitz, 1995; Boucher et al., 1997; Boucher et al., 2001).

The reversible switch from the ON (Bvg^+, Vir^+, or virulent state) to the OFF (Bvg^-, Vir^-, or avirulent state) is called "modulation." Temperature is an important switch for *B. bronchiseptica*, which only expresses virulence factors when grown at temperatures around 37°C, the body temperature of its mammalian hosts. Temperature is less of a regulatory factor for *B. pertussis*, which is never thought to persist outside of a human host and grows very poorly at temperatures much less than 37°C. The presence of high concentrations (10–40 mM) of SO_4 ions also causes modulation. Where in the human body *B. pertussis* could encounter such high concentrations of SO_4 ion and why this should serve as a signaling mechanism has been somewhat of a mystery. Recent studies suggest that *B. pertussis* can produce sulfate from sulfur-containing compounds in the growth medium (Bogdan et al., 2001). High concentrations of nicotinic acid and some analogues (Melton and Weiss, 1993) also can promote modulation. Whether the bacteria also produce this signaling compound or acquire it from some other source remains to be determined. Accumulation of sulfate and other compounds could serve as a density-sensing mechanism similar to or in conjunction with quorum-sensing mecha-

nisms (de Kievit and Iglewski, 2000) utilized by other bacteria.

In *B. pertussis*, the Bvg-activated genes include toxins (pertussis toxin, adenylate cyclase toxin and dermonecrotic toxin), adhesins (FHA, pertactin and BrkA), or colonization factors, such as tracheal colonization factor, and BrkA (which protects the bacterium from killing by complement). Interestingly, the product of one of the Bvg-activated genes is BvgR (Merkel et al., 1998a), which acts as a repressor for expression of a class of genes called "the Vir-repressed genes" (or Vrgs). In *B. bronchiseptica*, Vir-repressed genes are important for allowing the bacteria to survive in the environment outside of the mammalian hosts in a nonpathogenic state. Expression of flagella and the motile phenotype are only observed in the avirulent (Bvg–) state (Akerley and Miller, 1993), enabling the bacteria to cope in an aqueous environment. In addition, *B. bronchiseptica* are able to survive in a nutrient-poor environment in the avirulent state, but not the virulent (Bvg^+) state (Cotter and Miller, 1994).

The role of the Vir-repressed genes in *B. pertussis* is unclear. *Bordetella pertussis* isolates are nonmotile and have never been recovered from a source other than a human host and are unlikely to exist outside of the laboratory environment. Mutants in the Bvg-locus are incapable of surviving in mammalian models of disease (Weiss and Goodwin, 1989). In one study, expression of Bvg-induced virulent phase genes was reported to be necessary and sufficient for virulence in *B. pertussis* (Martinez de Tejada et al., 1998), but in another study, mutants lacking BvgR expression had a modest reduction in virulence (Merkel et al., 1998b). Interestingly, Bvg^- minus mutants have been recovered from human cases late in the infection (Kasuga, 1954), suggesting they occur in vivo.

Recently, a new class of Bvg-regulated genes (intermediate-phase genes) has been identified (Cotter and Miller, 1997). Some of these genes are only expressed in a very narrow range of conditions that bridge the fully induced Bvg-activated state and the fully OFF Bvg-repressed state (Stockbauer et al., 2001). Special adhesins that are not expressed in the fully ON Bvg virulent phase have been demonstrated in *B. bronchiseptica* (Register and Ackermann, 1997; Brockmeier, 1999; Stockbauer et al., 2001). These adhesins could enable *B. bronchiseptica* to adhere to the respiratory tract when the bacteria are transitioning from an environmental reservoir where they express the avirulent Bvg^- phenotype and before they have had time to express the virulent phase Bvg^+ adhesins like FHA.

Furthermore, recent studies suggest that the amount of BvgA protein influences the level of expression of the Bvg-activated genes (Kinnear et al., 2001), and some promoters require many copies of BvgA to be bound before expression will be activated. It has been demonstrated that the structural gene for FHA, *fhaB*, is an early gene, coming on within minutes of a switch from the Bvg^- to the Bvg^+ state. The genes in the pertussis toxin operon are late genes, requiring hours to be expressed when the bacteria are switched from the Bvg^- to the Bvg^+ state. Finally, pertactin has been shown to be an intermediate gene (Kinnear et al., 1999). In an elegant study, promoter swap mutants were constructed such that the late-gene pertussis toxin promoter drove FHA expression and the early-gene FHA promoter drove pertussis toxin expression (Kinnear et al., 2001). All of the promoter swap mutants had a modest reduction in virulence, supporting a role for qualitative differences in regulation in addition to the ON/OFF switch.

Together these studies suggest that the Bvg regulon functions in two ways. First, it can act as a switch to sense the conditions and alternate expression of two sets of genes. Each set of genes is designed to permit maximal adaptation to a single ecological niche. Thus, this ON/OFF switch prevents simultaneous expression of both sets of genes, which would be counterproductive. One set of genes is designed to promote colonization of a mammalian host, and the other is designed to promote survival as a free-living environmental microorganism. While it is clear that *B. bronchiseptica* can occupy these two ecological niches, little evidence suggests that *B. pertussis* can survive anywhere but in a human host, and the need for an ON/OFF switch is unclear.

Secondly, in addition to acting as an ON/OFF switch, the Bvg regulon can also act as a rheostat and can fine tune gene expression to promote optimal levels, even when committed to the pathogenic state, as demonstrated by the differential gene expression in response to quantitative difference in BvgA levels. The fine-tuning function performed by the Bvg regulon is likely to be important in *B. pertussis*.

Bvg regulation appears to be a general adaptation in the genus *Bordetella*. The presence of a Bvg-like regulon has been demonstrated in the distantly related *B. avium* (Gentry-Weeks et al., 1991). Temperature has been identified as an environmental signal that controls motility in *B. avium* (Temple et al., 1998), and avirulent mutants in *B. avium* (as in *B. bronchiseptica*) arise at a very high frequency, suggesting a global regulatory switch occurs in these pathogens as well. The presence of an analogous system in the most distantly related of the *Bordetella* species suggests a system like Bvg is likely to be present in other *Bordetella*.

New evidence from the pertussis genome project suggests that the Bvg regulon is only part

of a very large regulatory network in *B. pertussis*. Homologies with 15 complete two-component regulatory systems have been identified, as well as 7 sensors and 5 regulators and 15 σ factors (Locht et al., 2001). Clearly, we have only begun to understand this very complex process.

Virulence Factors of *B. pertussis*

As a successful pathogen, *B. pertussis* has evolved complex mechanisms to maintain itself in the human respiratory tract, even in the presence of a vigorous immune response. A one-to-one correspondence between a human mucosal immune defense and a specific counter defense elaborated by the bacteria is now being identified, and we have only begun to appreciate the subtleties in this complex relationship between host and bacterial pathogen.

ADHESINS Like other *Bordetella*, *B. pertussis* preferentially attaches to the ciliated cells of the trachea. Filamentous hemagglutinin (FHA; Locht et al., 1993), pertactin (Leininger et al., 1991), fimbriae (van den Berg et al., 1999b), and BrkA (Fernandez and Weiss, 1994) have been directly shown to promote attachment to mammalian cells. In addition, tracheal colonization factor (Finn and Stevens, 1995) and Vag8 (Vir-activated gene 8; Finn and Amsbaugh, 1998) may play a role in this process. As a result of the redundancy of adhesins, with the exception of BrkA (Weiss and Goodwin, 1989), mutants deficient in production of a single adhesin are often as virulent as the wildtype strain in animal models of disease (Weiss and Goodwin, 1989; Goodwin and Weiss, 1990; Khelef et al., 1994), and only mutants lacking more than one adhesin are reduced in virulence.

Filamentous Hemagglutinin A large protein of about 220 kDa, FHA is synthesized from a 367-kDa precursor. The N-terminus is modified and most of the C-terminus is cleaved from the mature peptide (Jacob-Dubuisson et al., 1996). Molecules of FHA are found both attached to the bacterial surface and secreted in the culture medium. Mediating attachment by several mechanisms (Locht et al., 1993), FHA uses an RGD (arginine, glycine, and aspartic acid) integrin receptor motif to bind to mammalian cells, and it also binds to carbohydrate and heparin sulfate groups on lipids and proteins.

Autotransporter Protein Family (Pertactin, BrkA, Tracheal Colonization Factor and Vag8) *Bordetella pertussis* possesses several outer membrane proteins in the autotransporter family, several of which have been shown to promote adherence. Pertactin has been shown to mediate attachment via an RGD tripeptide (Leininger et al., 1991). It is processed (Charles et al., 1989) from a large precursor to a form with an apparent molecular mass of 69 kDa and a 30-kDa form. The larger N-terminal portion acts as the adhesin. The 30-kDa C-terminal fragment acts as an autotransporter that promotes translocation of the N-terminal portion to the outer membrane.

The crystal structure of the N-terminal portion of pertactin has been determined, and it has a unique β-helical structure (Emsley et al., 1996; Fig. 4). The structure of the autotransporter has not been determined.

BrkA is 29% identical to pertactin and it is processed in a similar manner. The N- and C-terminal portions of BrkA are 73 and 30 kDa, respectively. The role of the 30-kDa fragment as an autotransporter domain with a role in translocation is supported by studies that demonstrate it can form pores in the outer membrane (Shannon and Fernandez, 1999). Like pertactin, BrkA has two RGD sequences. BrkA mediates adherence to cells (Fernandez and Weiss, 1994). BrkA, but not pertactin, can protect the bacteria from the bactericidal activity of complement, and this additional activity is thought to be its major contribution to virulence (Fernandez and Weiss, 1994). This will be discussed in more detail in the section, "BrkA and Complement Resistance."

Other autotransporter proteins have been characterized. Tracheal colonization factor (TCF) has been shown to promote bacterial

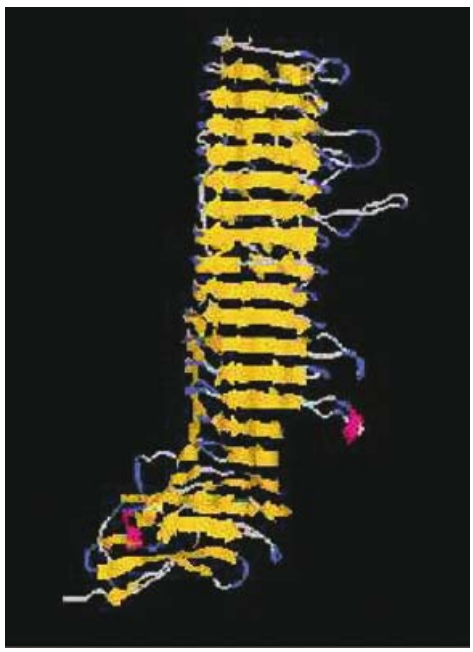

Fig. 4. Cartoon diagram of the crystal structure of pertactin showing the parallel (yellow) β-strands forming one of the three faces in the unique β-helix structure (Emsley et al., 1996).

growth in the trachea (Finn and Stevens, 1995), and Vag8 has no known function (Finn and Amsbaugh, 1998). Both of these proteins possess RGD motifs and could be adhesins. In addition to these confirmed proteins, at least 10 other genes containing autotransporter motifs have been identified in the genome sequence of *B. pertussis* (Locht et al., 2001).

Fimbriae *Bordetella pertussis* also produces several antigenically distinct fimbriae (or pili) that promote adherence (Geuijen et al., 1998; van den Berg et al., 1999b). The minor fimbrial protein, FimD, acts as the adhesin (Hazenbos et al., 1995). The major fimbrial proteins, which form the bulk of the pilus structure and determine the fimbrial serotype, include the serotype 2 and 3 fimbrial proteins. Fimbriae can undergo phase variation, and a single bacterium can express one fimbrial type, two fimbrial types, or no fimbriae (Willems et al., 1990).

Other Roles for Adhesins

Adhesins as Toxins The act of binding to a cell and engaging certain cell receptors can activate signaling pathways that, in the end, result in toxicity. Clear examples of the lethal potential of molecules that mediate toxicity through binding activities are the superantigens, for example, toxic shock syndrome toxin (TSST) of *Staphylococcus aureus*. These toxins bind to the T cell receptor and major histocompatibility complex (MHC) class II molecules (Swaminathan et al., 1992), inducing T cell proliferation and overexpression of cytokines, which can lead to shock and even death. It has recently become apparent that virulence factors of *Bordetella*, which were once considered to only act as adhesins, can also promote toxicity.

Toxic activities have been described for FHA. For example, FHA can be toxic to macrophages by inducing apoptosis (Abramson et al., 2001). In addition, FHA can suppress IL-12 expression by macrophages, resulting in a suppression of Th1-helper T cell-mediated immune responses (McGuirk and Mills, 2000b). Th1-mediated immunity can result in faster clearance of the bacteria (Mills et al., 1993; Redhead et al., 1993; Ryan et al., 1998). Preventing development of a more effective immune response would confer an obvious advantage to the bacteria. It should not be surprising if future studies demonstrate that the other purported adhesins of *B. pertussis* possess additional activities with toxic potential, and there may be a need to inactivate adhesins included in pertussis vaccines.

BrkA and Complement Resistance Complement is present in serum, but it is not generally appreciated that complement is also extruded from the blood to the mucosal surfaces (Persson et al., 1991; Brandtzaeg, 1995) and can play a role in defending against bacteria present on mucosal surfaces. For example, like *B. pertussis*, *Vibrio cholerae* is a mucosal pathogen that produces a potent protein toxin, cholera toxin. Immunity to cholera toxin does not correlate well with the immune response to cholera toxin; instead it correlates with the vibriocidal assay, which measures the ability of serum to kill *V. cholerae* by antibody-mediated complement fixation (Glass et al., 1985; Losonsky et al., 1996). Until recently, the role of bactericidal activity in immunity to *B. pertussis* has been largely overlooked.

The "*Bordetella* resistance to killing" (BrkA) protein can confer resistance to killing by complement (Fernandez and Weiss, 1994). Past reports have suggested that *B. pertussis* is sensitive to killing by complement (Friedman et al., 1992). Explanations for the discrepancies could be due to differences in conditions used to culture the bacteria. *Bordetella pertussis* has been shown to automodulate expression of the Bvg-regulated genes during growth in vitro (Bogdan et al., 2001). The BrkA protein is only expressed in the virulent state, and downregulation of the bvg-locus would in turn downregulate BrkA, resulting in a complement-sensitive phenotype. In addition, growth phase has been shown to influence susceptibility to complement, and stationary phase bacteria are much more resistant to complement (Barnes and Weiss, 2002). It is important for investigators who wish to examine the role of bacterial virulence factors to ensure that the cultures they are using are in the Bvg-positive, virulent state.

Complement has several antibacterial activities. The proteolytic products of complement activation (C4a, C3a and C5a) can upregulate the immune response. Also, C3b deposited on the bacterial surface is a powerful opsonin and can promote phagocytosis by neutrophils and macrophages. Finally, the terminal complement components (C5–C9) form the membrane attack complex. Insertion of the membrane attack complex into the membranes of Gram-negative bacteria leads to bacterial lysis and death (Taylor, 1992; Moffitt and Frank, 1994). *Bordetella pertussis* has several defenses against complement. The surface of *B. pertussis* does not appear to activate the alternative pathway of complement (Fernandez and Weiss, 1994). Antibodies bound to *B. pertussis* can activate the classical pathway, but the BrkA protein protects against the bactericidal activity of complement and antibody (Fernandez and Weiss, 1994). Specifically, BrkA inhibits activation of the complement cascade after the initial step of C1 binding to the bacterial surface (Barnes and Weiss, 2001). In addition to

preventing formation of the membrane attack complex, inhibition of the complement cascade at this initial step prevents activation of other antibacterial activities of complement, including generation of signaling molecules (C4a, C3a and C5a) and deposition of the opsonin C3 on the bacterial surface.

Vaccination with acellular pertussis vaccines does not appear to induce bactericidal activity (Weiss et al., 1999) and may even antagonize an existing bactericidal response (Weingart et al., 2000b). However, some individuals have been shown to mount an immune response that overcomes this bacterial defense (Weiss et al., 1999). Bactericidal antibodies to lipopolysaccharide (LPS) appear to be important in overcoming the BrkA-mediated resistance to complement killing (Weiss et al., 1999).

Toxins of *B. pertussis*

In whooping cough, the bacteria remain localized to the ciliated cells of the respiratory tract for the duration of the infection, causing considerable local damage through the action of tracheal cytotoxin. However, whooping cough also has aspects of a toxin-mediated disease (Pittman, 1979; Pittman, 1984), and *B. pertussis* produces several protein toxins. Two toxins of *B. pertussis*, adenylate cyclase toxin and pertussis toxin, have been shown to be essential for virulence in experimental models of disease. Interestingly, the two toxins act at different times in the disease (Goodwin and Weiss, 1990). Adenylate cyclase toxin appears to be needed immediately upon infection of the host, since adenylate cyclase toxin mutants fail to establish infection and are rapidly cleared from the lungs. Adenylate cyclase toxin has been shown to target the innate immune defenses, especially neutrophils. In contrast, pertussis toxin appears to exert its effects later in infection, since mutants lacking pertussis toxin survive and multiply in the lungs of infected mice for about 10 days, but are then rapidly eliminated by the immune defenses. These results suggest pertussis toxin is needed to disrupt the acquired immune responses. The role of a third toxin in whooping cough, the dermonecrotic toxin, is unclear, but it has been shown to play a role in disease caused by other *Bordetella*.

Tracheal Cytotoxin

The first immune defenses to challenge *B. pertussis* are the mucociliary defenses. The mucociliary escalator is essential for keeping the lungs and lower respiratory tract sterile. Mucus in the respiratory tract traps bacteria, and the beating action of cilia moves the mucus and bacterial particles to the back of the throat where the mucus is swallowed and sterilized by stomach acid. Following attachment by *B. pertussis*, ciliated cells stop beating and eventually die. This process is in part mediated by a factor called "tracheal cytotoxin," which is derived from the bacterial peptidoglycan. Tracheal cytotoxin, in conjunction with LPS, promotes production of interleukin 1-alpha and nitric oxide (Flak et al., 2000), leading to death of the ciliated cells and negating mucociliary clearance.

Pertussis Toxin

Pertussis toxin is a member of the AB_5 toxin family, which also includes cholera toxin and Shiga toxin. With five different protein subunits (termed "S1–S5"), it is the most complex bacterial toxin known. The enzymatic or A subunit, S1, catalyzes ADP-ribosylation of G proteins in the target mammalian cell and blocks signaling through these G proteins. Transfection of mammalian cells with only the S1 gene suggests that the S1 subunit alone is sufficient to confer toxicity (Castro et al., 2001). The B, or binding subunit (composed of subunits S2–S5), is needed to ensure delivery of S1 to the cytoplasm of the target mammalian cells by inducing receptor-mediated endocytosis and retrograde transport through the Golgi (el Baya et al., 1997; el Baya et al., 1999).

Pertussis toxin is essential for bacterial virulence (Weiss et al., 1984; Goodwin and Weiss, 1990). Pertussis toxin interferes with the mechanisms used by host cells to remain in communication with the rest of the body. Introduction of pertussis toxin in experimental animals can also cause the pancreas to secrete too much insulin. Pertussis toxin can also cause weight loss, elevated IgE production, and increased sensitivity to histamine, serotonin and cold. While pertussis toxin alters the behavior of many human and animal cells, its ability to inhibit activation and recruitment of the cells of the immune system (neutrophils, macrophages, monocytes, natural killer (NK) cells, and lymphocytes) in response to infection best explains its role in human whooping cough. Pertussis toxin-induced lymphocytosis is second only to coughing in defining pertussis.

Assembly and secretion of pertussis toxin is a complex process. Each pertussis toxin subunit is synthesized with a signal peptide and is secreted to the periplasm by a Sec-mediated process, where the subunits fold and are assembled into the holotoxin. Finally, nine pertussis toxin liberation (Ptl) proteins, PtlA–H, are required for secretion of the holotoxin past the outer membrane (Craig-Mylius and Weiss, 1999; Craig-Mylius et al., 2000; Weiss et al., 1993). The Ptl-secretion system is a member of the type IV secretion system (Burns, 1999). Interestingly, most of the type IV systems are involved in bacterial conjugation. Each of the nine Ptl proteins

is highly homologous to proteins in bacterial conjugation systems, and the closest matches are to the VirB system of *Agrobacterium* (Weiss et al., 1993). The 11 VirB proteins mediate the transfer of protein-coated DNA from the cytoplasm of the bacteria into the cytoplasm of plant cells. In spite of the strong structural similarities, the job performed by these two systems is very different. The conjugation systems mediate the transfer of DNA past as many as four bacterial membranes, whereas the Ptl complex only moves an AB_5 toxin past the outer membrane. However, in conjugation the DNA is transferred as a protein-coated complex, and this complex may bear some structural resemblance to the protein complex of assembled pertussis toxin.

Pertussis Toxin B Subunit Activities The B subunit of pertussis toxin is the most complex of all AB_5 toxins. For most toxins, the B subunit is formed from five identical proteins. However, for pertussis toxin, four different protein subunits associate to form the B pentamer. There is a single copy of S2, S3, and S5, and two copies of S4.

Dissociation studies performed by sequential treatments with increasing concentrations of urea have separated the B-subunit into different functional units (Tamura et al., 1982). Subunits S1 and S5 have the lowest affinity for the pertussis toxin complex and dissociate first. Addition of more concentrated urea releases two heterodimers. One copy of S4 remains associated with S2, and the second copy of S4 remains associated with S3 (Tamura et al., 1982). The heterodimers are able to bind to mammalian cells and the receptors appear to be present on S2 and S3, but not S4. The S2 and S3 heterodimers have different substrate specificities for glycoproteins and glycolipids on mammalian cells (Witvliet et al., 1989). Additional urea dissociates the heterodimers, but binding activity is lost, suggesting S4 is needed to stabilize the conformation of the binding sites.

The B subunit has been shown to contribute to the disease process in the absence of A-subunit (ADP-ribosylation) activity. The B-subunit possesses T-cell mitogenic activity in the absence of A-subunit enzymatic activity (Gray et al., 1989). In a recent study the purified dimer S3-S4 was shown to have mitogenic activity, as well as a new activity. Purified S3-S4 dimer was shown to cause a reversal in the ratio of CD_4^+/CD_8^+ in T-cells cultured from lymph nodes (Latif et al., 2001). This activity could have important implications in the generation of autoimmune disease, as well as therapeutic potential.

Several different experimental approaches have been used to identify the sites on pertussis toxin that mediate binding to host cells. These include mutational analysis, using synthetic peptides to directly block toxin activity, using synthetic peptides to elicit neutralizing antibodies (Loosmore et al., 1990; Loosmore et al., 1993), and solving the crystal structure of pertussis toxin (Stein et al., 1994a; Stein et al., 1994b). Together these studies suggest that at least three distinct regions on pertussis toxin are responsible for binding to host cells. Two amino acids (tyrosine 102 and tyrosine 103) present on both S2 and S3 bind sialic acid (Fig. 5, shown in red). Since sialic acid is common on mammalian cells, this serves as a universal receptor for pertussis toxin, a notion supported by the nearly universal susceptibility of cells to pertussis toxin. Mutations in the sialic acid-binding domain of S3 reduce toxicity to Chinese hamster ovary (CHO) cells by 10-fold, but did not alter lymphocytosis-promoting activity in mice (Loosmore et al., 1990; Loosmore et al., 1993). A second domain of S3 was shown to be important for the development of lymphocytosis. Lymphocytosis, or elevated white cell counts, occurs when pertussis toxin blocks the ability of lymphocytes to migrate from the blood to the lymphatics. Tyrosine 82 (Fig. 5, shown in orange) present on S3 appears to be required for optimal CHO cell toxicity, as well as lymphocytosis-promoting activity, since mutants in this region are deficient in both toxicity assays.

In vaccine production, methods must be developed that will inactivate toxins, but still preserve enough structure to elicit a good immune response. Ideally, inactivation of pertussis toxin should be done in a way that preserves important functional domains, such as the binding regions. Several different methods are currently used to

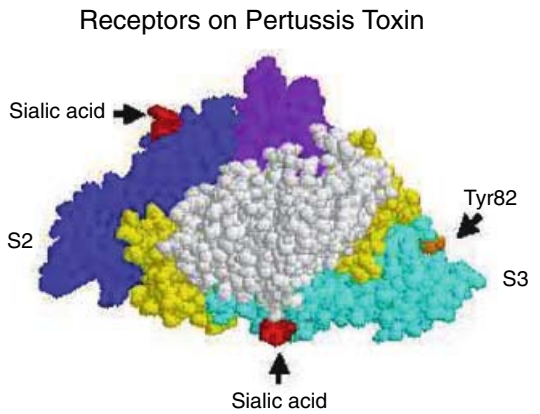

Fig. 5. Amino acids of pertussis toxin necessary for recognition of host cell receptors. A space-filling representation of the crystal structure of pertussis toxin is shown looking down from the top. Subunit S1 is white, S2 is blue, S3 is cyan, S4s are yellow and S5 is purple. Sialic acid residues (from the host) are shown in red. Tyrosine 82 is shown in orange.

inactivate (or make a toxoid from) pertussis toxin. Tetranitromethane (used in the Massachusetts Public Health Labs vaccine) modifies tyrosine residues. Hydrogen peroxide (used in the Amvax vaccine) oxidizes methionine, cysteine (cystine), tryptophan and tyrosine residues. Both of these methods alter tyrosines, the amino acids that are important for toxin binding. These methods are highly likely to completely inactivate the toxin, but are unlikely to elicit antibodies that neutralize the toxin by binding to the receptor binding sites. Formalin (used in the SmithKline, Connaught and other vaccines) covalently modifies primary amines and lysine residues. Interestingly, the S1 subunit of pertussis toxin lacks lysine residues, and formalin treatment primarily affects the B-subunit. However, formalin treatment should not affect the receptor-binding sites. The Chiron vaccine uses genetically inactivated pertussis toxin. In this vaccine, only two critical amino acids in the enzymatic S1 domain of pertussis toxin are altered, and the B subunits remain intact. This vaccine should elicit the broadest antibody response. In human trials, the genetically inactivated pertussis toxin was shown to be the most immunogenic when comparable doses of antigen were compared (Keitel and Edwards, 1999).

The ability of pertussis toxin to bind nearly every type of mammalian cell (el Baya et al., 1999) raises an interesting question. Pertussis toxin is produced by *B. pertussis* in the respiratory tract, but in disease, pertussis toxin is known to affect cells far from the site of infection. How does the toxin reach the distal cell targets when it is capable of being bound and internalized by all of the cells in between? It has been shown that some types of mammalian cells bind pertussis toxin more readily and are more susceptible to the toxin (el Baya et al., 1999). It is also interesting that pertussis toxin can bind to a soluble serum protein, haptoglobin (Witvliet et al., 1989; Latif et al., 2001). This question has not been directly examined, but it is possible that pertussis toxin circulates in the bloodstream loosely bound to haptoglobin and is only able to intoxicate cells that have a higher affinity for pertussis toxin than that of haptoglobin.

Adenylate Cyclase Toxin The adenylate cyclase toxin is a 177 kDa, single chain toxin (for a recent review, see Ladant and Ullmann, 1999). In contrast to pertussis toxin, which is secreted, adenylate cyclase toxin appears to remain on the bacterial surface and only affects the cells that come in contact with the bacteria.

As its name suggests, the adenylate cyclase toxin is an enzyme that can catalyze the conversion of ATP to cyclic AMP (cAMP). Interestingly, the toxin requires the eukaryotic calcium-binding regulatory protein, calmodulin, for maximal activity. Calmodulin regulates the endogenous mammalian adenylate cyclase, and it has been suggested that the bacteria might have originally acquired the gene for the toxin from a eukaryotic source.

The adenylate cyclase toxin can also elevate intracellular cAMP levels in target cells (Gray et al., 1998), an activity referred to as "adenylate cyclase toxin activity" because following exposure to the toxin, cytoplasmic cAMP levels can far exceed what can be achieved by normal cellular mechanisms. While this may not affect the viability of the cell, it can lead to dire consequences for the host. In the case of neutrophils, elevated cAMP inhibits their ability to phagocytose and kill microorganisms (Confer and Eaton, 1982).

A pore-forming activity of the toxin allows the catalytic domain to traverse the cytoplasmic membrane of eukaryotic cells. The toxin is synthesized as an inactive precursor, and posttranslational addition of a fatty acid group (palmitoylation) is needed for the toxin to interact with the mammalian target cells and promote pore formation (Hackett et al., 1995). Unmodified toxin retains enzymatic activity and produces cAMP if supplied with ATP, but it cannot form pores and cannot elevate cAMP inside mammalian cells, and hence it lacks toxin activity (Ehrmann et al., 1992). Pore-forming ability confers a second toxic activity; the adenylate cyclase toxin can cause hemolysis, or lysis of red blood cells. Toxin molecules lacking the palmitoyl modification are unable to mediate hemolysis. This activity is independent of enzymatic activity, since mutants unable to generate cAMP can still cause hemolysis (Gray et al., 1998).

Adenylate cyclase toxin has been shown to be essential for bacterial virulence (Weiss et al., 1984; Goodwin and Weiss, 1990). Several cells of the immune system, including neutrophils and monocytes, are programmed to shut down in response to high cyclic AMP levels, and toxin-treated cells are inhibited for chemotaxis, phagocytosis, superoxide generation, and microbial killing (Confer and Eaton, 1982). Adenylate cyclase toxin also induces macrophages to undergo apoptosis, or programmed cell death (Khelef and Guiso, 1995).

Dermonecrotic Toxin All members of the *B. bronchiseptica* cluster as well as the distantly related *B. avium* group produce dermonecrotic toxin (DNT). The conservation of this toxin would apparently attest to its importance in disease; however, this has not been the case, and its role in disease remains enigmatic. Purified DNT can be lethal to animals when injected; however, DNT is primarily localized to the cytoplasm of

the bacteria (Cowell et al., 1979), and less toxin is delivered when intact bacteria are injected into experimental animals than when sonicated bacteria are injected. To date, no secretion mechanism has been identified that would allow for efficient delivery of the toxin to cells. Also, DNT has been shown to be a virulence factor in some experimental infections, and these will be discussed. However, *B. pertussis* mutants lacking expression of DNT appear to be as virulent as wildtype bacteria when delivered intranasally to infant mice (Weiss and Goodwin, 1989), and a role for the toxin in simple respiratory infection by *B. bronchiseptica* has not been established.

DNT has been shown to play a role in turbinate atrophy in swine atrophic rhinitis. Atrophic rhinitis only affects piglets, not adults, because disease is not due to tissue damage, but rather failure of the turbinates to develop properly in the growing piglet. In particular, DNT has been shown to impair proper development of osteoblasts in culture, resulting in failure of proper bone development (Horiguchi et al., 1991; Horiguchi et al., 1995).

Mutants of *B. avium* lacking expression of the dermonecrotic toxin were avirulent in a turkey model of disease (Temple et al., 1998), but the nature of the genetic lesion in these mutants was less well characterized than in the *B. pertussis* mutants.

The N-terminus of DNT protein has been determined (Kashimoto et al., 1999). The actual start codon for the DNT gene appears to be a GTG, 39 base pairs upstream from a previously predicted ATG start, resulting in a 160-kDa protein (Kashimoto et al., 1999). The N-terminus of DNT mediates binding and internalization into the target cell, and the C-terminus possesses the catalytic activity. DNT has been shown to deaminate glutamine 63 on the mammalian protein, Rho, a GTPase that regulates actin cytoskeletal rearrangements. Deamination by DNT results in decreased GTPase activity, leaving Rho in a constitutively active state (Kashimoto et al., 1999; Schmidt et al., 1999). The connection between molecular activity of DNT leading to activation of Rho and dermonecrotic lesions is not entirely clear, but cytoskeletal rearrangements and shape changes could lead to breaking of tight junctions in the endothelium, resulting in vascular leakage and the blue-black bruise-like lesions seen in experimental models. Other roles for the toxin in disease remain to be determined.

Lipopolysaccharide

Lipopolysaccharide (LPS) serves as an important bacterial defense against toxic compounds; however, it is also a major target for the immune system. The members of the *Bordetella bronchiseptica* cluster produce structurally and antigenically different forms of LPS, and these differences are likely to play an important role in host-range specificity and resistance or susceptibility to the innate immune defenses.

The structure of the LPS of *B. pertussis* has been determined (Caroff et al., 2000), and the genetic basis for the different LPS structures among the members of the *B. bronchiseptica* cluster has been elucidated (Allen and Maskell, 1996; Allen et al., 1998). When LPS from *B. pertussis* is separated on acrylamide gels and subjected to silver staining, two low molecular weight bands are typically observed. The slower-migrating band has been designated "band A" and the faster-migrating band has been designated "band B" (Peppler, 1984). Monoclonal antibodies that distinguish between the two forms have been developed. Band B has been shown to consist of lipid A, linked to one molecule of 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) and a branched oligosaccharide containing heptose, glucose, glucuronic acid, glucosamine, and galactosaminuronic acid (Lebbar et al., 1994; see Fig. 6, double-lined box). Band A contains an additional three sugars, *N*-acetylglucosamine (Glc*N*Ac), a mannuronic acid residue (Man2*N*Ac3*N*AcA), and a fucosyl residue (Fuc2*N*Ac4*N*Me; see Fig. 6, triple-lined box).

Bordetella bronchiseptica produces LPS with the identical band A and B structure, but in addition, it synthesizes an O-antigen structure consisting of polymerized 2,3-dideoxy-di-*N*-acetyl-galactosaminuronic acid (2,3-di-*N*AcGalA; Preston et al., 1999; see Fig. 6, large box). However, some isolates of *B. bronchiseptica* express different antigenic forms of LPS (Gueirard et al., 1998b). The genes for O-antigen synthesis have been deleted from the *B. pertussis* chromosome, and they lack this modification (Preston et al., 1999). *Bordetella parapertussis* synthesizes O-antigen, but the core LPS does not react with the antibodies that recognize band A or band B, and the structures corresponding to band A and band B in *B. parapertussis* have been termed A′ and B′ (Harvill et al., 2000). Twelve genes, *wlbA-L* in the *wlb* gene cluster, have been shown to be required to complete the synthesis of band A, starting from the band B precursor, and the different structure in *B. parapertussis* is due to a difference in the activity of one of the enzymes needed to synthesize this structure (Allen et al., 1998).

The different forms of LPS confer different properties. *Bordetella bronchiseptica* isolates expressing full length LPS are more resistant to the action of antimicrobial peptides than are mutants expressing only truncated B-band LPS (Banemann et al., 1998), and they are also more

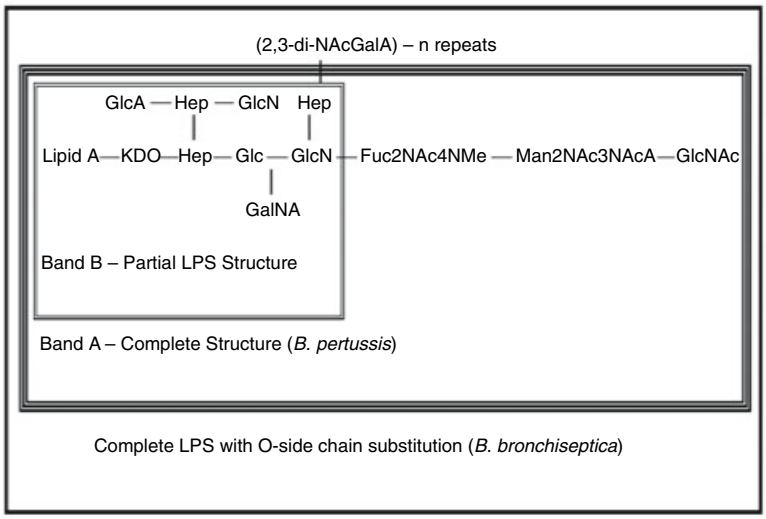

Fig. 6. Structure of lipopolysaccharide (LPS) from *B. pertussis* and *B. bronchiseptica*.

resistant to the action of antimicrobial peptides than *B. pertussis* is (Banemann et al., 1998), a phenotype that could be due to the expression of the O-side chain, although this has not been tested directly. The virulence of mutants of *B. bronchiseptica*, *B. parapertussis* and *B. pertussis* deleted for the *wlb* locus, expressing only band B LPS, was studied (Harvill et al., 2000). Compared to the wildtype strain, the LPS mutants of all three species were defective in the ability to colonize mice in experimental infections and were more susceptible to the bactericidal activity of serum complement from rabbits (Harvill et al., 2000). However, the virulence of each strain was the same in wildtype and C5-complement deficient mice, suggesting complement does not play a role in clearing the infection. Mice have been shown to express inhibitors of complement (Appelmelk et al., 1992), and this may have been a factor in these studies. Interestingly, wildtype LPS was shown to protect against the innate immune defenses for *B. pertussis* and *B. parapertussis*, but LPS appeared to have a role in protecting *B. bronchiseptica* from the acquired immune response (Harvill et al., 2000).

Molecular Basis of Whooping Cough

Innate Immune Defenses in the Respiratory Tract

Immune defenses can be classified as innate (constitutive) or acquired. Acquired defenses (with T cells and B cells as the primary effector cells) mediate the immune responses to microbial agents that the host has encountered previously. Acquired defenses are immunologically specific for each pathogen and are qualitatively improved by repeated exposure to the agent. In contrast, the innate defenses represent initial lines of defense that do not depend upon prior exposure to a microbe. These defenses provide protection against pathogens in the first few minutes of infection (versus 3–5 days for adaptive immune responses) and act to localize the infection and promote adaptive immunity.

Phagocytosis and killing by neutrophils is one of the most important parts of the innate immune defenses. A role for neutrophils in immunity to pertussis has been demonstrated in mouse models of disease. A significant neutrophil infiltration was observed in the lungs of naïve mice and mice immunized with the whole cell pertussis vaccine following aerosol challenge (McGuirk and Mills, 2000a). Neutrophil recruitment was associated with efficient clearance of the bacteria. Adenylate cyclase toxin was shown to be essential for *B. bronchiseptica* to counter neutrophils (Harvill et al., 1999b). Wildtype organisms, but not adenylate cyclase toxin mutants, caused a lethal infection in T- and B-cell-deficient mice. However, both wildtype and adenylate cyclase toxin mutants were lethal in neutropenic mice, suggesting that neutrophils play a critical role in resolving the infection and adenylate cyclase toxin can block clearance by neutrophils.

A similar role for adenylate cyclase toxin could not be demonstrated for *B. pertussis* because even wildtype *B. pertussis* lacks the ability to cause a lethal disease in adult mice (Harvill et al., 1999a), and until recently, the contribution of neutrophils to immunity in human disease had not received much consideration. Early reports suggested that *B. pertussis* was capable of surviving following phagocytosis by neutrophils (Steed

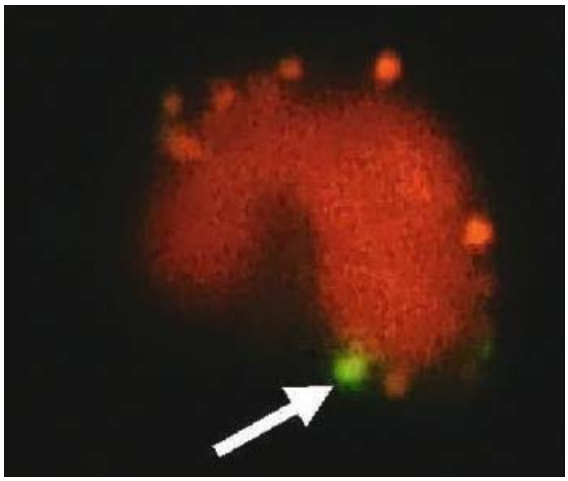

Fig. 7. Method to distinguish extracellular adherent from phagocytosed, intracellular *B. pertussis*. Bacteria expressing the green fluorescent protein (GFP) were incubated with human neutrophils for 1 hour, followed by staining with ethidium bromide. Extracellular bacteria accumulate the stain and appear orange by fluorescence microscopy; however, intracellular bacteria resist staining and remain green (see arrow). Micrograph courtesy of Christine Weingart. From Weingart et al. (1999).

et al., 1991; Torre et al., 1994), but recent quantitative studies report that 98% of the intracellular bacteria are killed by neutrophils (Lenz et al., 2000), suggesting this could be a powerful clearance mechanism (Fig. 7). However, two virulence factors (FHA and adenylate cyclase toxin) alter phagocytosis of *B. pertussis*. Bacteria expressing FHA, but not FHA mutants, attach efficiently to neutrophils but remain extracellular (Weingart and Weiss, 2000a), suggesting that FHA mediates attachment of *B. pertussis* to a site on neutrophils that fails to provoke phagocytosis. Adenylate cyclase toxin inhibits phagocytosis, since mutants lacking adenylate cyclase toxin were efficiently phagocytosed, but only when the bacteria were opsonized with human immune serum (Weingart et al., 1999; Weingart and Weiss, 2000a; Weingart et al., 2000c). Addition of neutralizing antibodies to adenylate cyclase toxin promoted phagocytosis of wildtype *B. pertussis* (Weingart et al., 2000c). Adenylate cyclase toxin is not included in any of the acellular pertussis vaccines and addition of an inactivated form of adenylate cyclase toxin might promote clearance by neutrophils.

Recently, more attention has been directed toward understanding the molecular nature of the innate immune defenses. It has become clear that the innate immune system has evolved receptors (pattern recognition receptors) to detect pattern recognition molecules, or highly conserved microbial structures. Examples of these pattern recognition molecules include peptidoglycan, carbohydrates such as glycans and mannans, bacterial DNA (specifically the CpG motif), teichoic acids (produced by Gram-positive bacteria) and LPS. It is thought that there are up to 100 different pattern recognition receptors. This contrasts sharply with the 10^{18} possible immunoglobulin and T-cell receptors that can be generated by gene rearrangement and somatic mutation. In addition, engagement of the pattern recognition receptors triggers immediate effector responses, and proliferation is not required. The pattern recognition receptors can be secreted or expressed on cell surfaces (primarily macrophages, dendritic cells and B cells).

Secreted pattern recognition receptors target microorganisms for phagocytosis or complement activation. Complement protein C1q, a member of the collectin family, has been shown to activate the alternative pathway of complement in the absence of antibody for some Gram-negative bacteria, but this does not occur with *B. pertussis* (Barnes and Weiss, 2001). As pathogens of the respiratory tract, the *Bordetella* must resist the action of other collectins, surfactant protein A (SP-A) and surfactant protein D (SP-D; Ofek et al., 2001). SP-A has a high affinity for LPS (McCormack, 1998) and can promote phagocytosis and upregulate the immune response. Different types of LPS differ in their ability to interact with SP-A (Song and Phelps, 2000), and it would seem reasonable that respiratory pathogens such as the *Bordetella* would evolve to have minimal susceptibility to this immune defense.

Other receptors of LPS have been described, for example, the soluble LPS-binding protein (LBP). Cells of the myeloid-lineage have a surface bound receptor, CD_{14}, which detects LPS via interaction with LPB. A glycosylphosphatidylinositol anchors CD_{14} to the membrane (Beutler, 2000). Since it lacks a cytoplasmic domain, it cannot signal by itself. Intracellular signaling by LBP and CD_{14} is mediated through interactions with TLR4 (toll-like receptor 4; Lien et al., 2000), and binding of LPS through these molecules initiates a cytokine cascade that in humans can lead to lethal shock following septicemia. Mice lacking TLR4 expression are resistant to endotoxic shock (Beutler, 2000). It will be interesting to determine how efficiently the LPS of *B. pertussis* activates this pathway. In addition, a synergistic interaction of tracheal cytotoxin and LPS has been described (Flak et al., 2000), and it is likely that this occurs through another pathway mediated by pattern recognition receptors.

Other molecules on the mucosal surface such as lactoferrin and transferrin act by limiting the availability for the essential nutrient, iron. Members of the *B. bronchiseptica* cluster are able to

synthesize an iron-scavenging siderophore (Brickman and Armstrong, 1999). Interestingly, mutants of *B. pertussis* deficient in siderophore synthesis were as virulent as the wildtype strain in a mouse model of disease (Pradel et al., 1998), whereas mutants of *B. bronchiseptica* lacking siderophore synthesis were shown to colonize animals, but lacked full virulence in a piglet model of atrophic rhinitis (Register et al., 2001). It is not clear if these differences are due to the mammalian host, the bacterial strain, or both.

The antimicrobial peptides produced on mucosal surfaces are also important in conferring protection from infection. In general, they are small (3–5 kDa) cationic peptides that bind to the microbial surfaces and have a detergent-like action on the membrane. Different antimicrobial peptides have different specificities, and the composition of the LPS plays a major role in conferring susceptibility or resistance to these peptides (Banemann et al., 1998). *Bordetella pertussis* has been shown to resist the action of some antimicrobial peptides via BrkA (Fernandez and Weiss, 1996).

There is considerable species variation in the number of different defensins produced and their tissue distribution. For example, the antimicrobial defensins are abundant in human neutrophil granules, as well as neutrophil granules from rabbits, guinea pigs, rats and hamsters. Interestingly, neutrophils of mice and pigs lack this class of antimicrobial peptide, but do express antimicrobial peptides of the cathelicidin family (Ganz, 1999; Ganz, 2001). This is unfortunate, since mice are the most commonly used species for experimental infections, and it is likely that susceptibility or resistance to defensins plays an important role in determining host specificity for the *Bordetella*.

In addition to these molecular defenses, other factors can play a role in limiting host specificity for *B. pertussis*. The respiratory tract is cooler than the core body temperature and *B. pertussis* prefers to grow at 35°C. Many mammalian species have a higher body temperature than humans, which could influence susceptibility. Anatomical differences could also be important. For example, *B. pertussis* attaches to the cilia of both mice and humans, but just considering relative size of the two species, there is over 1,000-fold difference in the amount of ciliated tissue. Lung infection is common in experimentally infected mice, but less common in humans, and it may be more difficult to access the lungs of humans. Since the innate defenses are often more variable between different species than the acquired immune response is, it is likely that resistance or susceptibility to the innate immune defenses plays an important role in determining host specificity of the *Bordetella*.

Transcriptional Responses to *B. pertussis*

In pioneering studies, microarray analysis was used to examine changes in the transcription program of a respiratory epithelial cell line following incubation with *B. pertussis* (Belcher et al., 2000). The data is {available electronically} [{at relman.stanford.edu}]. Sixty-five genes were downregulated following infection, including several transcription factors and cell adhesion molecules. Thirty-three genes were upregulated, and eight of these encoded proinflammatory cytokines, including neutrophil and monocyte chemoattractants. *Bordetella pertussis* isolates were shown to upregulate expression of mucin by the epithelial cells and to adhere to the mucin that was produced by the cells. Expression of both pro-apoptotic and anti-apoptotic genes was observed. The NF-κB pathway appeared to be activated, but there was no evidence of apoptosis. However, these experiments were performed in transformed cell lines, which may resist apoptosis. Pertussis toxin was shown to downregulate expression of several proteases and fibroblast activation protein. Such responses could alter the composition of the respiratory secretions and may be responsible for the thick mucus observed in pertussis patients.

Overall, it appears that the epithelial cells can detect the infection and send out cytokine signals to mobilize the inflammatory immune defenses, including recruitment of neutrophils. Presumably the bacteria rely on adenylate cyclase toxin and other bacterial defenses to prevent clearance by phagocytes. However, the bacteria, in part via the action of pertussis toxin, alter the transcriptional program to change the fluid composition of the airways in a way that would prevent mucociliary clearance. Future studies promise to yield even more secrets about this complex host-parasite interaction.

Acquired Immunity to Pertussis and the Pertussis Vaccine

The pertussis vaccine was first introduced in the 1940s and standardized in the 1950s. Use of the pertussis vaccine has reduced the incidence of the disease in the United States from 150,000 cases per year in 1925 to about 6,000 in 1998 (Anonymous, 1999). Worldwide, pertussis causes an estimated 400,000 deaths per year, most of which are believed to be vaccine preventable. Part of the problem is that multiple doses of pertussis vaccine are needed to confer protective immunity. A five-dose schedule at 2, 4, 6 and 18 months of age and a booster at school entry are used in the United States. Access to medical care in developing nations can be erratic at best, and immunizing every child after birth using this schedule requires constant access to medical care

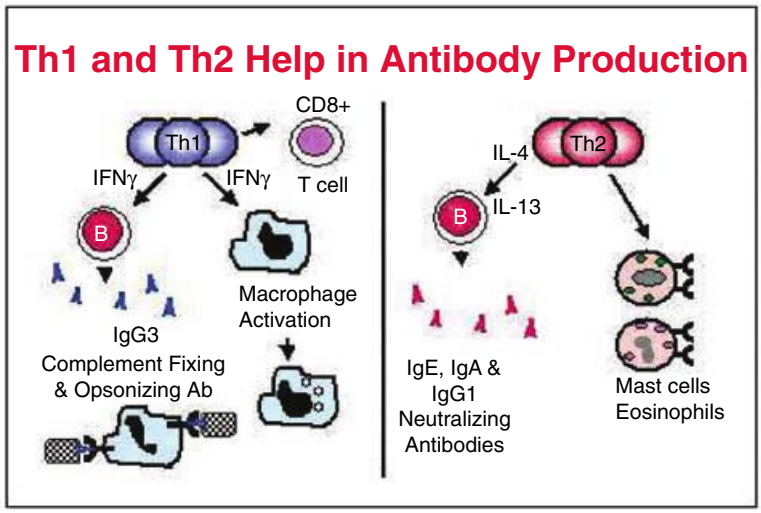

Fig. 8. Th1 and Th2 helper T cells promote antibody production. Th1 cells help promote production of IgG3, the class of antibody that works best with cells, such as macrophages (and neutrophils), that are upregulated by Th1 help. Th2 cells help promote high levels of antibody expression of many classes, including IgE, the class of antibody that works best with the cells that are upregulated by Th2 help, such as mast cells and eosinophils.

and may be more of a challenge than obtaining vaccine.

The whole-cell pertussis vaccine is made of killed *B. pertussis* organisms. Use of this vaccine is associated with adverse effects that are more common after age 6, and the whole-cell pertussis vaccine is not given after this age. The whole-cell vaccine is very inexpensive to manufacture and remains in use in countries where cost is an issue.

The newer acellular vaccines are made with purified protein and appear to have fewer side effects than the whole-cell vaccines. Several formulations have been approved for use. All contain inactivated pertussis toxin, and most are supplemented with varying combinations of the bacterial adhesins: FHA, fimbriae (one or two antigenic types), and pertactin. It is clear that immunization does not yield lifelong immunity, which may account for more frequent occurrence of pertussis in teenagers and adults. Licensing of booster doses for adults using the safer acellular vaccines is being considered.

Recent studies have shed a great deal of light on the benefits and limitations of the current pertussis vaccines. Two studies (Cherry et al., 1998; Storsaeter et al., 1998) designed to establish a serologic correlate of protection have shown that the level of the antibody response to pertactin (Prn), fimbriae (Fim) and pertussis toxin (PT) correlate with protection, but antibodies to FHA do not correlate with protection. A detailed study by Storsaeter et al. (1998) showed that following household exposure to whooping cough, greater than 80% of the unvaccinated individuals coughed for 21 days or more, but so did 24% of the individuals receiving the most efficacious acellular vaccine. In addition, about a third of these vaccinated individuals showed evidence of harboring the microorganisms and coughed for at least a day. Vaccinated individuals with mild disease are a potential source of transmission to those too young to vaccinate, and it would be desirable to develop a vaccine that is more effective at preventing colonization by *B. pertussis*.

The actual mechanisms by which the pertussis vaccines confer immunity have not been clearly elucidated. While there are limitations in using animal studies to study a human disease, experiments by Mills and colleagues using a mouse model for pertussis suggest that the immune system may successfully protect against disease using two different pathways, one involving a Th2 pathway and the other involving the Th1 pathway (Mills et al., 1998b).

The acellular pertussis vaccine induces primarily a Th2 response in mice and protection is correlated with antibody production. Antibodies to pertussis toxin presumably neutralize its toxic activities and antibodies to the adhesins (FHA, pertactin and fimbriae) block attachment and reduce, but do not totally prevent, colonization of the airways (Mills et al., 1998b). Mast cells and eosinophils, the effector cells of the Th2 pathway, do not appear to play a role in immunity to pertussis.

In contrast, the whole-cell vaccine and natural disease induce primarily a Th1 response. Production of the Th1 cytokines upregulates innate defenses (Mills et al., 1998a), recruits neutrophils to the site of infection, and mobilizes other defenses. Neutrophils and the other defenses mediate rapid clearance of the bacteria. In contrast to the Th2 pathway, a clear role for antibody to any specific antigen has not been determined for the Th1 pathway in mice (Mills et al., 1993; Mahon et al., 2000), but opsonizing antibodies may augment phagocytic clearance. The Th1 pathway appears to generate a more effective

immune response than the Th2 antibody-mediated immune response. There is some evidence to suggest that these results in mice correlate well with what is observed in human disease, but human disease presents a more complicated picture.

A difficulty in determining what constitutes a protective immune response in humans may be due to the long period for disease symptoms to develop following infection with *B. pertussis*. Following exposure, individuals may be asymptomatic for a week and have very mild symptoms for another two weeks before experiencing severe symptoms. A secondary immune response to the vaccine antigens will certainly be generated during the first week when no symptoms are apparent, and in the following weeks a primary immune response will be generated against other bacterial antigens not included in the vaccine. It is likely that in some cases, vaccine-induced immunity may be sufficient to protect from disease, but in other cases the immune response to the non-vaccine antigens may be needed to clear the infection. If so, it will be difficult to predict whether an individual will be susceptible or immune to pertussis following vaccination, considering the protective immune response may not have been generated yet.

The hypothesis that vaccine-induced immunity works to slow the growth of the bacteria, and that an immune response to antigens not included in the vaccine may be needed for clearing the infection, agrees well with what is known about the role of the vaccine antigens in the pathogenesis of the disease. The acellular vaccine should generate antibodies to block adherence and neutralize pertussis toxin. Antibodies to the adhesins reduce, but do not appear to eliminate, bacterial attachment to mammalian cells in culture (van den Berg et al., 1999a). Pertussis toxin exerts its effects late (after 15 days) in experimental models of pertussis infection (Goodwin and Weiss, 1990) and seems primarily to target development of the acquired immune response. To date, the vaccine has not been shown to elicit bactericidal immune responses that could eliminate the bacteria (Weiss et al., 1999; Weingart et al., 2000b; Weingart et al., 2000c). Two antigens, adenylate cyclase toxin and BrkA, have been shown to be important in protecting *B. pertussis* from bactericidal immune defenses of opsonophagocytosis and complement killing and should be considered for inclusion in future vaccine formulations.

Possible Development of Vaccine Resistant Strains

There has been concern that the use of only a limited number of antigens in the pertussis vaccines may allow the bacteria to develop antigenic variants that can escape vaccine control. Recently, polymorphisms have been observed in two vaccine antigens (pertussis toxin and pertactin) in clinical isolates of *B. pertussis* collected over several decades (Mooi et al., 1998). The polymorphisms in pertussis toxin involved up to three amino acid substitutions in the S1 subunit, and the polymorphisms for pertactin consisted of amino acid substitutions and changes in the number of GGxxP repeats. These are relatively minor changes in two very large proteins, but they did occur in regions known to be antigenic. Furthermore, it was noted that clinical isolates from 1950 to 1960 had the same antigenic types as in the pertussis vaccine, but more recent isolates expressed antigenic types different from those in the pertussis vaccine. During this same period, the incidence of pertussis was on the rise, and the disturbing hypothesis that *B. pertussis* undergoes antigenic variation to escape vaccine protection was put forward to explain these observations. It should be noted that for the most part, the strains used to develop the pertussis vaccines were isolated in the 1950s, and it should not be too surprising that isolates from this time more closely resemble the vaccine isolates than contemporary isolates.

A similar analysis was performed on isolates from Finland (Mooi et al., 1999), and a shift away from vaccine types was observed in this country as well. In contrast to the situation in the Netherlands, the pertussis vaccine program was successful in controlling the disease in Finland.

B. pertussis strains from the United States collected from 1935 to 1999 have been characterized for polymorphisms in pertussis toxin and pertactin (Cassiday et al., 2000) and by pulsed-field gel electrophoresis (PFGE; Bisgard et al., 2001). The PFGE profiles suggest that *B. pertussis* forms a relatively homogeneous bacterial population, but differences in subtypes and polymorphisms in pertussis toxin and pertactin were observed. Interestingly, decreasing genetic diversity was noted since vaccination was initiated in the United States. Variation in isolates from an epidemic in the Cincinnati area in 1993 suggests that this epidemic was not due to a single clone (which would be consistent with vaccine escape), but was more likely the result of a true increase in the disease rate, possibly due to the presence of more susceptible hosts (Bisgard et al., 2001).

Several studies have attempted to test directly the ability of antigenic variants to escape vaccine protection. In one study, a pertussis toxin variant with 42 amino acid differences was neutralized as effectively as the form of pertussis toxin used in the immunization, suggesting that neutralizing antibodies can tolerate many amino acid changes (Hausman and Burns, 2000). In another study,

mice immunized with a tri-component vaccine (pertussis toxin, FHA and pertactin) similar to human acellular vaccine formulations conferred good protection to strains expressing the same antigenic type used in the vaccine and to strains expressing different antigenic types than the vaccine (Boursaux-Eude et al., 1999).

Overall, these results are more consistent with the hypothesis that *B. pertussis* naturally undergoes population shifts. Polymorphisms in pertactin and pertussis toxin (as well as other genomic changes) have been seen over time for isolates from different countries on different continents regardless of vaccine usage or efficacy, suggesting that vaccine escape is not the primary driving force for these changes. Furthermore, the amino acid changes are so minor they do not appear to alter the ability to mount an effective immune response.

Virulence Factors of the *B. bronchiseptica* Cluster

Type III Secretion System

A type III secretion system has been identified in *B. bronchiseptica* (Yuk et al., 1998). Expression is regulated by the Bvg locus and occurs in the virulent phase. While the genes are present in all members of the *B. bronchiseptica* cluster, transcription has not been detected in most isolates of *B. pertussis* and *B. parapertussis*. Mutants of BscN, the protein believed to energize the secretion process, produced fewer extracellular polypeptides and displayed decreased cytotoxicity for macrophage and epithelial cell lines. Incubation with wildtype *B. bronchiseptica*, but not the BscN mutant, resulted in reduced tyrosine phosphorylation of mammalian cell proteins, an activity similar to that mediated by the YopH phosphatase of Yersinia. The BscN mutants were unable to maintain long-term colonization of the trachea of rats, but nasal colonization was unaffected (Yuk et al., 1998). Wildtype bacteria expressing the type III secretion system, but not mutants, were shown to induce apoptosis by a mechanism that seems to involve inappropriate localization of NF-κB (Yuk et al., 2000). Higher anti-Bordetella titers were observed in animals infected with the mutants in type III secretion system, suggesting it acts to inhibit an acquired immune response (Yuk et al., 2000).

FHA and Fimbriae While mutants in FHA or fimbriae did not display a strong phenotype in experimental infections of *B. pertussis*, *B. bronchiseptica* mutants lacking production of these adhesins were shown to have a more pronounced defect in virulence. In a rat model of disease, FHA is required for efficient colonization of the trachea, but is not required for nasal colonization (Cotter et al., 1998b). The efficient attachment mediated by FHA has been proposed to be important for overcoming mucociliary clearance mechanisms, since FHA was not needed for colonization of the trachea in anesthetized animals where the mucociliary defenses are compromised. Also, FHA was shown to play a role in persistence in the trachea (Cotter et al., 1998b). Fimbriae have also been shown to enhance tracheal colonization in rats and to be essential for persistence at this site (Mattoo et al., 2000).

Bordetella bronchiseptica expresses multiple fimbrial types, and certain types may play a role in host species specificity. A monoclonal antibody to the *B. pertussis* serotype 2 fimbriae was shown to bind to some, but not all, fimbriae expressed by intact bacteria (Burns et al., 1993). *Bordetella bronchiseptica* isolates from different species were characterized and reactivity with the monoclonal antibody was found to correlate with host species (Burns et al., 1993), suggesting that colonization of a specific host species may be enhanced by expression of a certain fimbrial serotype.

Role for the Intracellular State in *Bordetella* Infection Members of the *B. bronchiseptica* cluster have been shown to survive in mammalian cells following phagocytosis (Ewanowich et al., 1989). These studies were among the first to examine the intracellular fate of a bacterial species, which up to that point had been considered to be an exclusively extracellular pathogen. It is now clear that *Bordetella* are not particularly unique in this regard. *Escherichia coli*, *Staphylococcus aureus* (Guzman et al., 1994), and many other bacterial species have been shown to be capable of transient survival inside mammalian cells, and the existence of an intracellular niche for *Bordetella* will be critically examined.

A clinical study published in 1991 found *B. pertussis* associated with alveolar macrophages isolated from children with acquired immunodeficiency syndrome (AIDS) and pertussis, but the authors noted that their methodology could not distinguish intracellular from extracellular bacteria, and they failed to recover viable organisms from the patients, suggesting that intracellular bacteria might not be viable (Bromberg et al., 1991). Several in vitro studies published about this time suggested *B. pertussis* was capable of long-term survival, and perhaps replication, in professional phagocytes, including polymorphonuclear cells (neutrophils) and macrophages (Steed et al., 1991; Friedman et al., 1992; Torre et al., 1994), a surprising result con-

sidering the potent killing mechanisms available to these cells.

Technical issues plagued some of these early reports. Complement was used to kill the extracellular bacteria (Friedman et al., 1992), but *B. pertussis* expressing the BrkA protein resists the action of complement (Fernandez and Weiss, 1994). Inefficient killing by complement could have led to survival of extracellular bacteria, which were assumed to be intracellular. In other studies the bacteria were labeled with fluorescein isothiocyanate (FITC) to allow visualization by fluorescence microscopy (Steed et al., 1991). An amine-reactive probe, FITC covalently binds to the α-amino-groups at the N-terminus or the ε-amino groups of lysines on proteins that are exposed on the bacterial surface. The FITC-labeling was shown to inactivate the adenylate cyclase toxin, which has a lysine in the active site (Weingart et al., 1999). Adenylate cyclase toxin has long been known to be an important bacterial counter-defense, particularly for neutrophils (Confer and Eaton, 1982). As might be expected, neutrophils were shown capable of efficient phagocytosis of FITC-labeled bacteria (Weingart et al., 1999). In contrast, *B. pertussis* labeled cytoplasmically with green fluorescent protein (GFP) was found to efficiently adhere to neutrophils, but to resist phagocytosis (Weingart et al., 1999; Weingart and Weiss, 2000a; Weingart et al., 2000b; Weingart et al., 2000c). Of the few bacteria that were internalized, only about 1% of the bacteria remained viable (Lenz et al., 2000). These data suggest that the primary strategy of *B. pertussis* is to remain extracellular and resist phagocytosis through the action of adenylate cyclase toxin. Bacteria that are successfully phagocytosed are killed.

More recent reports suggest there is a clear difference in the kinetics of intracellular survival between *B. bronchiseptica* and *B. pertussis* strains. While neither species appears capable of replicating intracellularly, *B. bronchiseptica* remains viable for days within mammalian cells and *B. pertussis* has only transient intracellular survival (Friedman et al., 1992; Masure, 1992; Masure, 1993; Guzman et al., 1994; Schipper et al., 1994; Banemann and Gross, 1997; Chhatwal et al., 1997). In one study, fewer than 100 of the more than 100,000 *B. pertussis* internalized by macrophages survived for 24 hours, a rate not too different from that of the avirulent *E. coli*, DH5α (Banemann and Gross, 1997). Furthermore, other studies even suggest that the continued presence of viable *B. pertussis* induces apoptosis and kills mammalian cells (Khelef and Guiso, 1995; Gueirard et al., 1998a), making it unlikely that *B. pertussis* could persist as an intracellular pathogen. These results suggest that a role for intracellular survival is not likely to be important in the pathogenesis of *B. pertussis*. However, *B. bronchiseptica* are capable of long-term intracellular survival, but the role this may play in pathogenesis of the disease remains to be determined.

Conclusions

The genus *Bordetella* comprises a group of Gram-negative bacteria that are well adapted as respiratory pathogens of mammals and birds. They remain a significant threat to human health and agricultural activities. Controlling the diseases caused by the *Bordetella* has proven to be challenging, and there is a need to improve the human pertussis vaccine and to develop effective vaccines or other preventative measures for some of the agricultural diseases. While there is much to learn, the availability of the genome sequence for three members of the *Bordetella bronchiseptica* cluster should aid in this process.

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Prokaryotes (2006) 5:675–700
DOI: 10.1007/0-387-30745-1_28

CHAPTER 3.2.4

Achromobacter, *Alcaligenes* and Related Genera

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The genus *Alcaligenes* is the type genus of the family *Alcaligenaceae* (De Ley et al., 1986), which is affiliated with the "*Betaproteobacteria*." Other genera within this family, although not all formally described as members of the family, are *Achromobacter*, *Bordetella*, *Kerstersia*, *Pigmentiphaga* and most likely also *Oligella*, *Taylorella* and *Pelistega*. Phylogenetically, they form a homogeneous cluster separate from other taxa within the "*Betaproteobacteria*." Within *Alcaligenaceae*, the species of the genera *Oligella*, *Taylorella* and *Pelistega*, which were reported to have a relatively low G+C-content (36.5–47.5 mol%) of the genomic DNA (Sugimoto et al., 1983; Rossau et al., 1987; Vandamme et al., 1998; Jang et al., 2001), form a separate subbranch.

Like other members of the "*Betaproteobacteria*," species of the genera *Alcaligenes*, *Achromobacter* and *Pigmentiphaga* contain the quinone system ubiquinone Q-8 (Fletcher et al., 1987; Oyaizu-Masuchi and Komagata, 1988; Busse et al., 1992; Lipski et al., 1992; Yokota, et al., 1992; Ahrens et al., 1997; Blümel et al., 2001; Schroll et al., 2001) and a polyamine pattern that contains the predominant compound putrescine and the diagnostic polyamine, 2-hydroxyputrescine (Busse and Auling, 1988; Busse et al., 1992; Hamana and Takeuchi, 1998). Generally, fatty acids of members of these taxa are exclusively straight-chained, completely saturated or monounsaturated. Hydroxylated fatty acids and cyclopropane acids are present as well (Foss et al., 1998; Blümel et al., 2001; Coenye et al., 2003a; Coenye et al., 2003b; Table 1). Identification at the genus level can be easily obtained by comparison of 16S rRNA gene sequences. Unfortunately, no other features are known which are characteristic for a single genus within the family. So far, polar lipid profiles are the most promising tool for a reliable identification at the genus level. As indicated from the data available, the genera *Alcaligenes*, *Achromobacter* and *Pigmentiphaga* may be distinguished on the basis of their different polar lipid profiles but unfortunately, only profiles of *Al. faecalis* (Yabuuchi et al., 1995), *Ac. xylosoxidans* and *P. kullae* have been reported. Owing to this limited information, it is not clear whether the differentiating features in the polar lipid profiles are characteristics for only these species or for the corresponding genus. Thus, analyses of additional representatives of each genus are needed to clarify the specificity of the different polar lipid profiles. More information on the family *Alcaligenaceae* and affiliated genera is summarized by Busse and Auling (2004).

Genus *Alcaligenes*

The genus *Alcaligenes* was described by Castellani and Chalmer (1919). After numerous reclassifications, it now encompasses two species whose names have been validly published: the type species of the genus *Alcaligenes faecalis* with the two subspecies *Alcaligenes faecalis* subsp. *faecalis* and *Alcaligenes faecalis* subsp. *parafaecalis* (Schroll et al., 2001) and *Alcaligenes defragrans* (Foss et al., 1998). The third species [*Alcaligenes*] *latus* (Palleroni and Palleroni, 1978) cannot be considered a member of the genus *Alcaligenes* because phylogenetic studies have demonstrated that [*Al.*] *latus* is most closely related to *Rubrivivax gelatinosus* and *Leptothrix discophora* (Willems et al., 1991), which are not affiliated to the family *Alcaligenaceae*. Thus, this species is not dealt with in the context of this chapter.

Members of the genus are Gram-negative, strictly aerobic rods or coccobacilli that are motile by means of 1–9 peritrichous flagella. They possess oxidase and catalase. They grow well on complex media such as nutrient agar. The fatty acid profiles are predominated by $C_{16:0}$, $C_{16:1\omega7c}$, $C_{17:0cyclo}$, $C_{18:1\ \omega\ 7c}$ and/or 3-OH $C_{14:0}$ (Foss et al., 1998; Schroll, et al., 2001; Coenye et al., 2003a; Table 1). *Alcaligenes faecalis* has been reported to possess among its polar lipids an ornithine lipid (Yabuuchi et al., 1995). This characteristic differentiates it from representatives of *Achromobacter* and *Pigmentiphaga*. However, it still has to be examined whether this feature is characteristic for the species or the entire genus.

Table 1. Relative fatty acid compositions, polar lipid contents, and G+C contents of species of the genera *Alcaligenes*, *Achromobacter*, *Pigmentiphaga* and *Kerstersia*.

Characteristic	*Al. f.*[a]	*Al. def.*[b]	*Ac. xyl.*[a]	*Ac. den.*[a]	*Ac. pich.*[a]	*Ac. ins.*[c]	*Ac. spa.*[c]	*P. kul.*[d]	*K. gyi.*[a]
Dodecanal	—	4.5	—	—	—	—	—	—	—
3-OH $C_{10:0}$	—	—	—	—	—	—	—	2.9	—
$C_{12:0}$	2.1	6.0	Tr	Tr	Tr	1.0	Tr	—	—
2-OH $C_{12:0}$	2.3		3.5	2.5	3.4	3.8	4.6	—	Tr
$C_{14:0}$	1.4	0.4	1.1	5.3	5.6	5.6	4.2	—	5.7
2-OH $C_{14:0}$	—	—	4.0	—	—	—	—	4.0	4.3
3-OH $C_{14:0}$	10.4[e]	1.0	10.6[e]	8.6[e]	9.6[e]	12.2[e]	12.5[e]	4.6[e]	12.1[e]
$C_{16:1\ \omega7c}$	7.1[f]	28.6	9.7[f]	17.4[f]	5.3[f]	10.0[f]	18.1[f]	—	3.9[f]
$C_{16:0}$	36.8	24.6	38.9	39.7	34.3	35.3	28.8	39.9	33.8
$C_{17:0\ cyclo}$	28.8	7.6	24.4	17.3	31.8	22.9	20.0	21.9	23.6
2-OH $C_{16:0}$	—	—	Tr	Tr	1.3	Tr	2.4	4.8	—
$C_{18:1\ \omega7c}$	3.9	19.7[g]	1.8	3.3	4.3	3.5	5.2	9.8[h]	10.7
$C_{18:0}$	Tr	—	2.0	1.8	1.8	2.1	2.1	—	2.6
$C_{19:0\ cyclo\ \omega8c}$	Tr	0.2	Tr	Tr	Tr	Tr	—	12.2	2.0
Polar lipids[i]	PE (PG, OL)	—	DPG, PE, PG	—	—	—	—	PE (DPG, PG)	—
Mol% G+C	55.9–59.4	66.9	66.0–69.8	63.9–68.9	64.0–65.0	64.9–65.5	64.9	68.5	62.9

Symbols and abbreviations: Al. f., *Alcaligenes faecalis*; Al. def., *Alcaligenes defragrans*; Ac. xyl., *Achromobacter xylosoxidans*; Ac. den., *Achromobacter denitrificans*; Ac. pich., *Achromobacter piechaudii*; Ac. ins., *Achromobacter insolitus*; Ac. spa., *Achromobacter spanius*; P. kul., *Pigmentiphaga kullae*; K. gyi., *Kerstersia gyiorum*; —, not present; 3-OH $C_{10:0}$, 3-hydroxydecanoic acid; $C_{12:0}$, dodecanoic acid = lauric acid; 2-OH $C_{12:0}$, 2-hydroxydodecanoic acid; $C_{14:0}$, tetradecanoic acid = myristic acid; 2-OH $C_{14:0}$, 2-hydroxytetradecanoic acid; 3-OH $C_{14:0}$, 3-hydroxytetradecanoic acid; $C_{16:1\ \omega7c}$, cis-9-hexadecenoic acid = palmitinoleic acid; $C_{16:0}$, hexadecenoic acid = palmitic acid; $C_{17:0\ cyclo}$, Δ-*cis*-9,10-methylenehexadecanoic acid; 2-OH $C_{16:0}$, 2-hydroxyhexadecanoic acid; $C_{18:1\omega7c}$, cis-11-octadecenoic acid = vaccenic acid; $C_{18:0}$, octadecanoic acid = stearic acid; $C_{19:0\ cyclo\ \omega8c}$, Δ-*cis*-11,12-methyleneoctadecanoic acid; Tr, trace amounts present; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; OL, ornithine lipid; and DPG, diphosphatidyl glycerol.

[a]Coenye et al. (2003a).

[b]Foss et al. (1998). Cells were grown under denitrifying conditions with acetate as the sole source for carbon and energy.

[c]Coenye et al. (2003b).

[d]Blümel et al. (2001).

[e]Given as summed-in-feature 2 or 3 ($C_{16:1\ ISO\ I}$ and/or 3-OH $C_{14:0}$).

[f]Given as summed-in-feature 3 ($C_{16:1\ \omega7c}$ and/or 2-OH $C_{15:0\ ISO}$).

[g]Given as $C_{18:1\ \omega7c}$ or $C_{18:1\ \omega9t}$.

[h]Given as summed-in-feature 7 ($C_{18:1\ \omega7c}$, $C_{18:1\ \omega9t}$, and/or $C_{18:1\ \omega12t}$).

[i]Compounds in parentheses were reported to be present in moderate or minor amounts (Yabuuchi et al., 1974; 1995; Blümel et al., 2001).

Strains of *Al. faecalis* have been isolated from soil, water, feces, urine, blood, sputum, wounds, pleural fluid, nematodes and insects (Kersters and De Ley, 1984). The mol% G+C of genomic DNA in *Al. faecalis* subsp. *faecalis* and *Al. faecalis* subsp. *parafaecalis* is 55–59 (De Ley et al., 1970; Pichinoty et al., 1978; Schroll et al., 2001). Carbohydrates are not used as sole source of carbon and energy. The type strain of *Al. faecalis* subsp. *parafaecalis* and two strains of *Al. faecalis* subsp. *faecalis* (including the type strain) display almost identical Biolog GN patterns but differ in reaction for L-serine, L-ornithine and L-histidine (Schroll et al., 2001). Other distinguishing phenotypic traits between the two subspecies are nitrite reduction, gelatin liquefaction, assimilation of L-tryptophan and gentisate, growth at 42°C or in the presence of 7% NaCl, the low content of the diagnostic diamine 2-hydroxyputrescine, and the significantly higher content of $C_{16:1}$ in *Al. faecalis* subsp. *parafaecalis* (Schroll et al, 2001). However, the reliability of these distinguishing traits will have to be confirmed by examination of more strains of both subspecies.

Strains of *Al. defragrans* have been isolated under denitrifying conditions after enrichment from activated sludge samples and a ditch sample from a forest, with one of the monoterpenes ((+)-menthene, α-pinene, 2-carene, or α-phellandrene) as the sole source of carbon and energy (Harder and Probian, 1995). Strains in 2-ml portions of activated sewage sludge from a local wastewater treatment plant were enriched in 0.5 liter bottles that contained 400 ml of enrichment medium, 4 ml of degassed anoxic 2,2,4,4,6,8,8-heptamethylnonane (HMN), and 200 µl of liquid or 200 mg of solid substrate (monoterpenes) and in a N_2-CO_2 (90 : 10, vol/vol) atmosphere at 28°C in the dark (Harder and Probian, 1995). Numerous monoterpenes can serve as sources for carbon and energy. However, *Al. defragrans* also grows on simple complex media such as nutrient agar (Deutsche Sammlung von Mikroorganismen und Zellkulturen, 2001). The genomic DNA of the type strain of *A. defragrans* has a G+C-content of 66.9 mol%. The fatty acid profiles of strains of *A. defragrans* are strongly dependent on the growth conditions. When cells are grown on monoterpenes, the fatty acids $C_{17:0cylo}$ and $C_{16:0}$ and high amounts of $C_{19:0cyclo}$ are predominant. In contrast, when grown in the presence of acetate as sole source of carbon and energy, the contents of $C_{17:0cyclo}$ and $C_{19:0cyclo}$ decrease and the contents of $C_{16:1}$ and $C_{18:1}$ increase significantly.

Nutrient Agar

Peptone	5.0 g
Meat extract	3.0 g
Agar	15.0 g
Distilled water	1000 ml

Adjust pH to 7.0

Anoxic Medium for Enrichment of *Alcaligenes defragrans* (Harder and Probian, 1995)

NaCl	1.0 g
$MgCl_2 \cdot 7H_2O$	0.1 g
$CaCl_2$	0.04 g
KCl	0.5 g
NH_4Cl	0.125 g
Na_2SO_4	0.2 g
KH_2PO_4	0.4 g
K_2HPO_4	1.2 g
$NaNO_3$	0.85 g
Distilled water	1000 ml

After autoclaving and cooling the medium under an N_2/CO_2 atmosphere (90/10; vol/vol), add from sterile stock solutions 2 ml of nonchelated trace element mixture, 2 ml of a selenite-tungstate solution, 1 ml of riboflavin solution (25 mg/liter in 25 mM sodium phosphate buffer pH. 3.2), 1 ml of thiamine · HCl solution (100 mg/liter in 25 mM sodium phosphate buffer, pH 3.7), 1 ml of cyanocobalamin solution (vitamin B_{12}; 50 mg/liter), 1 ml of vitamin solution, and 20 ml of a 1 M $NaHCO_3$ solution and adjust the pH to 7.0.

Nonchelated Trace Element Solution (Widdel et al., 1983)

Distilled water	987 ml
100mM HCl	12.5 ml of a 25 % HCl
7.5 mM $FeSO_4 \cdot 7H_2O$	2100 mg
0.5 mM H_3BO_3	30 mg
0.5 mM $MnCl_2 \cdot 4H_2O$	100 mg
0.8 mM $CoCl_2 \cdot 6H_2O$	190 mg
0.1 mM $NiCl_2 \cdot 6H_2O$	24 mg
0.01 mM $CuCl_2 \cdot 2H_2O$	2 mg
0.5 mM $ZnSO_4 \cdot 7H_2O$	144 mg
0.15 mM $Na_2MoO_4 \cdot 2H_2O$	36 mg

Autoclave the trace element mixture in bottles tightly closed with rubber-fitted screw caps or fixed stoppers; leave a head space of approx. 1/3 of the volume (air for common use; N_2 for strictly anoxic procedures).

Selenite–tungstate Solution (from Gram-Negative Mesophilic Sulfate-Reducing Bacteria in the second edition; Widdel and Bak, 1992)

Distilled water	1000 ml
10 mM · NaOH	0.4 g
0.02 mM · $Na_2SeO \cdot 5H_2O$	6.0 mg
0.02 mM · $NaWO_4 \cdot 2H_2O$	8.0 mg

Autoclave the solution as described for the trace element solution.

Vitamin Solution (Aeckersberg et al., 1991)

D(+)-Biotin	15.0 mg
Folic acid	40.0 mg
Pyridoxamine dihydrochloride	150.0 mg
Nicotinic acid	100.0 mg
Ca-D-pantothenate	100.0 mg
4-Aminobenzoic acid	40.0 mg
D,L-a-Lipoic acid	15.0 mg
Sodium phosphate buffer (20 mM; pH 7.2)	1 liter

Filter sterilize.

Genus *Achromobacter*

The type species of the genus *Achromobacter* is *Achromobacter xylosoxidans* (Yabuuchi and Ohyama, 1971; Yabuuchi and Yano, 1981; Yabuuchi et al., 1998). Other species are *Achromobacter denitrificans*, *Achromobacter piechaudii*, *Achromobacter ruhlandii*, *Achromobacter insolitus* and *Achromobacter spanius* (Packer and Vishniac, 1955; Rüger and Tan, 1983; Kiredjian et al., 1986; Yabuuchi et al., 1998; Coenye et al., 2003b). The taxonomic status of *Ac. denitrificans* is still controversial. Originally described as *Alcaligenes denitrificans* (Leifson and Hugh, 1954; Rüger and Tan, 1983), it was combined with *Achromobacter xylosoxidans* in a single species with two subspecies, *Al. denitrificans* subsp. *denitrificans* and *Al. denitrificans* subsp. *xylosoxidans* (Kersters and De Ley, 1984). Owing to taxonomic priority, Kiredjian et al. (1986) proposed the new combination *Al. xylosoxidans* subsp. *denitrificans* and *Al. xylosoxidans* subsp. *xylosoxidans*. On the basis of polyphasic evidence that these two taxa are sufficiently different to classify them as separate species, Vandamme et al. (1996) proposed the two species *Al. denitrificans* and *Al. xylosoxidans*. Yabuuchi et al. (1998) recombined the two species, despite their phenotypic and genotypic heterogeneity, into the single species *Achromobacter xylosoxidans* with the two subspecies *xylosoxidans* and *denitrificans*. However, Coenye et al. (2003b) revived the species *Achromobacter denitrificans* because they could substantiate earlier results that *Achromobacter xylosoxidans* and *Achromobacter denitrificans* can be easily differentiated by results from DNA-DNA hybridizations, whole cell protein patterns, fatty acid profiles, and other phenotypic traits. Since this taxonomic conclusion conforms to present standards, we here refer to the latter taxonomic rearrangements.

The species of the genus *Achromobacter* are Gram-negative, strictly aerobic rods that are motile by means of 1–20 peritrichous flagella. They are positive for catalase and oxidase and grow well on nutrient broth agar. Predominant fatty acids are $C_{16:0}$, $C_{17:0\ cyclo}$, 3-OH $C_{14:0}$, and $C_{16:1\omega7c}$ (Coenye et al., 2003b; Table 1). Strains of *Ac. xylosoxidans* have been isolated from human clinical specimens (blood, spinal fluid, pleural fluid, peritoneal fluid, pus, urine, stools, and swabs of eyes, ears and pharynxes), distilled water, and chlorhexidine solutions in hospitals (Yabuuchi and Yano, 1981). *Achromobacter xylosoxidans* is also capable of persistent infection of the respiratory tract of persons with cystic fibrosis (Dunne and Maisch, 1995; Burns et al., 1998; Peltroche-Llacsahuanga et al., 1998) and can cause meningitis (Ramos et al., 1995; Pan et al., 1996) and bacteremia (Ramos et al., 1996). Because *Ac. xylosoxidans* is frequently confused with species within the *Burkholderia cepacia* complex (Blecker-Shelly et al., 2000; McMenamin et al., 2000), which is associated with significantly increased rates of morbidity and mortality in cystic fibrosis, a polymerase chain reaction (PCR) assay based on 16S rRNA gene sequences has been developed to enable more accurate identification of *A. xylosoxidans* (Liu et al., 2002). Owing to its pathogenic potential, *Ac. xylosoxidans* is classified as a safety level 2 organism. *Achromobacter ruhlandii* has been isolated from soil (Packer and Vishniac, 1955). Strains of *Ac. piechaudii* were isolated from pharyngeal swabs, a nose wound, blood, human ear discharge, and soil (Kiredjian et al., 1986). Strains of *Ac. insolitus* were recovered from wounds, urine and a laboratory sink, and strains of *Ac. spanius* were recovered from human blood (Coenye et al., 2003b). However, the pathogenic potential of these three species remains to be determined.

The species can be distinguished from each other on the basis of several physiological characteristics (Yabuuchi et al., 1998; Coenye et al., 2003b; Table 2), but *Ac. xylosoxidans* and *Ac. ruhlandii* differ in only few physiological traits (Yamasato et al., 1982; Yabuuchi et al., 1998; Table 2). *Achromobacter xylosoxidans* can be also distinguished from the other species of the genus (no data available for *Ac. ruhlandii*) by 2-OH $C_{14:0}$ in its fatty acid profile. Within the family *Alcaligenaceae*, this feature was only reported for species of *Bordetella* (Vandamme et al., 1995; Vandamme et al., 1996), *P. kullae* (Blümel et al., 2001) and representatives of *Kerstersia* (Coenye et al., 2003a).

Since the taxonomic status of the majority of *Alcaligenes* and *Achromobacter* strains examined for pathogenicity; potential for denitrification; degradation of natural, aromatic and xenobiotic compounds; heavy metal resistance; occurence in the environment; and biotechnologically useful enzymes is not clear, we are applying the nomenclature used in the original literature in the following text.

Alcaligenes and *Achromobacter* Strains as Human Pathogens

There are several reports about the isolation of *Alcaligenes* and *Achromobacter* strains from clinical material. Most of these strains were identified as *Alcaligenes denitrificans* subsp. *xylosoxidans* (Pickett et al., 1991). This taxon has been recognized as an opportunistic human pathogen capable of causing a variety of infections including bacteremia, pneumonia, wound and urinary tract infections, peritonitis and meningitis (Decre et al., 1992; Duggan et al., 1996;

Table 2. Selected physiological characteristics useful for differentiation between species of the genera *Alcaligenes*, *Achromobacter*, *Pigmentiphaga* and *Kerstersia*.

Characteristics	*Al. faecalis*	*Al. defragrans*	*Ac. xylosoxidans*	*Ac. denitrificans*	*Ac. ruhlandii*	*Ac. piechaudii*	*Ac. insolitus*	*Ac. spanius*	*P. kullae*	*K. gyiorum*
Denitrification	–	+	+	+	–	–	–	–	ND	–
Reduction	–	ND	–	–	+	+	+	+	ND	–
Nitrate → nitrite										
Assimilation of										
Gluconate	–	v	+	–	+	+	+	+	–	v
n-Capric acid	+	ND	+	–	+	–	+	–	ND	+
Adipate	–	–	+	+	+	+	+	+	+	–
Citrate	+	ND	+	+	+	–	+	+	+	+
Aconitate	v	ND	+	+	+	+	+	–	+	–
Mesaconate	–	ND	+	v	+	+	+	–	+	–
Itaconate	–	–	+	+	+	+	+	+	+	–
D-Glucose	–	–	+	–	+	–	–	–	–	–
D-Xylose	–	–	+	–	+	–	–	–	–	–

Symbols and abbreviations: +, positive; –, negative; v, strain dependent; and ND, not determined.
From Yamasato et al. (1982); Kersters and De Ley (1984); Yabuuchi et al. (1998); Blümel et al. (2001); Coenye et al. (2003a, b).

Knippschild and Ansorg, 1998; Liu et al., 2002). The organism has also been repeatedly isolated from the sputum of cystic fibrosis patients (Burns et al., 1998; Tan et al., 2002). Furthermore, *Achromobacter*/*Alcaligenes* strains (shown to survive in soaps, antiseptic solutions, respirators, tap water, and dialysis fluids) have been connected to several outbreaks of nosocomial infections (Lehours et al., 2002). Many clinical isolates are multiple resistant to major antibiotics (Decre et al., 1992; Decre et al., 1995). Although in the literature most opportunistic pathogen identification was by classical techniques and therefore some cases may be erronous, there are several contemporary examples of *Achromobacter xylosoxidans* strains isolated from nosocomial infections and cystic fibrosis patients identified by modern DNA-technology (Krzewinski et al., 2001; Ferroni et al., 2002; Lehours et al., 2002; Liu et al., 2002).

Denitrification by *Achromobacter*/*Alcaligenes* Strains

The most extensively studied trait of bacteria belonging to the genera *Alcaligenes* and *Achromobacter* is their ability to dissimilatorily denitrify under anaerobic conditions. This metabolic pathway, by which nitrogen oxide compounds are reduced to dinitrogen gas, has also been described in various other bacterial species such as *Pseudomonas stutzeri*, *Paracoccus denitrificans*, *Rhodobacter capsulatus*, *Thiobacillus denitrificans*, and *Thiosphera pantotropha* (Ferretti et al., 1999; Kukimoto et al., 2000). *Alcaligenes* bacteria are worldwide suggested to be the second most prevalent group (after pseudomonads) of denitrifiers (Gamble et al., 1977). The dissimilatory reduction of nitrate is initiated by nitrate reductase, and the nitrite produced in this reaction is reduced by nitrite reductases to NO, which is the first specific reaction in denitrification. These reactions involve the transfer of a single reduction equivalent to the substrate. Two different types of nitrite reductases are found in bacteria; members of the first group contain cd_1 heme, while members of the second group contain copper ions in their catalytic center. The latter enzymes obtain the required reducing equivalents from a group of blue periplasmatic copper containing reductases called "pseudoazurins." Nitric oxide (NO) is further reduced by NO reductase to nitrous oxide (N_2O) and finally converted by a N_2O reductase to N_2. The denitrification processes have been intensively studied in three putative members of the *Alcaligenaceae*. These organisms are *Alcaligenes xylosoxidans* NCIMB 11015, "*Achromobacter cycloclastes*" (ATCC 21921; IAM 1013), and *Alcaligenes faecalis* S-6. *Alcaligenes xylosoxidans* NCIMB 11015 was originally isolated and described by Iwasaki as "*Pseudomonas denitrificans*" (Inoue et al., 1998) and was used for an early intensive study of the heterotrophic denitrification process (Iwasaki and Mori, 1955; Iwasaki, 1960; Iwasaki et al., 1963).

"*Achromobacter cycloclastes*" (ATCC 21921; IAM 1013) was described first by Iwasaki and Matsubara (1972) as a denitrifying organism, but unfortunately no reference to the enrichment conditions or the reason to describe the organism as "*A. cycloclastes*" was given.

Alcaligenes faecalis S-6 was originally isolated by Kakutani et al. (1981a) using an enrichment with a nutrient broth (NB)-acetate medium with nitrate under a nitrogen atmosphere. The organism was characterized as Gram-negative, peritrichously flagellated, oxidase- and catalase positive. It showed no growth on glucose and had a G+C DNA content of 58 mol% (Kakutani et al., 1981a).

The dissimilatory nitrite reductases from *Alcaligenes xylosoxidans* NCIMB 11015, "*Achromobacter cycloclastes*" and *Alcaligenes faecalis* S-6 have been described as trimeric periplasmatic green or blue enzymes which contain two atoms of copper per enzyme subunit. The copper centers in the nitrite reductases are type 1 centers (giving rise to the green or blue color) and type 2 centers (not significantly contributing to the visible spectrum).

The nitrite reductase from *Alcaligenes xylosoxidans* NCIMB 11015 (formerly *Pseudomonas denitrificans*) was the first nitrite reductase studied in greater detail by Iwasaki and colleagues (Suzuki and Iwasaki, 1962; Iwasaki et al., 1963; Masuko et al., 1984) and later by Abraham et al. (1993). This enzyme was described as "blue" nitrite reductase. The reaction mechanism was analyzed in detail by steady state kinetics and electron spin resonance (EPR) spectroscopy (Abraham et al., 1997). The structure of the enzyme was determined by X-ray scattering in solution (Grossmann et al., 1993) and by analysis of protein crystals (Dodd et al., 1998). The corresponding gene was cloned and the deduced amino acid sequence shown to have about 48–77% sequence identity to previously cloned nitrite reductases (Prudêncio et al., 1999; Suzuki et al., 1999). The function of different amino acids in the nitrite reductase reaction has recently been analyzed by site-specific mutagenesis (Ellis et al., 2002; Prudêncio et al., 2002).

The nitrite reductase from *Alcaligenes faecalis* S-6 was purified and characterized as "green" nitroreductase by Kakutani et al. (1981c). The corresponding gene was cloned, and the enzyme was found to be translated with an amino-terminal signal sequence which was removed upon transport into the periplasm (Nishiyama

et al., 1993). The enzyme was crystallized in the reduced and oxidized form in the absence or presence of nitrite and the corresponding structures were determined (Kukimoto et al., 1994; Murphy et al., 1995; Murphy et al., 1997). Furthermore, this enzyme has been modified by site specific mutagenesis to analyze nitrite binding and catalysis and also the interaction between the nitrite reductase and the natural electron donor pseudoazurin (Kukimoto et al., 1996; Boulanger et al., 2000; Boulanger and Murphy, 2001).

The "green" nitrite reductase from "*A. cycloclastes*" was initially purified for comparison with the archetypal nitrite reductase from *A. xylosoxidans* NCIMB 11015 (then *P. denitrificans*) by Iwasaki et al. (Iwasaki et al., 1972; Iwasaki et al., 1975). The binding of the copper ions to the protein and to nitrogen monoxide was analyzed by resonance Raman spectroscopy and electron paramagnetic resonance spectroscopy (Dooley et al., 1988; Suzuki et al., 1989). The enzyme from "*A. cycloclastes*" was the first nitrite reductase for which a three-dimensional structure was determined (Godden et al., 1991; Adman et al., 1995). The encoding gene was cloned and a sequence homology to the isofunctional enzyme from *S. faecalis* S-6 of 81% has been described (Chen et al., 1996).

In general, it can be stated that the encoding genes for all the above described intensively studied nitrite reductases are clearly homologous, and these reductases and their copper centers also clearly resemble each other. Although these well studied denitrifying *Alcaligenes* strains all synthesize copper-containing nitrite reductases, some presumed *Alcaligenes* strains have been described that possess heme-containing nitrite reductases (Coyne et al., 1989).

Under physiological conditions, the reduction of the dissimilatory nitrite reductases is catalyzed by small blue copper proteins which belong to the cupredoxin family and are identifiable as so-called "azurins" or "pseudoazurins" by a characteristic amino acid sequence and optical spectrum. Thus a rather unique electron transfer chain is present in these organisms that involves the direct interaction of two different blue (or green) copper-containing enzymes. Also these pseudoazurins have been studied in parallel in *A. xylosoxidans* NCIMB 11015, "*A. cycloclastes*" and *A. faecalis* S-6 A, and it was suggested that pseudoazurins would deliver the electron to green nitrite reductase and azurins to the blue enzyme.

Alcaligenes xylosoxidans NCIMB 11015 synthesizes two biochemically distinct azurins, both of which are able to donate electrons to the purified nitrite reductase (Dodd et al., 1995). The single pseudoazurin from *A. faecalis* S-6 has been analyzed in more detail. The protein was originally purified and crystallized by Kakutani et al. (1981b), who demonstrated that under aerobic conditions the reduced form of pseudoazurin reacted with molecular oxygen to produce H_2O_2, which inactivated the nitrite reductase from the organism. The sequence of the protein was determined directly by Hormel et al. (1986) and deduced from the sequence of the encoding gene by Yamamoto et al. (1987). The structure of the pseudoazurin was determined independently in two laboratories (Petratos et al., 1987; Petratos et al., 1995; Adman et al., 1989), and the interaction sites of the protein with the nitrite reductase were investigated by site-specific mutagenesis (Kukimoto et al., 1995).

The pseudoazurin from "*A. cycloclastes*" was purified and characterized by Liu et al. (1986) and electrochemically and optically analyzed (Kohzuma et al., 1995), and the crystal structures of the oxidized and reduced forms were determined (Inoue et al., 1999). Recently, the protein was also analyzed by paramagnetic proton nuclear magnetic resonance (^{1}H NMR; Sato and Denisson, 2002).

Compared to the nitrite reductases and pseudoazurins, the other enzymes involved in denitrification in the three strains discussed here have been much less studied. The nitric oxide reductase was purified from "*A. cycloclastes*" and shown to form a membrane-bound cytochrome *bc* complex (Jones and Hollocher, 1993). The genes encoding a cytochrome-*b* and -*c* type protein and some accompanying genes which presumably encode parts of the nitric oxide reductase complex were subsequently cloned from *A. faecalis* S-6. It was found that the organization of the genes clearly resembled that in other denitrifying bacteria which do not belong to the *Alcaligenaceae* (Kukimoto et al., 2000).

The N_2O reductases from "*A. cycloclastes*" and *A. xylosoxidans* NCIMB 11015 have been biochemically characterized and shown to be similar to N_2O reductases from other organisms. They are soluble dimeric enzymes which contain about four copper atoms per subunit (Ferretti et al., 1999).

In addition to the well-characterized strains *Alcaligenes xylosoxidans* NCIMB 11015, "*Achromobacter cycloclastes*," and *Alcaligenes faecalis* S-6, the denitrification process has also been studied with some other presumed members of the *Alcaligenaceae*, e.g., *A. faecalis* IAM 1015 (Matsubara and Iwasaki, 1971), *A. denitrificans* NCTC 8582 (Baker, 1988; Hoitink et al., 1990; Romero et al., 1993; Salgado et al., 1996), *A. xylosoxidans* GIFU 1051 (Inoue et al., 1998), and *Alcaligenes* sp. STC1 (Ozeki et al., 2001).

Members of the *Alcaligenaceae* also have been intensively studied because of their ability to

perform heterotrophic nitrification under aerobic conditions. Thus, an *Alcaligene*s strain, which oxidizes under aerobic conditions pyruvic oxime and hydroxylamine to nitrite, has been described as the most active heterotrophic nitrifier. This organism is able to carry out both heterotrophic nitrification and denitrification of nitrite (Castignetti and Gunner, 1980; Castignetti and Gunner, 1981; Castignetti and Hollocher, 1982). The ability to oxidize pyruvic oxime has also been described for *Alcaligenes faecalis* ATCC 8750 (Castignetti and Hollocher, 1984). A heterotrophic nitrification of ammonia has been described for *Alcaligenes faecalis* OKK17 (the strain was identified by biochemical tests and the API test system [bioMérieux, France]; Nishio et al., 1994) and *Alcaligenes faecalis* TUD (LMD 89.147) was shown to produce high amounts of N_2O during denitrification as well as heterotrophic ammonia oxidation (Otte et al., 1996; Otte et al., 1999). An aerobic heterotrophic denitrification process, which produced N_2 and N_2O from ammonia and nitrite, was confirmed for *Alcaligenes faecalis* TUD by gas chromatography and ^{15}N mass spectrometry (Robertson et al., 1995).

Aerobic Degradation of Natural Compounds by Presumed *Alcaligenes* Strains

Bacteria belonging to the genera *Alcaligenes*/*Achromobacter* have often been reported to degrade isoprene derivatives under aerobic and also anaerobic conditions (see below). Thus, an enrichment with isoprene resulted in the isolation of strain JE 75, which was identified as *Alcaligenes denitrificans* subsp. *xylosoxidans* by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; German Collection of Microorganisms and Cell Cultures). This strain has been studied because of its ability to effectively cometabolize trichloroethene (TCE) in the presence of isoprene under aerobic conditions (Ewers et al., 1990). The aerobic degradation of α-pinene has been related to the presence of an *Achromobacter xylosoxidans* strain (identified using the BIOLOG test system; Kleinheinz et al., 1999) and the degradation of resin acids (tricyclic, diterpenoid carboxylic acids found in the bark of pine trees) has been associated with *Alcaligenes* sp. D11-13 (Morgan et al., 1996). Another *Alcaligenes* strain (*A. faecalis* MTCC3134) was shown to degrade lantadene (a group of pentacyclic triterpenoidins found in the leaves of a hepatotoxic plant; Singh et al., 1999).

Several *Alcaligenes xylosoxidans* strains (Cm1, Cm3, Cm4, and Ep3), identified by 16S rDNA sequencing, have been isolated from the rhizosphere of the garden peas and Indian mustard because they contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyzes the direct precursor of ethylene in plants. Bacterial strains with this ability are supposed to stimulate plant growth and are considered as plant growth promoting rhizobacteria. During this study it was observed that *A. xylosoxidans* strains with the screened phenotype were especially prevalent in loamy sod-podzolic gleyic arable soil (Belimov et al., 2001).

From *Alcaligenes faecalis* IFO 14479, an aromatic amine dehydrogenase (one of the two known bacterial enzymes that contain a tryptophan tryptophylquinone as prosthetic group) has been studied in some detail. The enzyme catalyzes the oxidative deamination of aromatic amines including tyramine and dopamine to the corresponding aldehydes (Iwaki et al., 1983; Nozaki, 1987; Govindaraj et al., 1994; Hyun and Davidson, 1995b). Surprisingly, it was observed in this context that an azurin (see Denitrification by Achromobacter/Alcaligenes strains) acted as physiological electron acceptor during the oxidative deamination of aromatic amines (Edwards et al., 1995; Hyun and Davidson, 1995a). Recently, the genes encoding the two subunits of the aromatic amine dehydrogenase from *Alcaligenes faecalis* were cloned and sequenced and it was shown by growth experiments and DNA-hybridization that a very similar activity is presumably present also in *A. xylosoxidans* ATCC 15175 (Chistoserdov, 2001).

Poly(3-hydroxybutyrate) (PHB) depolymerases have been described from two *Alcaligenes faecalis* strains. *Alcaligenes faecalis* T_1 was isolated from the activated sludge of a sewage treatment plant and only tentatively designated as *A. faecalis* (Tanio et al., 1982). The extracellular PHB depolymerase from *A. faecalis* T_1 hydrolyzed not only hydrophobic PHB but also the water-soluble trimer and larger oligomers of D-(–)3-hydroxybutyrate regardless of their solubility in water (Shirakura et al., 1983; Shirakura et al., 1986; Fukui et al., 1988). The gene encoding the PHB depolymerase was cloned and functionally expressed in *E. coli* (Saito et al., 1989) and it was subsequently demonstrated that the PHB depolymerase from *A. faecalis* T_1 clearly resembled the isofunctional enzyme from *Pseudomonas* (now *Paucimonas*) *lemoignei* (Shinohe et al., 1996). More recently, a structure-function relationship was established for the PHB depolymerase using different kinds of mutants (Nojiri and Saito, 1997).

Alcaligenes faecalis AE122 (identified by the National Collections of Industrial and Marine Bacteria Ltd, United Kingdom [NCIB]) was isolated from a coastal seawater sample by its ability to use PHB as sole source of carbon and

energy. The extracellular PHB depolymerase was purified and it was found that the enzyme activity was enhanced in the presence of seawater (Kita et al., 1995; Kita et al., 1997).

As indicated above, *Alcaligenes* strains have also been found in marine environments and some interesting enzymatic activities have been described from marine *Alcaligenes* strains. A well characterized marine strain is *Alcaligenes faecalis* M3A (identified by the Vitek [bioMérieux, Inc., Durham, NC] system, MIDI [Microbial ID, Inc., Newark, DE, USA] fatty acid analysis, and 16S rDNA sequencing), which was isolated from a salt marsh sediment. From this organism, a dimethylsulfide (DMS)-producing enzyme was studied. Quantitatively the most important biogenic sulfur compound emitted from oceans and salt marshes, DMS is biologically formed from the osmolyte of marine algae dimethylsulfoniopropionate (DMSP) by the action of a lyase, which converts DMSP to DMS and acrylate. The enzyme responsible for this reaction, a DMSP lyase, has first been isolated and characterized from the above-mentioned *Alcaligenes* strain (de Souza and Yoch, 1995; Ansede et al., 1999).

Isolated from a marine sediment, a putative *Alcaligenes* strain XY-234 (identified by a few biochemical tests and the G+C content of the DNA) was shown to excrete a β-1,3-xylanase at a high level into the culture medium. Because of its potential usefulness for the hydrolysis of the cell walls of seaweeds, the enzyme was purified and characterized and the corresponding gene cloned (Araki et al., 1998; Okazaki et al., 2002).

Degradation of Aromatic Compounds

Several enrichments from different groups with biphenyl as sole source of carbon and energy resulted in the isolation of *Achromobacter* or *Alcaligenes* species (which were in most cases identified according to classical taxonomic procedures; e.g., Ahmed and Focht, 1973; Furukawa and Matsumura, 1976; Furukawa et al., 1978). More recently, another strain of *Alcaligenes xylosoxidans xylosoxidans* (which was identified by the Czech Collection of Microorganisms) was isolated by enrichment with biphenyl from contaminated soil and used for the conversion of different polychlorinated biphenyls in soil microcosms (Haluska et al., 1995).

From these strains, *Achromobacter xylosoxidans* KF701 and *Alcaligenes* sp. KF711 were studied in greater detail, but unfortunately almost no information was given about the reasons to affiliate these strains to the relevant genera. They degrade biphenyl via the "standard pathway" which involves the extradiol ring-fission of 2,3-dihydroxybiphenyl and the intermediate formation of benzoate. This intermediate is subsequently converted to catechol which is subject to another extradiol ring-fission reaction. Hybridization studies with a gene probe containing the *bphABC* genes from a *Pseudomonas* strain demonstrated that the *bph* genes of strains KF701 and KF711 were clearly homologous to the genes from the pseudomonad (Furukawa et al., 1989). The genes encoding the catechol 2,3-dioxygenases from both organisms were later cloned and partly characterized. A sequence alignment demonstrated that the deduced sequence of the enzyme from *A. xylosoxidans* KF 701 highly resembled (up to 96% sequence identity) the catechol 2,3-dioxygenases from different pseudomonads (Chang et al., 1992; Moon et al., 1997). From *A. xylosoxidans* KF701 also the gene encoding a 2-hydroxymuconic acid semialdehyde dehydrogenase (*bphG*), which is part of the meta-cleavage pathway for catechol, was cloned and sequenced. Also with this enzyme a high degree of sequence identity (94%) was found with the isofunctional enzyme from *Pseudomonas putida* mt-2 (Kang et al., 1998).

Achromobacter xylosoxidans T7, which was isolated from soil after an enrichment with a mixture of toluidines (methylanilines), offers another example of a member of the *Alcaligenaceae* with a meta-cleavage pathway. The DSMZ identified this strain (DSM 11852) by biochemical tests and 16S rDNA sequencing. The organism was able to degrade all three isomers of toluidine and it was suggested that the substrates were degraded via an initial oxidative deamination to the corresponding methylcatechols followed by a classical meta-cleavage pathway (Hinteregger and Streichsbier, 2001).

Alcaligenes faecalis CCT 7145 was isolated from an Amazonian soil sample after an enrichment with phenol. The classification of the organism was done by the Biolog system (Hayward, CA, USA), fatty acid analysis using the MIDI system, and 16S rDNA sequencing, which demonstrated 97% sequence identity with *A. faecalis* ATCC 8750 (Bastos et al., 2000).

There is some evidence available that suggests that *Alcaligenes* strains are also able to degrade polycyclic aromatic hydrocarbons (PAK). Thus, Kiyohara et al. (1982) identified the phenanthrene degrading organism *Alcaligenes faecalis* AFK2 by a rather intensive classical taxonomic methodology which also involved the determination of the G+C-content (68.4–68.8 mol%) of the organism. (This high G+C content contradicts the affiliation of this strain with *A. faecalis* on the species level.) Later, a fluoranthene degrading organism was identified according to the API test system as *Alcaligenes denitrificans* (Weissenfels et al., 1990). Other naphthalene,

2,6-dimethylnaphthalene, or phenanthrene degrading *Alcaligenes* strains have been reported (Miyachi et al., 1993; Møller and Ingvorson, 1993; Guerin and Boyd, 1995) and there are some indications by 16S rDNA sequence analyses that uncharacterized *Alcaligenes* strains may become the dominant microorganisms in soils contaminated with C5+ (a complex mixture of aromatic hydrocarbons; Greene et al., 2000).

Anaerobic Degradation of Natural Compounds by Denitrifying *Alcaligenes*/*Achromobacter* Strains

As indicated above, *Al. defragans* has been obtained from anaerobic ennrichments with monoterpenes such as (+)-menthene or α-pinene as sole carbon source and nitrate as electron acceptor. α-Pinene and other monoterpenes are intermediately converted by *A. defragans* 54Pin to geranic acid (Heyen and Harder, 2000). The strain can grow under denitrifying conditions with natural essential oils (such as lemon, pine needle, parsley seed, or camphor oil) and its ability to grow under anaerobic conditions with terpenoid compounds might be environmentally important for the anaerobic turnover of these important plant derived volatile compounds (Harder et al., 2000).

Other references describe the anaerobic degradation of aliphatic sulfonic acids or aromatic compounds by *Alcaligenes* strains. Thus from an enrichment under strictly anoxic conditions starting with material from the anaerobic digestors of communal sewage works with taurine (2-aminoethanesulfonate) as electron donor and nitrate as electron acceptor, strain NKNTAU (DSM 11046) was isolated, which, according to 16S rDNA sequencing, was *Alcaligenes defragrans*. The strain could also grow aerobically with taurine (Denger et al., 1997; Ruff et al., 2003). Another putative *Alcaligenes* strain (LuBRes1) was obtained after an enrichment under anaerobic conditions with resorcinol and nitrate.The strain was classified by classical biochemical methods and the G+C content of the DNA (Gorny et al., 1992).

Aerobic Degradation of Xenobiotic Compounds by *Achromobacter* and *Alcaligenes* Strains

Enrichments with various aliphatic or aromatic compounds which carry rare substituents (such as chloro-, fluoro-, nitro-, or sulfo-groups) repeatedly resulted in the isolation of bacteria belonging to the genera *Achromobacter* or *Alcaligenes*. Unfortunately, the taxonomic affiliation of many of these strains is questionable because only very limited taxonomical data have been presented. Nevertheless, there is good evidence that indeed some bacterial strains with rare degradative abilities are *Achromobacter* or *Alcaligenes*.

Some of the presumed *Alcaligenes*/*Achromobacter* strains that degrade sulfonated compounds have been taxonomically studied in greater detail. The most intensively studied example is *Alcaligenes* sp. strain O-1 (isolated from a sewage treatment plant by an enrichment with orthanilic acid as sole source of carbon and energy), which degrades orthanilic acid (2-aminobenzenesulfonic acid; Thurnheer et al., 1986). Good evidence (i.e., ubiquinone [Q-8] is the main ubiquinone and 2-hydroxyputrescine and putrescine are the dominant polyamines) shows that this organism indeed belongs to the "*Betaproteobacteria*." Antibodies raised against the isolate crossreacted specifically with cells of *A. xylosoxidans*, and also the G+C content of the DNA (66.1 mol%) and DNA reassociation measurements suggested an affiliation of this organism to the genus *Alcaligenes* (Jahnke et al., 1990). However, on the basis of recent reclassifications (Yabuuchi et al., 1998), strain O-1 now can be considered to represent a novel species of the genus *Achromobacter*. *Alcaligenes* sp. strain O-1 degrades orthanilic acid by a rather unique pathway which involves an initial oxidative deamination of orthanilic acid to 3-sulfocatechol, an oxidative attack on 3-sulfocatechol by a "3-sulfocatechol dioxygenase" activity which results in ring-fission and desulfonation, and finally the formation of the ring-fission product 2-hydroxymuconic acid. The oxygenases of this pathway have been purified and characterized. They belong to the major groups of ring-hydroxylating or ring-cleaving dioxygenases that have been found in several other Proteobacteria (Thurnheer et al., 1990; Junker et al., 1994a; Junker et al., 1994b; Mampel et al., 1999). The genes encoding this degradative pathway are encoded (at least partly) on a 117-MDa plasmid in this strain (Jahnke et al., 1990; Jahnke et al., 1993).

The degradation of alkanesulfonates by *Achromobacter*/*Alcaligenes* strains has been reported. Thus, the taurine (2-aminoethanesulfonate)-degrading strain studied by Kondo and coworkers has been deposited as *A. xylosoxidans* NCIMB 10751 (Erdlenbruch et al., 2001). This strain degrades taurine via sulfoacetaldehyde to acetate plus sulfite (Kondo et al., 1971; Kondo et al., 1973; Kondo and Ishimoto, 1972; Kondo and Ishimoto, 1975). More recently, an ethanesulfonate degrading organism (strain AE4) was isolated from activated sludge (Erdlenbruch et al., 2001) and identified by some biochemical tests and 16S rDNA sequencing to

be related to *A. xylosoxidans* (98% sequence identity). For strain AE4, an initial oxidative desulfonation of ethanesulfonate by a monooxygenase to acetaldehyde was suggested (Erdlenbruch et al., 2001).

The evidence that certain *Alcaligenes*/*Achromobacter* strains can degrade aliphatic and aromatic chlorinated compounds is also good. Thus, Heinaru and coworkers demonstrated by 16S rDNA similarity analyses that the 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading organism EST4002 (previously isolated in their laboratory) had the highest identity (about 99%) with *A. xylosoxidans denitrificans*. This strain harbors a 78-kb plasmid, which contains a set of genes that resemble the genes of the chlorocatechol pathway from the well studied plasmid pJP4 (originally isolated from *Ralstonia eutropha* JMP134; Vedler et al., 2000).

A presumed strain of *Alcaligenes denitrificans* (NTB-1) was obtained from sewage sludge after an enrichment with 4-chlorobenzoate as sole source of carbon and energy. The substrate was converted by a hydrolytic mechanism to 4-hydroxybenzoate. The strain also grew with 4-bromo- and 4-iodobenzoate. Furthermore, 2,4-dichlorobenzoate was degraded, involving presumably an initial reductive conversion to 4-chlorobenzoate (van den Tweel et al., 1986; van den Tweel et al., 1987). The aerobic degradation of 2,4- and 2,5-dichlorobenzoate was also shown for two other bacterial strains (BRI 3010 + BRI 6011), which were tentatively identified by some standard taxonomic tests and the commercial API and Biolog test systems as *Alcaligenes denitrificans* (Miguez et al., 1990).

Another *Alcaligenes* species (strain L6) has been obtained from a freshwater sediment after an enrichment with 3-chlorobenzoate under a 2% O_2 atmosphere. The almost complete 16S rRNA gene showed the highest degree of sequence identity (94%) with the gene of *Alcaligenes xylosoxidans* subsp. *denitrificans*. This organism differed from most known aerobic 3-chlorobenzoate degraders because it did not metabolize 3-chlorobenzoate via the well-known chlorocatechol pathway and it was suggested that the strain used either the protocatechuate or the gentisate pathway for the degradation of 3-chlorobenzoate (Krooneman et al., 1996).

The metabolism of all three fluorobenzoate isomers was demonstrated for another tentative *Alcaligenes* strain (RHO22). This strain performed with 4-fluorobenzoate a hydrolytic dehalogenation reaction (Oltmanns et al., 1989) similar to the conversion of 4-chlorobenzoate to 4-hydroxybenzoate by *Alcaligenes denitrificans* NTB-1 (see above).

There are also some reports of degradation of aliphatic chlorinated compounds by *Alcaligenes*/*Achromobacter* strains. For example, strains ABIV and RS9 can degrade the herbicide Dalapon (2,2-dichloropropionate) and other short-chain chlorinated alkanoates. These organisms were identified by morphological, biochemical, and serological tests as *Alcaligenes*/*Achromobacter xylosoxidans* (*denitrificans*). The haloalkanoate halidohydrolase genes of both strains were found to be encoded on conjugative 60 kb-plasmids, which could be functionally transferred to a strain of *Pseudomonas fluorescens* (Brokamp and Schmidt, 1991; Brokamp et al., 1997b; Schwarze et al., 1997). The sequence of the D,L-haloalkanoate acid halidohydrolase of strain ABIV was found to be rather unique among previously known halidohydrolases (Brokamp et al., 1997a).

Alcaligenes strain CC1 (ATCC 49033) was isolated from sewage sludge after an enrichment with *trans*-3-chlorocrotonic acid and shown to degrade several α-chlorinated aliphatic acids (e.g., 2-chlorobutyrate, 2-chloropropionate, and chloroacetate) as well as β-chlorinated four-carbon aliphatic acids (3-chlorobutyrate, *cis*- and *trans*-3-chlorocrotonate) as sole source of carbon and energy. Direct attack of the 2-halo acid by a 2-haloacid dehalogenase and the necessity for intermediate formation of the corresponding CoA esters of the substrates to dehalogenate the β-chlorinated four-carbon compounds were suggested (Kohler-Staub and Kohler, 1989).

The similarities observed in the degradative plasmids of the 2-chloroalkanoate-degrading *Achromobacter xylosoxidans denitrificans* strains and the 2,4-D degrading *Achromobacter* species EST4002 with isofunctional plasmids from *Pseudomonas* or *Ralstonia* strains (Brokamp et al., 1997b; Brokamp et al., 1997a; Vedler et al., 2000) suggest that *Alcaligenes*/*Achromobacter* strains are able to take up broad host-range degradative plasmids and also functionally express the relevant enzymatic activities. This was also suggested by a report of McGowan et al. (1998), who within a collection of 2,4-D degrading organisms identified (by 16S rDNA sequencing) strain TFD9 to be highly related to *Alcaligenes xylosoxidans*. The sequence of the gene encoding TfdA (the first enzyme in the 2,4-D degradative pathway) was almost identical with that of *tfdA* from plasmid pJP4.

The metabolism of several types of non-natural nitrogen-containing compounds by true or presumed strains of *Achromobacter* and *Alcaligenes* has also been reported. Two strains (JS867 and JS871) with the ability to aerobically degrade 2,4-dinitrotoluene were found presumably to belong to the genus *Alcaligenes* using the Biolog test system and 16S rDNA sequencing (Nishino et al., 2000). According to 16S rDNA ribotyping, strain JS867 is most closely related to

Alcaligenes xylosoxidans (Smets and Mueller, 2001). The metabolism of 2,4-dinitrotoluene by strain JS867 was also studied under oxygen-limited conditions, and its conversion under oxygen-limited conditions was suggested to be by an oxygenolytic denitration pathway (Smets and Mueller, 2001).

Also N-containing heterocycles are degraded by presumed *Alcaligenes* strains. Thus an indole degrading bacterium has been tentatively identified as *Alcaligenes* spec. strain In3 (Claus and Kutzner, 1983). In addition, isoquinoline, or quinaldic acid (quinoline 2-carboxylic acid), kynurenic acid (4-hydroxyquinoline 2-carboxylic acid), and xanthine were utilized by *Alcaligenes faecalis* Pa and *Alcaligenes* sp. F-2, respectively, which were identified by standard tests according to *Bergey's Manual of Systematic Bacteriology* and *The Prokaryotes* (Röger et al., 1990; Bubeck et al., 1996). Also, a report suggests the presence of an atrazine dechlorinating activity in *Alcaligenes* sp. SG1 (Seffernick et al., 2000).

A bacterium that utilized the insecticide carbofurane (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) as nitrogen source was identified by different commercial test systems as *Achromobacter* sp. WM111 (Karns et al., 1986). The enzyme that hydrolyzed the carbamate linkage of various *N*-methylcarbamate insecticides was later purified (Karns and Tomasek, 1991) and shown to be encoded by a gene on a rather large plasmid (Tomasek and Karns, 1989).

An enrichment with the synthetic chelating agent iminodisuccinate (IDS) resulted in the enrichment of *Achromobacter xylosoxidans* subsp. *xylosoxidans* B3 (identified by standard methods and 16S rDNA sequencing), which was able to use IDS as sole source of carbon and energy (Reinecke et al., 2000).

An *N*,*N*-dimethylformamide degrading strain (KUFA-1) was identified by basic classical taxonomic studies as *Alcaligenes* sp. The genes encoding the two subunits of the formamidase have been cloned and sequenced and shown to be only distantly related to other amidases (Hasegawa et al., 1997; Hasegawa et al., 1999).

Another presumed *Achromobacter* strain, which degrades hydrazine and methylhydrazine (main components of hydrazine rocket fuels), has been described. This strain can degrade hydrazine to N_2 in the presence of a second nitrogen source (Ou, 1987; Ou, 1988). Recently, methylhydrazine-containing organic components present in waste waters from the Kennedy Space Center in Florida were found to be degraded by the addition of some defined bacterial strains, including *Achromobacter* strain M-30-Y (ATCC 21910; Nwankwoala et al., 1999).

Heavy-Metal and Arsenite-Resistant *Achromobacter*/*Alcaligenes* Strains

Although the best characterized bacterial strain which demonstrates several heavy metal resistance mechanisms (*Alcaligenes* strain CH34) has recently been transferred to the genus *Ralstonia* as *R. metallidurans* (Goris et al., 2001), "true" members of the *Alcaligenes*/*Achromobacter* group contain some heavy metal resistant strains. Thus, *Ac. xylosoxidans* 31A isolated in the presence of nickel ions was shown to possess a rather high degree of nickel resistance. The strain carries two megaplasmids (pTOM8 and pTOM9) that confer nickel resistance. The respective plasmids could be functionally transferred to *Ralstonia eutropha* by conjugation (Schmidt and Schlegel, 1989; Schmidt et al., 1991). Two distinct nickel resistance loci were found on plasmid pTOM9. One of these loci is homologous to other well characterized cation efflux pumps (e.g., in *R. metallidurans* CH34) and a second one (which confers a lower degree of nickel resistance) belongs to the major facilitator superfamily (Schmidt and Schlegel, 1994; Grass et al., 2001). A rather high degree of nickel tolerance has also been found in *Alcaligenes denitrificans* 4a-2 (DSM 5537; Kaur et al., 1990), and another cadmium-resistant isolate from soil has been identified by 16S rDNA sequencing as *Alcaligenes* sp. strain CT14. This strain also expressed a cation/proton antiporter highly homologous to the one from *R. metallidurans* CH34 (Kunito et al., 1996).

From *Al. faecalis* NCIB 8687, an arsenite oxidase has been characterized that oxidizes toxic arsenite (AsO_2^{-1}) to the less toxic arsenate (AsO_4^{-3}). The enzyme contains molybdenum bound to the molybdenum cofactor and it was suggested that azurin would serve as electron acceptor during the arsenite oxidase reaction (Anderson et al., 1992). The structure of this enzyme has been resolved, and in contrast to all other previously known molybdoenzymes, it contains a Rieske type (2Fe-2S) center (Ellis et al., 2001).

Detection of Presumed *Alcaligenes*/*Achromobacter* Strains in Various Environments

Alcaligenes/*Achromobacter* strains can be isolated from various soils, aquatic freshwater and marine environments, and also various living organisms. Recently, molecular ecology techniques have revealed that *Alcaligenes* strains may be rather frequent in various environments and found in some unexpected habitats. Thus amplified ribosomal DNA restriction analysis (ARDRA) and fatty acid analysis results showed

that in the summer about 6% of the culturable bacteria in sediments of a forested pristine stream in Tennessee are *Alcaligenes* strains (Halda-Alija and Johnston, 1999). Strains of *Alcaligenes xylosoxidans* (H151, HR5, and HR6) and *Alcaligenes faecalis* (HR4) (identified by Biolog and 16S rDNA sequencing) have also been identified in the feces of the microarthropods (collembolans [springtails]) as putative acceptors of conjugative plasmids (Hoffmann et al., 1998). The 16S rDNA sequencing of cultured microorganisms from a deep (224-m) subsurface environment revealed that *Alcaligenes* strains also seem to be quantitatively important members of this environment (Boivin-Jahns et al., 1995).

Potential Uses of *Alcaligenes*/*Achromobacter* Strains or Enzymes in Biotechnological Processes

Enzymes from *Achromobacter*/*Alcaligenes* strains have been used for the production of several industrially relevant chemicals. From the number of reports, most attention has been paid to the ability of various *Alcaligenes*/*Achromobacter* strains to produce D-aminoacylases. D-Aminoacylases (*N*-acyl-D-amino acid amidohydrolases) are zinc-containing enzymes that catalyze the hydrolysis of *N*-acyl-D-amino acids to the corresponding D-amino acids (used as intermediates in the preparation of pesticides, bioactive peptides, and antibiotics). The enzymes from the *Achromobacter*/*Alcaligenes* strains were shown to possess higher specific activities with several *N*-acyl-D-amino acids than the isofunctional enzymes from other bacterial strains possessed. The most intensively studied strain in this respect is *Al. xylosoxidans* A-6 which produces three *N*-acyl-D-amino acid amidohydrolases with strictly different substrate specificities. A D-aminoacylase specific for N-acyl derivatives of neutral amino acids and two D-aminoacylases specific for acidic *N*-acetyl-D-amino acids (such as *N*-acetyl-D-glutamate or *N*-acetyl-D-aspartate), in contrast to previously described D-aminoacylases, were found to be present in this organism (Sakai et al., 1990; Sakai et al., 1991a; Sakai et al., 1991b; Moriguchi et al., 1993a; Moriguchi et al., 1993b). The genes encoding D-aminoacylase, *N*-acyl-D-glutamate deacylase and *N*-acyl-D-aspartate amidohydrolase were cloned and sequenced, and the deduced amino acid sequences of the three enzymes were found to share more than 40% sequence identity with each other (Wakayama et al., 1995a; Wakayama et al., 1995b; Wakayama et al., 1995c). More recently, the amino acids involved in the binding of the catalytically important zinc ions by the D-aminoacylase have been identified by site-specific mutagenesis techniques (Wakayama et al., 2000).

D-Aminoacylases have also been characterized to some extent from *Alcaligenes faecalis* strains DA1 and DA181. These strains were identified by classical biochemical tests and the determination of the G+C content of the DNA (57.8 mol% and 65.1 mol%, respectively; Tsai et al., 1988; Tsai et al., 1992; Yang et al., 1991; Yang et al., 1992). For the D-aminoacylase from *Al. faecalis* DA-1, not only the deacetylation of *N*-acetylmethionine, but also the reverse reaction has been described (Chen et al., 1994). The gene encoding the enzyme from *Al. faecalis* DA1 was recently cloned and analyzed by mutational studies (Hsu et al., 2002). Other D-aminoacylases have also been described from *Al. denitrificans* MI-4 (Sakai et al., 1991a; Sakai et al., 1991b) and from some uncharacterized strains of *Al. denitrificans* and *Al. faecalis* (Tripathi et al., 2000).

The ability of *Alcaligenes*/*Achromobacter* strains to synthesize industrially relevant *N*-acylase activities has also been reported for *Achromobacter xylosoxidans* NCIMB 40407, which exhibited the ability to selectively hydrolyze glutaric acid from glutaryl-3-deacetoxy-7-aminocephalosporanic acid (Franzosi et al., 1995).

Alcaligenes/*Achromobacter* strains or their enzymes used to synthesize amino acids have also been reported. Thus, a novel 5-oxoprolinase, which converts pyroglutamate (an unwanted tasteless side product formed during the brewing of soy sauce) to the flavor compound glutamate, has been described from a putative *Al. faecalis* strain N-38A. (Unfortunately this strain was only preliminarily taxonomically characterized.) The gene encoding the 5-oxoprolinase was recently cloned, sequenced and heterologously expressed (Murao et al., 1995; Nishimura et al., 1999; Nishimura et al., 2000).

Other potentially useful organisms with the ability to convert amino acid derivatives include *Al. xylosoxidans* strain IFO 12669, which has a low-specificity D-threonine aldolase that can produce a key intermediate in the synthesis of a drug used to treat parkinsonism (Liu et al., 2000), and "*Achromobacter cycloclastes*" ATCC 21921 (see Denitrification by Achromobacter/Alcaligenes strains), which can be used to transform γ-butyrobetaine to L-carnitine (Naidu et al., 2001).

Some nitrilases of industrial relevance have been described from *Alcaligenes* strains. *Alcaligenes faecalis* ATCC 8750^T synthesizes a nitrilase that highly enantioselectively forms *R*-mandelic acid from racemic mandelonitrile (Yamamoto et al., 1991). Another strain of *Al. faecalis* (LU 1650) has been mentioned in a more recent patent (German patent application DE 198 48 129 A1) that described the enantioselective

hydrolysis of substituted (*R*,*S*-)mandelonitrile(s) to substituted *R*-mandelic acid(s). *Alcaligenes faecalis* JM3 (isolated because of its ability to hydrolyze indole-3-acetonitrile) has been shown to preferentially hydrolyze arylacetonitriles. The strain was identified by classical physiological tests and its G+C content (57–59 mol%) as *Al. faecalis*. The activity and specificity of nitrilase as well as the corresponding gene were subsequently characterized (Mauger et al., 1990; Nagasawa et al., 1990; Kobayashi et al., 1993).

The utilization of a nitrilase from another *Alcaligens faecalis* strain for the industrial production of some heterocyclic carboxylic acids has been described in some patents by Lonza AG (Switzerland; Liese et al., 2000).

A cell-wall degrading activity from "*Achromobacter lyticus*" M497-1 (ATCC 21456, IFO 12725) was originally described because of its ability to lyse cells of *Micrococcus* (now *Deinococcus*) *radiodurans* and *Staphylococcus aureus* in the presence of detergents. This activity was related to enzymes that degrade peptide-moieties of cell wall peptidoglycans (Nakamura et al., 1973; Horinouchi et al., 1977). This commercially available bacteriolytic agent known as "Achromopeptidase" has a broader bacteriolytic spectrum and higher bacteriolytic activity than lysozyme and has bacteriolytic activities towards several lysozyme-resistant pathogenic strains of *Staphylococcus*, *Streptococcus* and *Clostridium*. The Achromopeptidase activity is due to at least two proteases: the so-called "α-lytic protease" is a mammalian type serine protease, which hydrolyzes peptides at the carboxyl side of small hydrophobic amino acids such as alanine and valine. The enzyme cleaves the *N*-acetylmuramoyl-L-alanine amide bond and also D-Ala-Gly and Gly-Gly bonds in the peptidoglycan of *Staphylocoocus aureus* (Li et al., 1997). The so-called "β-lytic protease" is a zinc-containing metal protease that has been described as the most active part of the Achromopeptidase. This enzyme preferentially hydrolyzes the D-Ala-Gly/Ala and the Gly-Gly bonds at the linkage between the peptide subunit and the interpeptide chain and within the interpeptide bridge (Li et al., 1998). More recently, another bacteriolytic activity with *N*-acetylmuramoyl-L-alanine amidase activity was purified from the Achromopeptidase preparation (Li et al., 2000).

A third protease (= protease 1) was purified from "*Achromobacter lyticus*" M497-1 and its ability to specifically cleave proteins at lysine residues was studied (Masaki et al., 1978; Masaki et al., 1981a; Masaki et al., 1981b). This enzyme will cleave proteins specifically for the production of peptides during the sequencing of proteins. The sequence of the enzyme was determined, the encoding gene cloned, and several amino acids exchanged by site-specific mutagenesis to identify the amino acids responsible for the lysine specificity and the broad pH optimum of the enzyme in the alkaline region (Tsunasawa et al., 1989; Ohara et al., 1989; Norioka et al., 1994; Shiraki et al., 2002a; Shiraki et al., 2002b).

Alcaligenes sp. DS-S-7G (identified by classical physiological tests) was shown to preferentially assimilate *R*-3-chloro-1,2-propanediol (monochlorhydrin). The strain was able to convert racemic 3-chloro-1,2-propanediol to almost enantiomerically pure *S*-3-chloro-1,2-propanediol (Suzuki et al., 1992). An electron acceptor-dependent halohydrin dehydrodehalogenase was identified as the responsible enzyme for the resolution of the racemate. Later, it was demonstrated that the strain was also able to enantioselectively convert nonchlorinated diols (e.g., 1,2-butanediol or 1,2-hexanediol; Suzuki et al., 1994b; Suzuki et al., 1994a).

Very recently, a novel subspecies of *Al. faecalis* has been described (*Al. faecalis* subsp. *parafaecalis*) which produces PHB from the by-products of the acetone-butanol fermentation of *Clostridium beijerinckii*. The potential usefulness of this organism for the production of higher-value PHB from the waste of the acetone-butanol fermentation has been suggested (Schroll et al., 2001).

Some *Alcaligenes*/*Achromobacter* strains also seem to excrete some products that interact with other organisms. Thus from Kalimantan (Indonesia), a strain producing three antibiotics (Kalimantacin A, B and C) was identified by a thorough classical taxonomic analysis (including the determination of the G+C content of the DNA) as *Alcaligenes* sp. Yl-02632S (FERM-12694; Kamigiri et al., 1996; Tokunaga et al., 1996). *Alcaligenes* strain MFA1 was isolated from carnation (*Dianthus caryophillus* L.) roots and suppresses a vascular wilt of carnation caused by the fungal pathogen *Fusarium oxysporium* f. sp. *dianthi*. The bacterium inhibits the colonization of the plant roots by the fungus but also inhibits microconidial and chlamydospore germination of the fungal pathogen. The germination inhibition is presumably mediated by a siderophore produced by *Alcaligenes* sp. MFA1. The results of fatty acid analysis using gas chromatography (GC) suggested that the strain might be closely related to *Alcaligenes xylosoxidans* subsp. *denitrificans* (Yuen and Schroth, 1986; Martinetti and Loper, 1992). The proposed production of a siderophore by the vascular wilt-suppressing *Alcaligenes* strain MFA1 is in accordance with the description of a dihydroxamate siderophore (Alcaligin) from *Alcaligenes xylosoxidans* subsp. *xylosoxidans* KN 3-1, which was identified using the API test system and the

tests suggested by *Bergey's Manual* (Nishio et al., 1988; Nishio and Ishida, 1990).

Alcaligenes/*Achromobacter* strains are also reported to produce antibiotic resistance factors. Thus, a bacterial isolate resistant to the antibiotic Albicidin, which is produced by a phytopathogenic *Xanthomonas* strain and inhibits DNA replication in plastids and bacteria, was identified by a series of biochemical tests and the determination of the G+C content (69.5–70 mol%) as *Al. denitrificans subsp. denitrificans* (Basnayake and Birch, 1995). Furthermore, a penicillin acylase from *Alcaligenes faecalis* has been studied because of its high potential for enzymatic modification of β-lactam antibiotics and was found to have a very high affinity for natural and semisynthetic substrates (Vedas et al., 1997; Verhaert et al., 1997; Alkema et al., 1999).

Genus *Kerstersia*

The genus *Kerstersia* with the single established species *Kerstersia gyiorum* has been described as an *Alcaligenes faecalis*-like organism isolated from human clinical samples (Coenye et al., 2003a). Members of the genus are Gram-negative coccoid cells, which occur singly, in pairs, or in short chains. Cells grow on complex media such as nutrient broth. They possess catalase, but no oxidase, urease, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, gelatinase, amylase, DNase, or β-galactosidase. Cells grow at temperatures between 28°C and 42°C. The fatty acid profile contains predominantly $C_{16:0}$, $C_{17:0\ cyclo}$, 3-OH $C_{14:0}$ and $C_{18:1\ \omega\ 7c}$ (Table 1). *Kerstersia gyiorum* shares with *Pigmentiphaga kullae* combined presence of 2-OH $C_{14:0}$, relatively high amounts of $C_{18:1\ \omega\ 7c}$, and trace amounts of 2-OH $C_{12:0}$. *Kerstersia gyiorum* can be distinguished from other species of the family also on the basis of a set of physiological characteristics. Although reported to be isolated from different wounds, no pathogenic potential has been reported for *K. gyiorum*.

Genus *Pigmentiphaga*

So far the description of the genus *Pigmentiphaga* and the species *Pigmentiphaga kullae* is based on a single strain (Blümel et al., 2001). Cells are Gram-negative, motile and rod-shaped. They grow on complex media such as Luria-Bertani medium and at temperatures between 30°C and 42°C but not at 4°C. They are positive for catalase and oxidase. The type strain uses numerous organic acids as sole source of carbon and energy, but sugars are not utilized. The polyamine pattern consists of the predominant diamines putrescine and 2-hydroxyputrescine and a quinone system ubiquinone Q-8, which are "*Betaproteobacteria*"-specific characteristics. The polar lipid profile consists of the phosphate-containing lipids, phosphatidyl ethanolamine (predominant) and phosphatidyl glycerol and diphosphatidyl glycerol (minor compounds). The fatty acid profile of *Pigmentiphaga kullae* (Table 1) contains the majority of characteristics of the family *Alcaligenaceae* except for the presence of 2-OH $C_{12:0}$ and relatively high amounts of $C_{19:0cyclo\omega 8c}$. Recently, close association of a 2,6-naphthalenedisulfonic acid degrading strain with *Pigmentiphaga kullae* has been reported which was based on 16S rRNA gene sequence comparison and phenotypic characterization (Uchihashi et al., 2002).

Pigmentiphaga kullae^T K24 has been isolated during a continuous adaptation experiment with the model azo dye 1-(4′-carboxyphenylazo)-4-naphthol (carboxy-Orange I) as sole source of carbon and energy. It is one of the very few microorganisms described that are able to grow with azo dyes. The strain was originally described as *Pseudomonas* sp. K24 (Kulla, 1981; Kulla et al., 1984) and recently described as a new genus within the *Alcaligenaceae* (Blümel et al., 2001). The aerobic azoreductase from this organism has been purified and characterized (Zimmermann et al., 1984), and recently the sequence of the gene encoding the azoreductase has been determined and shown to be unrelated to previously described aerobic azoreductases (Blümel and Stolz, 2003). Some other recent work suggested that *Pigmentiphaga* strains were also obtained after enrichment with naphthalene-2,6-disulfonate (Uchihashi et al., 2002; Uchihashi et al., 2003).

Genus *Taylorella*

The genus *Taylorella* (Sugimoto et al., 1983) encompasses two species, *Taylorella equigenitalis*, which originally was described as *Haemophilus equigenitalis* (Taylor et al., 1978), and *Taylorella asinigenitalis* (Jang et al., 2001). Cells of the two species are Gram-negative, nonmotile, short rods. Growth occurs under microaerobic conditions in an atmosphere with 5–10% CO_2 at 37°C. They are catalase and oxidase positive and do not produce acid from carbohydrates. Compared to other members of the family, the G+C-content of the genomic DNA is relatively low (36.5–37.8 mol%). Fatty acid profiles consist of predominant acids summed-in-feature (SIF) 7, $C_{16:0}$, $C_{18:0}$ and SIF 3 (Table 3). *Taylorella equigenitalis* (an inhabitant on the external genitalia of stallions) is usually transmitted venereally to

Table 3. Relative fatty acid compositions and G+C contents of species of *Taylorella*, *Pelistega* and *Oligella*.

Characteristic	*T. equi.*[a,c]	*T. asin.*[b]	*P. euro.*[a]	*O. uret.*[c]	*O. urea.*[c]
$C_{10:0}$	—	Tr	—	—	—
$C_{12:0}$	—	1.1	4.4	—	—
2-OH $C_{12:0}$	—	—	—	—	—
SIF 1[d]	—	—	Tr	—	—
$C_{14:0}$	Tr	1.1	9.7	4.4–5.3	5.0–8.0
$C_{15:1\ \omega8c}$	—	1.6	—	—	—
$C_{15:0}$	—	—	—	0.2–2.0	0.2–1.2
3-OH $C_{14:0}$	10.7[e]	5.5[e]	12.7[e]	4.7–7.3	4.6–7.3
SIF 4[f]	Tr	1.8	21.7	2.2–3.6[g]	1.2–3.4[g]
$C_{16:1\ \omega5c}$	—	—	5.2	—	—
$C_{16:0}$	36.8	29.4	15.7	25.7–30.6	19.5–30.5
$C_{17:0}$	—	—	—	0.1–1.3	0.1–0.5
$C_{17:0\ cyclo}$	—	—	—	—	—
3-OH $C_{16:0}$	Tr	—	1.2	—	—
SIF 6[h]	—	1.4	—	—	—
SIF 7[i]	41.6	46.5	27.8	45.3–49.6[j]	37.8–47.9[j]
$C_{18:1\ \omega9c}$	—	1.8	—	0.0–0.5	Tr–0.2
$C_{18:0}$	6.8	6.9	Tr	0.9–1.4	0.5–1.8
$C_{19:0\ 10\text{-methyl}}$	2.2	1.6	Tr	—	—
$C_{20:1\ \omega9t}$	—	Tr	—	—	—
Mol% G+C	38	37.8	42.0–43.8	46–47.5	46–47

Abbreviations: *T. equi.*, *Taylorella equigenitalis*; *T. asin.*, *Taylorella asinigenitalis*; *P. euro.*, *Pelistega europaea*; *O. uret.*, *Oligella urethralis*; *O. urea.*, *Oligella urealytica*; SIF, summed in feature; and for other abbreviations, please refer to the footnote in Table 1.

[a]Vandamme et al. (1998).

[b]Rossau et al. (1987).

[c]Jang et al. (2001).

[d]$C_{14:1\omega5c}$ and/or $C_{14:1\ \omega5t}$.

[e]Given as summed-in-feature 3 ($C_{16:1iso\ I}$, 3-OH $C_{14:0}$, an unidentified fatty acid with equivalent chain-length value of 10.928 and/or $C_{12:0\ alde}$).

[f]$C_{16:1\omega7c}$ and/or 2-OH $C_{15:iso}$.

[g]Given as $C_{16:1}$.

[h]$C_{18:2\omega6,9}$/$C_{18:0\ anteiso}$ and/or $C_{18:0anteiso}$/$C_{18:2\omega\ 6,9c}$.

[i]$C_{18:1\omega9c}$, $C_{18:1\omega7c}$, $C_{18:1\omega9t}$ and/or $C_{18:1\omega12t}$.

[j]Given as $C_{18:1\Delta11}$.

mares. *Taylorella equigenitalis* is the causative agent of contagious equine metritis (CEM), a venereal disease in horses causing vaginal discharge, infertility, or early abortion (Wada et al., 1983). This disease has been detected in many countries all over the world in various breeds of horses (Taylor et al., 1978; Kagawa et al., 2001). Diagnosis of CEM is not possible on the basis of clinical signs because of similarity of the disease to other bacterial infections of the reproductive tract (Powell, 1981). To detect *T. equigenitalis* in clinical samples several PCR assays have been developed (Bleumink-Pluym et al., 1994; Anzai et al., 1999; Arata et al., 2001; Kagawa et al., 2001; Premanandh et al., 2003). *Taylorella equigenitalis* is sensitive to penicillin G, ampicillin, carbenicillin, cephaloridine, erythromycin, tetracycline, kanamycin, gentamicin, chloramphenicol, and polymyxin B and resistant to clindamycin, lincomycin, trimethoprim and sulfamethoxazole (Sugimoto et al., 1983). Strains of *T. asinigenitalis* have been isolated from the urethral fossae of donkeys, but they were not associated with the development of signs of disease in mares (Jang et al., 2001).

Genus *Pelistega*

Phylogenetically, the only representative of the genus *Pelistega*, *Pelistega europaea*, is the nearest relative of the genus *Taylorella* (Vandamme et al., 1998). Cells are Gram-negative and nonmotile with variable shape. They grow under microaerobic conditions on conventional media such as nutrient broth at 37°C and 42°C but not at 24°C and grow aerobically but not anaerobically. Cells are positive for catalase and oxidase. Acid is not produced from carbohydrates. The low G+C content (42–43 mol%) is in agreement with their close relatedness to the genus *Taylorella*.

In the fatty acid profile, SIF 7, 4, and 3 and $C_{16:0}$ and $C_{14:0}$ predominate. Strains of *P. europaea* have been isolated from samples of lungs, air sac

exudate, trachea mucosa, liver, spleen, and swabs taken from the palatine cleft or trachea of living acutely diseased pigeons. On the basis of clinical observations, *P. europaea* is considered to be pathogenic and involved in pathogenesis of respiratory diseases in pigeons.

Genus *Oligella*

Described by Rossau et al. (1987), the genus *Oligella* encompasses the species *Oligella urethralis* (formerly *Moraxella urethralis*) and *Oligella ureolytica* (formerly CDC group IVc2). Cells are Gram-negative, aerobic, small rods or coccobacilli. Incubation in the presence of 5% CO_2 enhances growth. They are oxidase and usually catalase positive. They grow on nutrient agar, but growth is enhanced by addition of serum, blood, or yeast autolysate. Few organic acids and amino acids are oxidized or used as sole source of carbon. The G+C content of the genomic DNA of the two species is in the range 46–47.5 mol%. *Oligella urethralis* contains a quinone system with the predominant compound Q-8 (Moss et al., 1988) and a polyamine pattern with the predominant polyamines putrescine and 2-hydroxyputrescine (H.-J. Busse, unpublished results). The fatty acid profiles consist of the major acids $C_{18:1\omega\ 7c}$ and $C_{16:0}$ and the hydroxylated acids 3-OH $C_{14:0}$ and 3-OH$C_{16:0}$ are detected (Jantzen et al., 1987; Rossau et al., 1987; Moss et al., 1988; Table 3).

Oligella urethralis has been isolated from human urine, genitourinary tract, and ear. *Oligella ureolytica* has been isolated from human urine. Both species have been reported to cause urosepsis (Rockhill and Lutwick, 1978; Pugliese et al., 1993).

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Prokaryotes (2006) 5:701–709
DOI: 10.1007/0-387-30745-1_29

CHAPTER 3.2.5

The Genus *Spirillum*

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Introduction

The genus *Spirillum* contains only a single species—*Spirillum volutans* (Krieg, 1984b). Prior to 1973, however, the genus *Spirillum* included other aerobic/microaerophilic, chemoheterotrophic spirilla as indicated in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (Krieg, 1974). Hylemon et al. (1973b) divided the genus into the three genera *Spirillum*, *Aquaspirillum*, and *Oceanospirillum* on the basis of their DNA base composition and certain physiological characteristics. The genus *Spirillum* was reserved for large, microaerophilic, freshwater spirilla having a DNA base composition of 38 mol% G+C (thermal denaturation method) or 36 mol% G+C (buoyant density method).

Within the Proteobacteria, *S. volutans* is a member of the beta division, based on oligonucleotide cataloguing of 16S rRNA (Woese et al., 1982; Woese et al., 1984; Woese, 1987). Its nearest neighbor is *Thiobacillus thioparus*, according to the *Bergey's Manual* revision of the Ribosomal Database Tree. Other fresh-water spirilla (i.e., *Aquaspirillum* species) are not closely related to *S. volutans*, although very few species have been analyzed. They have been placed in subgroups 1 and 2 of the beta subdivision and in the alpha subdivision of the Proteobacteria (Woese et al., 1984; Woese, 1987).

Other organisms provisionally named "*Spirillum*" are not included in the genus. These include "*Spirillum minus*," *Spirillum pulli*," and "*Spirillum pleomorphum*." These names have no official standing in nomenclature, and the taxonomic placement of these organisms is uncertain. They may be related to aquaspirilla or to campylobacters (Krieg, 1984a). "*S. minus*" is a causative agent of rat-bite fever in humans; "*S. pulli*" causes a diphtheroid stomatitis in chickens. Neither has been grown on artificial media. "*S. pleomorphum*" is a psychrophile isolated from Antarctic soil.

A large spirillum called "*Aquaspirillum voronezhense*" was described by Grabovich et al. (1987), which has a number of features similar to those of *S. volutans*. These include helical cells 1.5–2.1 to 3.0 μm wide, bipolar flagellar fascicles with up to 50 flagella in each fascicle, poly-beta-hydroxybutyrate granules, and inability to use carbohydrates. However, the organisms differ by being aerobic, catalase-positive, and urease-positive and by having a mol% G+C of the DNA of 58.5–60.0.

Habitat

S. volutans is widely distributed in many stagnant freshwater environments. Only two strains have been isolated in pure culture, one from hay infusions prepared with water from a pond in Virginia (Wells and Krieg, 1965) and the other from the cooling water of a sugar refinery in England (Rittenberg and Rittenberg, 1962). In hay infusions, the organisms are most abundant after several days. The spirilla accumulate in the microaerobic region just beneath the surface scum of aerobic bacteria.

Isolation

Selective Enrichment

Mixed cultures of the organism occurring in hay infusions or other sources can be enriched by inoculating Pringsheim's soil medium (Rittenberg and Rittenberg, 1962). One wheat or barley grain is placed in a large test tube and covered with 3–4 cm of garden soil. The tube is then filled almost to the top with tap water and is sterilized by autoclaving for 30 min. After inoculation, *S. volutans* multiplies to approximately 1–2 $\times$ 10^6 cells/ml with incubation at room temperature, while the total bacterial population reaches approximately 1 $\times$ 10^9 cells/ml (Rittenberg and Rittenberg, 1962).

Isolation Procedure

Isolation is difficult, even with enrichment, because *S. volutans* is greatly outnumbered by

other organisms and because no selective media are available. To date, the only successful method for isolation is a mechanical method first described by Giesberger (1936) and used successfully with *S. volutans* by Rittenberg and Rittenberg (1962). This method is also useful for isolating other freshwater and marine spirilla (Giesberger, 1936; Jannasch, 1965). In this procedure, the center of a short section of sterile cotton-plugged 5-mm glass tubing is softened in a flame and pinched with square-ended forceps until almost closed. The flattened portion is reheated and drawn out rapidly to form a long, thin-walled capillary tube, 15–30 cm long and 0.1–0.3 mm in diameter, with oval cross-section. The ends of the capillary are sealed in a flame and the tubes are stored in a dust-free environment. Before use, the capillary is broken near the tip with sterile forceps and 10–20 cm of sterile medium are drawn into it, followed by 2–4 cm of enrichment culture, with no air space between the two liquids. Leaving a small air space at the tip of the capillary, the tip is sealed in a flame. The tube is mounted horizontally on the stage of a microscope so that it can be examined along its length at a magnification of about 100×. Because of the rapid motility of *S. volutans*, the organism will frequently be able to reach the distal portions of the capillary before other motile bacteria. In many instances an aerotactic band of *S. volutans* may form and travel down the length of the tube; however, one should look for spirilla travelling ahead of this band, because the band itself may be contaminated by other highly motile bacteria. As soon as some spirilla have migrated far enough along the capillary to have outdistanced the contaminants, the capillary is broken behind the spirilla and sealed in a flame. The outside of the sealed capillary is sterilized in strong hypochlorite solution, followed by sterile thiosulfate solution. The tip is then broken again and the contents expelled into a dialysis sac containing sterile Pringsheim's medium (soil plus supernatant). The dialysis sac is suspended in a beaker of similar medium that has been inoculated with some of the original mixed culture. The main function of the mixed culture outside the dialysis sac appears to be that of decreasing the level of dissolved oxygen or possibly of destroying toxic forms of oxygen in the medium, thereby allowing the microaerophilic *S. volutans* organisms to grow in pure culture inside the sac. The dialysis sac system is incubated at room temperature until *S. volutans* has multiplied within the sac. Purity of the culture within the sac should be verified microscopically. When a pure culture has been obtained, it should be transferred to a fresh dialysis sac system every few days because some of the organisms growing in the mixed culture outside the sac may cause the sac to disintegrate through cellulolytic activity. Eventually, transfers should be made to tubes of a suitable medium such as semisolid modified peptone-succinate-salts (MPSS) or casein hydrolysate-succinate-salts (CHSS) medium.

Because *S. volutans* grows readily in semisolid MPSS and CHSS media, it is possible that the need for the intermediate dialysis sac cultures in the above procedure could be avoided by expelling the cells from the capillary tube directly into the semisolid medium.

Media for *Spirillum volutans*

MPSS Broth for *S. volutans* (Caraway and Krieg, 1972)

Succinic acid (free acid)	1.0 g
$MgSO_4 \cdot 7H_2O$	1.0 g
Bacto Peptone (Difco)	5.0 g
$(NH_4)_2SO_4$	1.0 g
$FeCl_3 \cdot 6H_2O$ (0.2% aqueous solution)	1.0 ml
$MnSO_4 \cdot H_2O$ (0.2% aqueous solution)	1.0 ml

The first four ingredients are dissolved in 1 liter of distilled water and the pH is adjusted to 7.0 with 2 N KOH. The $FeCl_3$ and $MnSO_4$ solutions are added and the medium is sterilized by autoclaving. The medium is stored in the dark to avoid photochemical production of hydrogen peroxide.

Semisolid MPSS medium is prepared by adding 0.15% agar to the liquid medium. MPSS agar is prepared by adding 1.5% agar to the liquid medium.

CHSS Broth for *S. volutans* (Padgett and Krieg, 1986)

Succinic acid (free acid)	1.0 g
$MgSO_4 \cdot 7H_2O$	1.0 g
Acid-hydrolyzed casein, "vitamin-free, salt-free" (ICN Nutritional Biochemicals, Cleveland, OH)	2.5 g
$(NH_4)_2SO_4$	1.0 g
NaCl	0.1 g
KH_2PO_4	0.14 g
$FeCl_3 \cdot 6H_2O$ (0.2% aqueous solution)	1.0 ml
$MnSO_4 \cdot H_2O$ (0.2% aqueous solution)	1.0 ml
Potassium metabisulfite	0.05 g

The first eight ingredients are dissolved in 1 liter of distilled water and the pH is adjusted to 7.0 with 2 N KOH. The potassium metabisulfite is added and the pH readjusted to 7.0. The medium is sterilized by autoclaving and stored in the dark.

Semisolid CHSS medium is prepared by adding 0.15% agar to the medium after addition of the potassium metabisulfite, boiling to dissolve the agar, and then autoclaving. CHSS agar is prepared by adding 1.5% agar to the liquid medium.

Goatcher et al. (1984) reported that Bactocasitone (Difco) could substitute for the indi-

cated casein hydrolysate and that $MnSO_4$ could be omitted under these conditions. Bowdre and Krieg (1974) did not include NaCl in their original formulation of CHSS broth. However, Padgett et al. (1982) found that the casein hydrolysate used by Bowdre and Krieg was not completely salt-free. When salt-free batches of casein hydrolysate were used, a low level of NaCl was required for growth. Levels of NaCl greater than 0.02% inhibited growth. Bowdre and Krieg (1974) did not include KH_2PO_4 in their original formulation of CHSS broth. However, its addition to the medium has since been found to increase the growth response of *S. volutans* (Friedman, 1987).

Colony Count Medium (CCM) (Alban and Krieg, 1996)

Succinic acid (free acid)	1.0 g
$MgSO_4 \cdot 7H_2O$	1.0 g
Acid-hydrolyzed casein, "vitamin-free, salt-free" (ICN Nutritional Biochemicals, Cleveland, OH)	2.6 g
$(NH_4)_2SO_4$	1.0 g
NaCl	0.04 g
KH_2PO_4	0.12 g
Sodium pyruvate	0.3 g
$FeCl_3 \cdot 6H_2O$ (0.2% aqueous solution)	1.0 ml
$MnSO_4 \cdot H_2O$ (0.2% aqueous solution)	1.0 ml
Potassium metabisulfite	0.05 g

The first nine ingredients are dissolved in 1 liter of distilled water and the pH is adjusted to 7.3 with 2 N KOH. The pH is adjusted to 7.3 with KOH and the potassium metabisulfite is added. The pH is readjusted to 7.3. The medium is sterilized by autoclaving and stored in the dark. The pH of the cooled medium should be 6.8.

For semisolid medium and for colony counts, 0.7 g of agar is added after addition of the potassium metabisulfite, and the medium is boiled to dissolve the agar prior to autoclaving.

Identification

S. volutans can be differentiated from other chemoheterotrophic, aerobic or microaerophilic, motile, curved or helical or vibrioid bacteria by the characteristics indicated in Table 1. Of particular importance are the large cell size, the exceptionally large bipolar fascicles of flagella (which can be seen readily by phase contrast microscopy), and the failure of the organism to grow aerobically on any ordinary solid medium. Verification of identification is best obtained by comparing an isolate with the type strain, ATCC strain 19554.

S. volutans cells have the form of a left-handed, or counterclockwise, helix (Swan, 1985). The cells have 1–5 turns, a wavelength of 16–28 μm, a helix diameter of 5–8 μm, and a helix length of 14–60 μm. Numerous refractile intracellular granules of poly-β-hydroxybutyrate are present. The bipolar flagellar fascicles consist of many individual nonsheathed flagella of long wavelength (Fig. 1). Flagellar hooks and basal structures have been described by Swan (1985). *S. volutans* swims in straight lines, and during swimming, the fore fascicle is bent toward the aft end of the cell and describes a wide bell; the aft fascicle extends behind the cell and describes a wide goblet. A cell often reverses its direction of swimming, and when this happens both flagellar fascicles reorient their configuration simultaneously, so that what was previously the fore fascicle now becomes the aft fascicle and vice versa. Cells that are flagellated at only one pole can reverse their swimming direction, indicating that either a fore or aft fascicle alone can propel the cells (Swan, 1982). Ramia and Swan (1994) have used high-speed cinemicrography to record the swimming of unipolarly flagellated cells. The geometry of these cells was numerically modeled with curved isoparametric boundary elements (from the measured geometrical parameters), and an existing boundary element method (BEM) program was applied to predict the mean swimming linear and angular speeds. A helical cell shape is not required for motility, as straight-cell variants of *S. volutans* are able to swim at nearly the same speed as wild-type helical cells (Padgett et al., 1983). The aerotactic behavior of *S. volutans* has been described by Caraway and Krieg (1974).

Electron microscopy of ultrathin sections of *S. volutans* reveals an intracellular "polar membrane," which is attached to the cytoplasmic membrane (Coulton and Murray, 1978). This structure has been observed mainly in helical bacteria, such as *Aquaspirillum*, *Campylobacter*, *Rhodospirillum*, *Selenomonas*, and *Vibrio*, but it has also been detected in nonhelical bacteria such as *Methanococcus* and *Chromatium*. The polar membrane of *Campylobacter jejuni* consists of an assemblage of ATPase molecules (Brock and Murray, 1988).

Round cells, called coccoid bodies, such as occur commonly in old cultures of other helical or curved bacteria, such as *Aquaspirillum itersonii* and *Campylobacter jejuni*, do not occur in cultures of *S. volutans*.

Cultivation

Optimal growth occurs between 30 and 36°C and at pH values of 7.0–8.2 (Moore, 1984). Although the organism is a microaerophile, cultures in semisolid media can be incubated in an air atmosphere due to stratification of the medium. Growth begins as a thin disk some distance

Table 1. Differential characteristics of the genus *Spirillum* and of other motile, aerobic or microaerophilic, Gram-negative, helical or curved bacteria.

	Cell shape	Cell diameter (μm)	Requires host for growth	Oxygen requirement	Usual arrangement of flagella	Catalase	Requires sea water or >1% NaCl	Inhibited by 35% NaCl	Carbohydrates catabolized	Nitrogenase activity (microaerobic conditions)	G+C content (mol%)	Habitat
Spirillum volutans	Helical	1.4–1.7	–	Micr	Large tufts, BP	–	–	+	–	–	36–38	Stagnant freshwater
Aquaspirillum	Helical[b]	0.2–1.4	–	Aer	Tufts, BP[e]	+[d]	–	+	–[e]	–[f]	49–66	Stagnant freshwater
"Aquaspirillum voronezhense"[g]	Helical	1.5–3.0	–	Aer	Large tufts, BP	+	–	+	–	–	58–60	Sewage treatment air tank
Arcobacter	Vibrioid	0.2–0.9	–	Aer[h]	1, SP	+[i]	D	D	–	D	27–30	Humans and other animals; roots of salt marsh grasses
Asospirillum	Vibrioid[j]	0.9–1.5	–	Aer	1, SP[k]	+	–	D	+	+	68–70	Soil and plant roots
Bdellovibrio	Vibrioid	0.2–0.5	+[l]	Aer	1, SP	D	D	D	–	–	33–52	Freshwater and marine
Campylobacter	Vibrioid[m]	0.2–0.9	–	Micr	1, SP or BP[n]	D	–	D	–	–	30–38	Humans and other animals
Helicobacter	Vibrioid[o]	0.5–1.0	–	Micr	Various types; some have sheathed flagella	+	–	+	–	–	35–44	Stomachs or intestines of humans and other animals
Herbaspirillum	Vibrioid[p]	0.6–0.7	–	Aer	1–3, SP or BP	+	–	+	+	+	66–67	Stagnant freshwater
Magnetospinillum	Helical	0.2–0.7	–	Micr	1, BP	D	–	+	–	D	64–71	Stagnant freshwater
Marinomonas communis	Curved	0.7–1.5	–	Aer	1, SP	+	+	–	+	–	45–48	Marine
Oceanospinillum	Helical[q]	0.3–1.4	–	Aer	Tufts, BP[r]	D	+	–	–[s]	–	42–51	Coastal marine waters

Pseudoalteromonas undina	Curved	0.7–1.5	–	Aer	1, SP	+	+	–	+	–	43–44	Marine
"Spirillumminus"	Helical	0.2	+		1 or more BP							Humans and rodents[l]
"Spirillumpulli"	Helical	1.0	+		1, SP or BP							Chickens[u]
"*Spinillum pleomorphum*"	Helical or curved	0.7–1.0	–	Aer	1, SP	+	–	–	+[t]	ND	603	Antarctic soil
Vampirovibrio	Vibrioid	0.3	+[w]		1, SP						50	Freshwater
Wolinella succinogenes	Helical	0.5–1.0	–	Micr	1, SP	–	–	ND	ND	–	47	Bovine rumen

[a]Symbols: +, all species positive except where noted; –, all species negative except where noted; D, differs among species; BP, both poles; SP, single pole; Aer, aerobic; Micr, microaerophilic; ND, no data.
[b]*A. delicatum* is mainly vibrioid.
[c]*A. delicatum* has mainly a single flagellum at one pole; *A. polymorphum* has mainly a single flagellum at each pole.
[d]*A. putridiconchylium* is catalase-negative or very weakly positive.
[e]*A. gracile*, *A. itersonii*, and *A. peregrinum* can catabolize only a very restricted variety of sugars.
[f]*A. peregrinum* and some strains of *A. itersonii* have nitrogenase activity.
[g]Described by Grabovich et al., 1987, Microbiologiya 56:666–672.
[h]May be microaerophilic on primary isolation. Although *A. nitrofigilis* is a microaerophilic nitrogen fixer, it can grow aerobically in complex media such as Brucella agar.
[i]*A butzleri* is weakly catalase positive.
[j]Some cells in *Azospirillum* cultures are straight rods.
[k]Some *Azospirillum* species form numerous lateral flagella in addition to the polar flagellum when cultured on solid media.
[l]All wild-type strains of *Bdellovibrio* upon initial isolation are dependent on intraperiplasmic growth in susceptible bacterial prey. Mutants capable of axenic growth ("prey-independent" strains) have been derived from the predacious strains, and some strains are facultative, i.e., capable of growth in the presence and absence of prey cells.
[m]Chains of *Campylobacter* cells may have a helical appearance. *C. rectus*, *C. showae*, and *C. gracilis* are straight rods.
[n]*C. showae* has a tuft of flagella at one pole.
[o]*H. trogontum* and *H. bilis* are fusiform straight rods. *H. bizzozeronii* and *H. felis* cells are long helices. *H. cholecystus* cells are coccoid to short curved rods.
[p]Some cells in cultures of *Herbaspirillum* are helical.
[q]*O. kriegii* and *O. jannaschii* are straight rods.
[r]*O. pusillum* has mainly a single flagellum at each pole. *O. kriegii* and *O. jannaschii* have a single flagellum at one pole.
[s]Causes one form of rat-bite fever in humans.
[u]Causes a diphtheroid stomatitis in chickens.
[t]Produces acid fiomxylose but not from other sugars that have been tested; it is also distinguished by failing to grow at temperatures above 20°C.
[w]Predacious on eukaryotic algae.

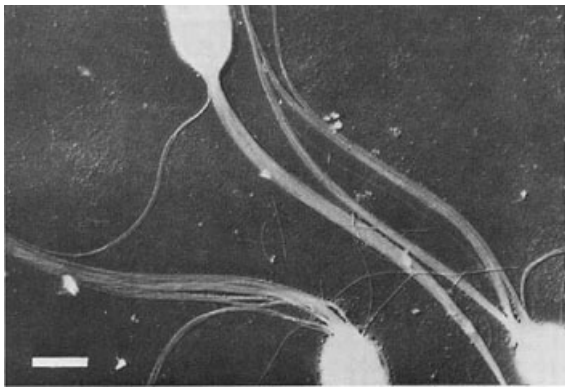

Fig. 1. Polar flagellar fascicles of *S. volutans* ATCC 19554 n by electron microscopy. Bar = 1 μm. (From Hylemon et al., 1973a.)

below the surface at a point where the respiratory rate of the cells matches the rate of diffusion of oxygen to the cells. As the cell numbers increase, the disk becomes denser and migrates toward the surface. After 48 h dense growth occurs just beneath the surface.

Cultures grown in MPSS broth (no agar added) or on solid media for colony development should be incubated under an atmosphere of 6% O_2 and 94% N_2. A convenient way to do this is to use a polycarbonate jar such as those that are used routinely for cultivation of anaerobes, provided that it has a vent to allow evacuation. After placing the inoculated media in the jar and sealing it, the jar is evacuated to 0.286 of the normal atmospheric pressure. Nitrogen gas is then added until the atmospheric pressure is restored.

Padgett et al. (1982) reported that cells inoculated into MPSS broth can grow under aerobic conditions if the medium is supplemented with either bovine superoxide dismutase (SOD) (4 units/ml), catalase (0.8 units/ml), potassium metabisulfite (0.02%), or norepinephrine (0.002%).

Cultures in CHSS broth (no agar added) can grow aerobically (Bowdre and Krieg, 1974). Five-ml volumes contained in 20 × 125-mm loosely screw-capped tubes are inoculated with several drops of culture and incubated at 30°C in a slanted position. Growth occurs near the surface of the medium in 24 h. Transfers must be made daily, with several drops of each previous culture serving as inoculum for the next tube. Cultures usually die within 48 h, possibly due to the high pH (>8.0) that develops as the result of oxidation of the succinate. Bowdre et al. (1976) used a chemically defined liquid medium containing norepinephrine, which, like CHSS broth, supports growth of *S. volutans* under aerobic conditions. Growth of *S. volutans* on the surface of solid media (MPSS or CHSS broth solidified with 1.5% agar) is difficult but can be achieved by methods described by Padgett et al. (1982). The agar media must be supplemented with potassium metabisulfite (0.002%), catalase (230 units/ml), or SOD (30 units/ml). If catalase or SOD is used, it is added aseptically to the molten medium at 45°C just before dispensing into Petri dishes, whereas potassium metabisulfite is added prior to autoclaving. The medium is dispensed into Petri dishes (20 ml per dish) under dim illumination or red photographic safelight (red light does not cause photochemical generation of toxic forms of oxygen in the medium). The plates are allowed to dry in the dark at room temperature for 24 h. In dim illumination, 0.1-ml portions of appropriate dilutions of a broth culture of S. volutans are spread onto the agar surface with a glass rod. The plates are incubated for 5 days at 30°C in the dark in a sealed vessel containing 6–12% O_2 (the balance being N_2). It is essential that the vessel be lined with moistened filter paper to maintain a high humidity. Even with these precautions, only 22–72% of the cells inoculated onto the plates develop into colonies.

A method for obtaining reproducible colony counts was reported by Alban and Krieg (1996). The method uses a semisolid version of colony counting medium (CCM) as an overlay for a thicker layer of sterile medium. The medium contains pyruvate, which destroys hydrogen peroxide. Cells to be used for colony counts are grown in 5.0 ml of CCM broth contained in slanted 20 × 120-mm loosely screw-capped tubes incubated aerobically for 24 h at 30°C. Sterile semisolid medium (CCM plus 0.7% agar) is dispensed into Petri dishes (15 ml per dish) and allowed to gel for 30 min. A 0.1-ml volume of an appropriate dilution of the inoculum is added to 10 ml of semisolid CCM at 45°C and is poured onto the plates as an overlay. After this has gelled, the plates are incubated at 30°C in an atmosphere of 6% O_2 and 94% N_2 for 3–4 days.

Preservation

Preservation of *S. volutans* can be accomplished by the following procedure (Pauley and Krieg, 1974): Cells from a turbid 24-hour-old broth culture are centrifuged at 3,500 × g and washed once in nutrient broth. The cells are then suspended by gentle agitation in a small amount of nutrient broth containing 10% dimethyl sulfoxide (DMSO). The resulting dense suspension is allowed to incubate for 30 min at room temperature and then 0.4-ml volumes are dispensed into plastic or glass vials suitable for liquid nitrogen preservation. The vials are sealed, frozen in a mixture of dry ice and alcohol, and then stored by submersion in liquid nitrogen. For recovery of

the organisms, the vials are thawed in water at 30°C and transferred to semisolid MPSS or CHSS medium.

Physiology

S. volutans has a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. It is microaerophilic in ordinary liquid media, such as nutrient broth (in which it grows poorly) and MPSS broth, and on solid media, such as CHSS agar supplemented with bisulfite. Also, it can grow aerobically in special liquid media such as CHSS broth or the chemically defined medium of Bowdre et al. (1976). Growth is inhibited by very low concentrations of H_2O_2 (0.29 μM or greater), and the organism is rapidly killed by concentrations of 10 μM or greater (Alban and Krieg, 1988). The microaerophilic nature of the organism has been attributed to the high susceptibility of the organism to toxic forms of oxygen which occur spontaneously in MPSS or CHSS broth under aerobic conditions, particularly when the media are illuminated (Padgett et al., 1982). Destruction of toxic forms of oxygen by media supplements such as SOD, catalase, potassium metabisulfite, or norepinephrine allows the organism to grow aerobically in broth (Padgett et al., 1982). However, even with these supplements, *S. volutans* can form colonies on MPSS, CHSS, or CCM agar only under microaerobic conditions (12% O_2 or less). It will not grow even under microaerobic conditions unless the media supplements are present. Padgett and Krieg (1986) described two variant strains of *S. volutans* which, unlike the wild type, could grow in an air atmosphere (i.e., 21% O_2) on CHSS agar containing potassium metabisulfite. Cell extracts of those aerotolerant variants exhibited greater *o*-dianisidine peroxidase activity than did the wild type, and this enzyme may be important for aerotolerance. The catalase-negative microaerophile *Spirillum volutans* is killed rapidly by levels of H_2O_2 greater than 10 μM. Alban and Krieg (1998) described a mutant isolated by single-step mutagenesis with diethyl sulfate that was able to survive and grow after exposure to 40 μM H_2O_2 and was effective in eliminating H_2O_2 added to the medium. Nevertheless, the mutant was no more tolerant to O_2 than the wild type. The only apparent phenotypic difference between the wild type and the mutant was that the mutant had high NADH peroxidase activity (0.072 IU per mg protein) whereas the wild type had no detectable activity (<0.0002 IU per mg protein). Alban et al. (1998) found that the mutant constitutively expresses a 21.5 kDa protein that is undetectable and noninducible in the wild type cells. Part of the gene that encodes the protein was cloned and the deduced 158 amino acid polypeptide showed high relatedness to rubrerythrin and nigerythrin—proteins previously described only in certain anaerobic members of the Bacteria and Archaea.

S. volutans is relatively inert biochemically in most routine characterization tests but is oxidase- and phosphatase-positive and produces H_2S from cysteine (Hylemon et al., 1973a). It is negative for the enzymes catalase, DNase, RNase, urease, and arylsulfatase; gelatin liquefaction; hydrolysis of casein, starch, and esculin; indole production; reduction of nitrate and selenite; anaerobic growth with nitrate; and growth in the presence of 1% bile or 1% glycine. Carbohydrates are not catabolized. Carbon and energy sources include the salts of various organic acids. Succinate, fumarate, malate, oxaloacetate, and pyruvate are oxidized readily, whereas citrate, aconitate, isocitrate, and α-ketoglutarate are oxidized weakly (Caraway and Krieg, 1974). Vitamins are not required for growth (Bowdre et al., 1976). In CHSS medium very low levels of NaCl are required, but growth is inhibited by levels greater than 0.02% (Padgett et al., 1982). Oxygen uptake by cells suspended in a chemically-defined motility medium is inhibited by 50 mM phosphate (pH 6.8) but not by 10 mM phosphate (Caraway and Krieg, 1974). Crude cell extracts possess an iron-containing superoxide dismutase and very low levels of peroxidase (Padgett and Krieg, 1986).

The response of *S. volutans* ATCC 19554 to 44 antibiotics and other chemotherapeutic agents was determined by Friedman (1987). The organism was inhibited by each of the agents tested except clindamycin, cloxacillin, lincomycin, and oxacillin. It is interesting that, although *S. volutans* respires with oxygen, its growth is completely inhibited by as little as 0.05 μg/ml of metronidazole, a compound that is selectively toxic toward anaerobes. As indicated by Padgett and Krieg (1986), this provides circumstantial evidence for the occurrence of ferredoxins or flavodoxins in *S. volutans*, because metronidazole reduction is preferentially linked to these electron carriers.

Applications

Growth in low concentrations of Zn^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, and various other toxic substances results in formation of cells that have either an aft flagellar fascicle at each pole or a fore fascicle at each pole, with consequent inhibition of motility (Krieg et al., 1967; Caraway and Krieg, 1972). This is the basis of a simple method for biological detection of toxicants in the effluents from industrial plants (Bowdre and Krieg, 1974). The

method essentially involves combining a sample of the material to be tested with cells of *S. volutans*; samples are removed periodically for microscopic examination to determine whether motility has been affected. The method has since been modified and evaluated by several investigators (Dutka et al., 1983; Goatcher et al., 1984; Moore, 1984; Cortes et al., 1996; Ghosh et al., 1996; Lacava and Ortolono, 1997) and has been found to be a useful screening procedure. Although this method cannot identify the pollutants present in an industrial effluent, it can detect the presence of toxic substances before the effluent is discharged into a receiving stream or river. Chemical and physical methods can then be used to identify the particular substances responsible. The method also has been used to detect the presence of toxic chemicals that may affect the normal operation of sewage treatment plants (McElroy, 1983).

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Prokaryotes (2006) 5:710–722
DOI: 10.1007/0-387-30745-1_30

CHAPTER 3.2.6

The Genus *Aquaspirillum*

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Helical bacteria have been found in nature under very diverse circumstances. For over 300 years people have admired these graceful forms of life under the microscope. Van Leeuwenhoek (1670) mentioned helical shapes when he described the first bacteria (animalcules). In contrast to their easy detection, spirilla have been very difficult to isolate and to maintain in pure culture. Müller (1773, 1786) described eight species of the genus *Vibrio*, three of which were spirilliforms. According to Williams (1959), the present type species of the genus *Aquaspirillum*, *Aquaspirillum serpens*, can be recognized in Müller's original description of "*Vibrio serpens*," but as noted by Terasaki (1980) and Krieg and Hylemon (1976), it is very unlikely that identification by modern standards can be made from the descriptions and drawings made by Müller.

The genus *Spirillum* was created by Ehrenberg (1832), with *Spirillum volutans* as the type species. Migula (1894, 1895, 1900) described many species within the genus *Spirillum*, mostly, however, based on mixed cultures. The genus *Spirillum* was reviewed thoroughly by Williams and Rittenberg (1957). They retained 19 species, mainly on the basis of morphology and a few nutritional and physiological characteristics. By cross-agglutination tests of cells of all 29 *Spirillum* strains, using antisera specific for *Spirillum* thermolabile antigens, McElroy and Krieg (1971) found 17 serogroups. Their subdivision was the basis of the classification of the genus *Spirillum* presented in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (Krieg, 1974). However, Hylemon et al. 1973 proposed that the genus *Spirillum* be split into the genera *Aquaspirillum*, *Oceanospirillum*, and *Spirillum* on the basis of DNA base composition and a limited number of physiological characteristics. Large obligately microaerophilic spirilla with large bipolar tufts of flagella and having a DNA base composition of 38 mol% GC constituted the redefined genus *Spirillum* (Hylemon et al., 1973), for which only one species, the type species of the original genus *Spirillum* (*Spirillum volutans* [Ehrenberg, 1832]), was described (see The Genus *Spirillum* in Volume 5). All marine spirilla requiring seawater for growth and having a GC content of 42 to 48 mol% were included in the new genus *Oceanospirillum*, containing five species (see The Genus *Oceanospirillum* in the second edition). *Oceanospirillum* was later extended with eight new species (Terasaki, 1973; Bowditch et al., 1984). The genus *Aquaspirillum*, with 13 species, was created for all aerobic freshwater spirilla having a low salt tolerance and a GC content of 50 to 65 mol%. Later the genus was also extended by addition of five new species (Aragno and Schlegel, 1978; Kumar et al., 1974; Maratea and Blakemore, 1981; Strength et al., 1976; Terasaki, 1973, 1979). As mentioned by Krieg (1981), this was an improvement over the previous classification, but it was still not entirely satisfactory. Indeed, on the basis of phenotypic characteristics, there seems to exist a continuum between the spirilla and a variety of other oxidative, Gram-negative bacteria, resulting in rather vague generic definitions. A vast polyphasic approach including genotypic studies was obviously needed to obtain a better insight in the relationship between these species. As a result *Oceanospirillum* has been redefined (Pot et al., 1989); it is described elsewhere (see The Genus *Oceanospirillum* in the second edition).

Results of DNA-rRNA hybridization studies (Pot et al., 1989) and 16S oligonucleotide cataloging (Woese et al., 1982, 1984b) incited us to explore further the inter- and intraspecific relationships within the genus *Aquaspirillum*. A very heterogeneous group of bacteria, spread over rRNA superfamilies III and IV, was found, indicating that *Aquaspirillum* can no longer be considered as one genus. The original genus name should be restricted to the species *Aquaspirillum serpens*, *A. bengal*, and *A. fasciculus*. As no definite proposals have been made yet to rename the other, misnamed, *Aquaspirillum* species, we will indicate their current (misnamed) status by square brackets.

All *Aquaspirillum* species described in *Bergey's Manual of Systematic Bacteriology* (Krieg, 1984) share the following characteristics: they are all rigid helical cells, except for *[A.] delicatum* which is vibrioid and *A. fasciculus* which is a straight rod. All species so far examined by electron microscopy have a polar membrane underlying the cytoplasmic membrane. They generally have bipolar tufts of flagella, but one single flagellum at each pole may be present (as in *[A.] polymorphum* and *A. magnetotacticum*, the latter being the only named *Aquaspirillum* species which was not included in our taxonomic investigations; for detailed information see Blakemore et al., 1989). In addition, *[A.] delicatum* possesses one or two flagella at one pole only. Intracellular poly-β-hydroxybutyrate is formed (except in *[A.] gracile* and *[A.] psychrophilum*) or is presumably formed (*A. magnetotacticum*). Coccoid bodies which predominate in old cultures (three to four weeks) may be formed (*[A.] peregrinum* subsp. *peregrinum, [A.] polymorphum*, *[A.] itersonii* and *A. fasciculus*). Aquaspirilla are typically aerobic, although some species grow in microaerophilic conditions (*[A.] peregrinum* and *A. fasciculus*), under which these species may exhibit nitrogenase activity (Strength et al., 1976). They have a respiratory type of metabolism with oxygen as terminal electron acceptor although some species can also grow anaerobically using nitrate. They are chemoorganotrophic; however, one species (*[A.] autotrophicum*) is facultatively autotrophic, as it can oxidize hydrogen. Only a few species can catabolize a limited number of carbohydrates; amino acids or organic acids usually serve as the major carbon source. Since the original description, the range of GC content of the genus has been

This chapter was taken unchanged from the second edition.

extended to vary between 49 and 66 mol%. An overview of differentiating phenotypic features is listed in Table 1.

Habitats

Freshwater spirilla are widely distributed in stagnant waters, and *Aquaspirillum* species have been isolated from various freshwater sources, including distilled water (Leifson, 1962), ditch and canal water (Giesberger, 1936), and sewage (Myers, 1940). *[A.] autotrophicum* was isolated from a eutrophic lake in Switzerland (Aragno and Schlegel, 1978). Some aquaspirilla have been isolated from putrid infusions of freshwater mussels, but according to Terasaki (1980) and Krieg (1976) it is more likely that these bacteria derived from the adherent mud rather than from the mussels. Giesberger (1936) also reported the isolation of a spirillum from horse or pig manure after it had been in contact with the stable floor ("*Spirillum pleomorphum*," presently regarded as a spirillum possibly belonging to the genus *Aquaspirillum*; Krieg, 1984). Presumably the bacteria were derived from the (wet) floor rather than from the (anaerobic) manure, as soil is a source of spirilla (Inoue, 1976; Inoue and Komagata, 1976).

In 1973, Scully and Dondero used the most-probable-number method to estimate the number of spirillum-like bacteria in samples of different origins. In nonmarine samples, spirilla numbers ranged from approximately 4% of the total bacterial population in algal samples to less than 0.01% in samples of cultivated field soil and drainage or forest soil. Generally the population density varied between 0.1 and 0.6% for pond mud, pond water, stream water, and cow manure.

Our new insights into the taxonomic relationships with and within the aquaspirilla (see "Identification and Taxonomy," The Genus *Aquaspirillum* in Volume 5) make it probable that the variety of habitats from which spirillum-like organisms will be isolated in the future will enlarge considerably. Indeed, B. Pot et al. (unpublished observations) found *[A.] autotrophicum* to be related at the intrageneric level with unnamed, slightly helical bacteria isolated from wounds and blood and with a group of generically misnamed [*Pseudomonas*] species pathogenic for sugarcane plants. *[A.] aquaticum* was found to have a DNA-DNA homology of at least 55% with a second group of unnamed, slightly helical bacteria of clinical origin (isolated from wounds, sputum, etc.), indicating a relationship at the species level. Although both [*Aquaspirillum*] species have to be renamed (see below), it indicates that clinical material can also be a source for the isolation of spirilla. Table 2 gives details on the isolation of all *Aquaspirillum* species presently included in the genus.

Isolation

Although spirilla have a very typical morphology, are widespread in nature, and were already described in the very early days of microbiology, they remained for a long time one of the least characterized groups of bacteria, due to 1) the lack of medical importance; 2) the difficult isolation of the organisms; and 3) the difficulties encountered for preservation (Hylemon et al., 1973). These obstacles have partly been removed since the publication of several techniques for enrichment, isolation, and pure culture preservation.

Enrichment is a necessary step in the isolation of spirilla since they are relatively slow-growing and never occur in predominant numbers.

Enrichment, Isolation and Maintenance of the Aquaspirilla

Since freshwater spirilla are rarely the predominant bacteria in any particular natural source (Scully and Dondero, 1973), for successful isolation several enrichment and isolation techniques (almost as many as there are species in the genus *Aquaspirillum*) had to be developed. A historical overview is given below.

GIESBERGER (1936) Giesberger (1936) used the following technique for the enrichment of freshwater spirilla from polluted water (Table 2):

> To a 300-ml Erlenmeyer flask, containing 200 ml of the water sample, was added:
>
> | | |
> |---|---|
> | Calcium malate or lactate | 2.0 g |
> | NH_4Cl | 0.1 g |
> | K_2HPO_4 | 0.05 g |
> | $MgSO_4 \cdot 7H_2O$ | 0.05 g |
>
> The pH was brought nearly to neutrality, but within 24 h the reaction became acidic and the pH had to be readjusted to 7.3. Spirilla began to develop from the third day, usually reaching a maximum after a week. Incubation at 40°C rather than 30°C resulted in good growth in 24 h. Giesberger also replaced the chemicals by a hay infusion (a handful of hay per liter of the water sample). Such infusions incubated at 30°C also gave good growth of spirilla after one week. The enriched cultures were streaked on peptone agar plates on which the excess moisture was removed by placing a filter paper soaked in glycerol in the cover. Although this helped to prevent contaminating bacteria from spreading too quickly over the plates, the colonies of the slow-growing spirilla were often rapidly overgrown by contaminants or were inhibited. For some spirilla, the addition of 0.5 to 1.0% sodium pyruvate to the peptone agar plates improved the isolation and culturing; for others Giesberger (1936) had to start by picking up the very small colonies with the aid of a microscope and a micromanipulator, after which they were blown out onto fresh peptone agar plates.

Table 1. Differentiating characteristics for all species presently included in the genus *Aquaspirillum*.

Differentiating characteristics	*A. serpens*[a]	*A. bengal*[a]	*A. fasciculus*[a]	*[A.] putridiconchylium*[a]	*[A.] dispar*[a]	*[A.] autotrophicum*[a]	*[A.] aquaticum*[a]	*[A.] delicatum*[a]	*[A.] gracile*[a]	*[A.] metamorphum*[a]	*[A.] psychrophilum*[a]	*[A.] sinuosum*[a]	*[A.] giesbergeri*[a]	*[A.] anulus*[a]	*[A.] magnetotacticum*[b]	*[A.] polymorphum*[c]	*[A.] itersonii* subsp. *itersonii*[c]	*[A.] itersonii* subsp. *nipponicum*[d]	*[A.] peregrinum* subsp. *peregrinum*[c]	*[A.] peregrinum* subsp. *integrum*[c]
Cell diameter (μm)[e]	0.6–1.1	0.9–1.2	0.7–0.9	0.7–1.2	0.5–0.7	0.6–0.8	0.5–0.6	0.3–0.4	0.2–0.3	0.7–1.3	0.7–0.9	0.6–0.9	0.7–1.4	0.8–1.4	0.2–0.4	0.3–0.5	0.4–0.6	0.5–0.8	0.5–0.7	0.5–0.7
Shape	H	H	SR	H	H	H	H	V	H	H	H	H	H	H	H	H	H	H	H	H
Type of helix	C	C	HA	C	C	C	C	HA	C	C	C	C	C	C	C	CC	CC	CC	CC	CC
Wavelength of helix (μm)	3.5–12.0	4.6–8.1	HA	4.5–7.0	2.0–3.5	3.0–4.0	2.0–5.0	HA	2.8–3.5	7.5–12.0	5.5–6.5	8.6–10.5	4.5–8.4	5.0–13.0	1.0–2.0	4.0–5.0	2.5–6.0	2.5–6.0	3.0–4.5	3.0–4.5
Helix diameter (μm)	1.2–4.2	1.7–2.3	HA	1.2–2.0	1.0–2.1	ND	0.8–1.0	0.4–0.7[g]	0.5–2.1	2.2–3.5	1.0–1.4	1.4–3.5	1.2–5.0	1.7–4.5	ND	1.0–1.5	1.0–2.2	1.0–2.2	1.4–2.0	1.4–2.2
Length of helix (μm)	3.5–42.0	5.2–22.0	3.6–43.0[f]	4.0–23.0	2.1–6.5	2.0–5.0	2.5–13.0	3.0–5.0[g]	3.5–14.0	3.5–11.0	1.5–14.0	5.0–42.0	4.0–40.0	4.0–52.0	4.0–6.0	3.5–8.4	2.0–10.0	2.0–10.0	1.5–22.0	1.5–22.0
Polar membrane present	+	+	+	+	+	ND	+	+	+	+	ND	+	+	+	ND	+	+	+	+	+
Poly-β-hydroxybutyrate formed	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	+	+
Flagellar arrangement	BT	BT	BT	BT	BT	BT	BT	U(1–2)	BT	BT	BT	BT	BT	BT	BS	BS	BT	BT	BT	BT
Coccoid bodies predominant at 3–4 weeks	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−
Acid produced from sugars[h]	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	+	+
Optimum temperature is 20°C	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Optimum temperature is 41°C	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Obligately microaerophilic	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Phosphatase	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	+
Urease[i]	d	−	+	−	−	+	−	−	+	−	ND	+	+	−	−	−	−	−	+	+
Indole test	−	−	−	−	−	−	−	−	−	−	−	−	−	−	ND	−	−	−	−	−
Nitrate reduced only to nitrite	d	−	+	−	−	−	+	+	+	−	−	−	−	−	−	+	−	−	−	−
Anaerobic growth with nitrate	−	−	+	−	+	−	−	−	−	−	+	−	−	−	−	−	+	+	−	−
Denitrification	−	−	−	−	+	−	−	−	−	−	+	−	−	−	+	−	+	+	−	−
Hydrolysis of esculin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+
Growth factors required	−	−	−	−	−	−	+[j]	−	+[j]	−	ND	−	−	−	ND	−	−	−	−	−
Glutamate as sole C-source	+	+	+	+	d	+	+	−	ND	+	ND	−	−	+	ND	+	+	+	+	+
Histidine as sole C-source	−	−	−	+	−	−	−	−	ND	−	ND	−	−	−	ND	−	+	+	−	−
Tryptophan and glycine as sole C-source	−	−	−	−	−	+	−	−	ND	−	−	ND	−	−	ND	−	−	−	−	−
Nitrogenase activity[i]	−	−	+	−	−	ND	−	−	ND	−	ND	−	−	−	+	−	d	d	+	+

Hydrogen autotrophy[i]	ND	ND	ND	ND	–	+	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–	–	–
Temperature range for growth, °C	12–42	15–42	ND	8–40	ND	10–35	12–42	9–40	ND	3–38	2–26	9–37	9–36	3–36	ND	14–36	12–42	12–42	11–40	11–40
pH range for growth	6.0–9.0	6.0–8.4	5.5–8.5	5.5–8.5	ND	5.0–8.0	5.5–9.0	5.5–8.5	ND	6.0–9.0	5.5–9.0	6.0–9.0	6.0–9.0	6.0–8.5	ND	6.0–8.5	5.5–9.0	5.5–9.0	5.5–9.0	5.5–9.0
Growth in presence of:																				
1% oxgall/1% glycine/3% NaCl	+/–/–	+/–/–	+/–/–	+/–/–	+/+/–	+/–/–	+/+/–	–/–/–	+/–/–	+/–/–	ND/ND/–	+/–/–	+/–/–	–/–/–	–/+/–	+/–/–	+/–/–	ND/ND/–	+/–/–	ND/ND/–
Water-soluble brown pigment formed in the presence of:																				
0.1% tyrosine	–	+	–	–	–	ND	–	–	–	–	ND	–	–	–	–	–	+	ND	–	ND
0.1% tryptophan	–	+	–	–	–	ND	–	–	–	–	ND	–	–	–	–	–	+	ND	–	ND
Water-soluble yellow-green fluorescent pigment formed	d	–	W	–	+	–	+	–	d	+	ND	–	–	–	–	+	+	ND	+	ND
Alkaline reaction in litmus milk	–	–	–	–	–	ND	–	–	–	–	ND	–	–	–	–	–	–	ND	+	ND
Growth on:																				
Eosin methylene blue agar	+	–	ND	+	+	ND	+	–	+	+	ND	–	–	–	ND	+	+	ND	+	ND
MacConkey agar	d	–	ND	–	+	ND	+	–	–	–	ND	–	–	–	ND	+	+	ND	–	ND
Triple-sugar iron agar	d	+	ND	+	+	ND	+	+	–	+	ND	–	–	–	ND	–	+	ND	+	ND
Methyl red-Voges Proskauer broth	d	ND	ND	–	+	ND	+	+	–	–	ND	–	+	–	ND	–	+	ND	–	ND
Reduction of 0.3% H_2SeO_3	–	–	ND	–	ND	ND	+	–	–	–	ND	–	–	–	ND	+	d	ND	+	ND
H_2S from 0.2% cysteine in PSS broth, 7 days	+	+	– or W	+	+	–	+	+	+	+	ND	+	d	+	ND	+	+	+	+	+
H_2S from 0.01% cystein in nutrient broth, 7 days	+	ND	ND	+	ND	ND	+	–	ND	+	–	–	W	W	–	+	+	+	+	+
Deoxyribonuclease	d	+[k]	ND	–	–	ND	+	–	+	–	ND	+	+	–	ND	–	+	ND	–	ND
Ribonuclease	d	–	ND	+	–	ND	+	+	+	+	ND	–	+	d	ND	–	+	ND	–	ND
GC content of DNA (mo1%)	49–51	52	62–65	52	63–65	60–62	64–65	63	64–65	63	65	57–59	57–58	58–59	65	61–62	60–64	66	60–62	64

[a]This taxon was found to be in rRNA superfamily III (see The Proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition).
[b]The taxonomic status of this species was not investigated by DNA-rRNA hybridizations. For taxonomic status see Blakemore et al. (1989).
[c]This taxon was found to be in rRNA superfamily IV (see The Proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition).
[d]The taxonomic status of this species was not investigated by DNA-rRNA hybridizations. For taxonomic status see Krieg (1984).
[e]By phase-contrast microscopy of 24- to 48-h-old broth cultures.
[f]*A. fasciculus* is a straight rod; the helix length refers to the length of the rod.
[g]*[A.] delicatum* is vibrioid rather than helical, thus wavelength can not be determined; the helix diameter refers to the width of the vibrio, and length of helix refers to the length of the vibrio.
[h]For *[A.] gracile*, acid from glucose, galactose and arabinose (aerobically). For *[A.] itersonii*, acid from glycerol (aerobically), fructose (aerobically and anaerobically) and glucose (anaerobically only). For *[A.] peregrinum*, acid from fructose (aerobically and anaerobically). Peptone concentrations must be kept low (0.2% or less) in order to detect change in pH indicator. Although *[A.] itersonii* and *[A.] peregrinum* acidify sugar media anaerobically, turbid growth does not occur and the organism should be considered to have mainly a respiratory rather than fermentative type of metabolism.
[i]See "Procedures for Testing Special Characters" in Krieg (1984).
[j]*[A.] gracile* requires biotin and *[A.] aquaticum* requires niacin.
[k]Positive at 37°C but not at 41°C.
Misnamed *Aquaspirillum* species are indicated between straight brackets.
Symbols: +, positive for all strains; –, negative for all strains; ND, not determined; H, helical (one or more complete turns or twists); V, vibrioid (less than one complete turn or twist); SR, straight rod; C, clockwise or right-handed helix (indicated by the pattern //// when focusing on the bottom of the cells); HA, helix absent; CC, counterclockwise or left-handed helix (indicated by the pattern \\\\ when focusing on the bottom of the cells); BT, bipolar tufts; U(1–2), 1 or 2 flagella at only one pole; BS, single flagellum at each pole; W, weak reaction; d, differs among strains.
Based on Krieg (1984) and Blakemore et al. (1989).

Table 2. List of type strains for all species presently included in the genus *Aquaspirillum*.

Name of organism according to Krieg (1984)[a]	Strain number of the type strain	Synonymous strain number	Source and place of isolation	References
[A.] anulus	NCIB 9012	LMG 5404	Freshwater pond, USA.	Williams and Rittenberg, 1957; Hylemon et al., 1973
[A.] aquaticum	ATCC 11330	LMG 2370	Small lake, USA.	Hylemon et al., 1973; Kropinski, 1975
[A.] autotrophicum	ATCC 29984	LMG 4326	Eutrophic lake Le Loclat, St. Blaise, Switzerland.	Aragno and Schlegel, 1978
[A.] bengal	ATCC 27641	LMG 6234	Freshwater pond, West Bengal.	Kumar et al., 1974; Boivin et al., 1985
[A.] delicatum	NCIB 9419	LMG 4327	Stored distilled water.	Leifson, 1962; Hylemon et al., 1973
[A.] dispar	ATCC 27510	LMG 4329	Fresh water, USA.	Hylemon et al., 1973
[A.] fasciculus	ATCC 27740	LMG 6233	Pond water hay infusion, USA.	Strength and Krieg, 1971; Strength et al., 1976
[A.] giesbergeri	ATCC 11334	LMG 4332	Freshwater pond, USA.	Williams and Rittenberg, 1957; Hylemon et al., 1973
[A.] gracile	ATCC 19624		Surface waters, USA.	Canale-Parola et al., 1966; Hylemon et al., 1973
[A.] itersonii subsp. *itersonii*	ATCC 12639	LMG 4337	Freshwater pond.	Giesberger, 1936; Hylemon et al., 1973
[A.] itersonii subsp. *nipponicum*	ATCC 33333	LMG 6237	Freshwater shellfish, Japan.	Terasaki, 1973; Terasaki, 1979
A. magnetotacticum[b]	MS-1		Fresh water from Cedar Swamp, Woods Hole, Mass.	Blakemore et al., 1979; Maratea and Blakemore, 1981
[A.] metamorphum	NCIB 9509	LMG 4338	Putrid infusion of freshwater mussel (*Corbicula japonica* Prime).	Terasaki, 1961b; Hylemon et al., 1973
[A.] peregrinum subsp. *peregrinum*	NCIB 9435	LMG 4340	Primary oxidation pond.	Pretorius, 1963; Hylemon et al., 1973
[A.] peregrinum subsp. *integrum*	IFO 13617	LMG 5407	Putrid infusion of freshwater mussel.	Terasaki, 1973; Terasaki, 1979
[A.] polymorphum	ATCC 11332	LMG 4392	Freshwater pond, USA.	Williams and Rittenberg, 1957; Hylemon et al., 1973
[A.] psychrophilum	IFO 13611	LMG 5408	Antarctic moss.	*Terasaki, 1973; Terasaki, 1979*
[A.] putridiconchylium	ATCC 15279	LMG 4342	Putrid infusion of freshwater mussel (*Semisulcospira bensoni* Philippi).	Terasaki, 1961a; Hylemon et al., 1973
[A.] serpens	ATCC 12638		Pond water.	Giesberger, 1936; Hylemon et al., 1973
[A.] sinuosum	ATCC 9786	LMG 4347	Freshwater pond, USA.	Williams and Rittenberg, 1957; Hylemon et al., 1973

[a]Generically misnamed *Aquaspirillum* species are indicated by brackets.
[b]The taxonomic status of this species was not investigated by DNA-rRNA hybridizations.

WILLIAMS AND RITTENBERG (1957) Williams and Rittenberg (1957) used the following techniques for the isolation of spirilla from stagnant water samples from small ponds (Table 2).

> The water was collected in wide-mouthed jars along with an amount of algae from the same source. After 10 to 14 days incubation at room temperature, the spirilla were collected near the surface of the infusion.
>
> A 100-ml water sample could also be supplemented with 1 g of peptone or yeast autolysate rather than algae. After three days incubation at room temperature the spirilla appeared, reaching a maximum development at seven days. Further enrichment could be achieved by a successive nutrient depletion. Therefore, part of the initial enrichment culture was diluted with an equal volume of the source water and autoclaved; the unsterilized part of the enrichment culture was then used for inoculation of this sterilized portion. After proper incubation, this procedure could be repeated one to three times until the spirilla were predominant.

Both Giesberger (1936) and Williams and Rittenberg (1957) added 1% of calcium malate or lactate to the water samples. NH_4Cl or other external sources of nitrogen were not used because other bacteria then overgrew the spirilla. Subsequent transfers (three to four times) of grown enrichment cultures into sterile source water samples containing malate or lactate also resulted in cultures predominated by spirilla.

> Isolation was achieved by diluting the enriched cultures 1:100 to 1:100,000 with sterile tap water. The dilution bottles were shaken and allowed to stand for 20 min, during which the spirilla accumulated near the surface. A loopful of the surface water was then streaked onto nutrient agar plates supplemented with 0.3% yeast autolysate or onto solidified Giesberger's medium containing 1% malate or lactate. The plates were incubated for 24 h at 30°C and were examined microscopically for characteristic colonies: umbonate (larger spirilla) or pulvinate (smaller spirilla) with a distinctive granular and ground-glass appearance and a typical wavy, interlaced texture with fimbriated edges. After another 24 h of incubation, the presence of spirilla was confirmed by the preparation of wet mounts. The colonies were then streaked until uniform in appearance and until microscopic examination indicated a uniform population.

Williams and Rittenberg (1957) established three principles important for successful enrichment and isolation of spirilla: 1) spirilla tend to grow well in diluted media while in concentrated media they are rapidly overgrown by other bacteria; 2) they grow relatively well in media with low levels of nitrogen, although infusions can give good results as well; and 3) the salts of organic acids (e.g. calcium malate or lactate) tend to selectively improve the growth of spirilla. These principles have been used by many other investigators for the successful isolation of spirilla.

TERASAKI (1961a, 1970) Terasaki isolated a spirillum (Table 2) from a putrified freshwater snail, *Semisulcospira bensoni* (Philippi), and cultivated it for about two years in vitro by the following methods:

> Broken putrid shellfish together with a teaspoon of mud were incubated at 27 to 28°C in a Petri dish filled with a 0.1% sodium chloride solution. Regular microscopic observation revealed large numbers of various spiral organisms, moving around together with other micro-organisms. The isolation medium contained:
>
> | | |
> |---|---|
> | Peptone | 5 g |
> | Yeast extract | 3 g |
> | NaCl | 1 g |
> | Water | 800 ml |
> | Shell fish extract | 200 ml |
>
> pH adjusted to 7.0 to 7.2 before sterilization.
>
> The shellfish extract was prepared by boiling 250 g broken shellfish in 500 ml of water for 20 min. The extract was filtered before use.
>
> Isolation was achieved by successively streaking on five isolation medium plates with a loop dipped into a small drop of the enrichment culture. The plates were incubated at 20°C for several days. Colonies were then transferred to tubes containing liquid isolation medium. Pure cultures were subcultured on isolation medium, in nutrient broth, or on nutrient agar containing beef extract (0.5%) and peptone (0.3%). For the preparation of nutrient agar either 0.7% agar or 12% gelatin was added; the pH was brought to 7.0 to 7.2.

TERASAKI (1961b, 1970) Terasaki used roughly the same method (Terasaki, 1961b) for the isolation of *[A.] metamorphum* (Table 2) from the freshwater shellfish *Corbicula japonica.* The shellfish extract was prepared; the pH of the culture medium was 8.0 to 8.2, and the incubation temperature was 30°C. According to Terasaki (1980) and Krieg (1976), the mud used during isolation is more likely to be the source of the spirilla, rather than the shellfish.

LEIFSON (1962) Leifson isolated a spirillum (*[A.] delicatum*) from a distilled water supply using the following medium, containing per l:

> | | |
> |---|---|
> | Casitone (Difco) | 3.0 g |
> | Yeast extract | 1.0 g |
> | K_2HPO_4 | 1.0 g |
> | Agar | 15 g |
>
> pH 7.1.
>
> The plates were dried overnight at 37°C and 0.1 ml of diluted water samples were spread over the plates. The plates were incubated at 20°C for three to five days.

PRETORIUS (1963) For enrichment, Pretorius used three different media:

> (a) An infusion of dried grass was prepared by boiling 1 g of small pieces of grass in 100 ml distilled water for 1 h, keeping the volume constant; the infusion was used as

such or as the filtrate; (b) a defined medium as described by Myers (1940) with calcium lactate as the carbon source and NH_4Cl as the nitrogen source; and (c) a basal medium containing per liter distilled water 1.0 g each of K_2HPO_4, $(NH_4)_2SO_4$, NaCl, $MgSO_4 7H_2O$, peptone, ethanol, glycerol, calcium lactate and cellulose powder and one drop of a 1% solution of $MnSO_4$ and $FeCl_3$. The medium was sterilized at 121°C for 15 min. Glucose, fructose, and cellobiose at 1.0% solutions were filter-sterilized and added to the heat-sterilized medium. After mixing with the inoculum, the pH was brought to 7.0. For the three media, the incubation temperature was 30°C. Isolation was achieved with the capillary tube method as described by Rittenberg and Rittenberg (1962).

Jannasch (1965) Jannasch mixed equal volumes of eutrophic water and double-strength Giesberger's medium to which he added portions of dead or fresh subsurface plants. When the organic content of the medium was high the proportion of spirilla was low. Jannasch further enriched the cultures by transferring a portion of the scum and underlying layer into a second flask, supplemented with lactate or malate, K_2HPO_4 and $MgSO_4$, without addition of organic material. In cases where spirilla reached 80 to 90% of the population, he isolated the spirilla by streaking undiluted enrichment cultures on a suitable medium containing 0.1% peptone, 0.01% yeast extract, 0.05% asparagine, and 1.8% agar; when population densities of spirilla were lower in the initial cultures, dilutions were streaked on the agar medium or small drops were placed on sterile cover slips (with the edges sealed with vaseline to prevent evaporation) which were inverted over a depression slide. The unicellular isolations made in this way could not be brought to multiply on agar plates, but were subcultured in a hanging drop culture containing enrichment medium supplemented with 0.005% ascorbic acid, before being transferred with a Pasteur pipette to agar plates.

Canale-Parola et al. (1966) The authors described a selective isolation procedure for a specific morphological and physiological type of spirillum from surface waters (*[A.] gracile*, Table 2).

The medium used for isolation from nature and for routine culturing (medium A) contains per l tap water:

Peptone	5 g
Yeast extract	0.5 g
Tween 80 (sorbitol monooleate polyoxyethylene)	20 mg
K_2HPO_4	0.1 g
Agar	10 g

pH 7.2.

The yeast extract could be replaced in medium B by:

Thiamine ·HCl	0.5 mg
Biotin	0.05 mg
$MgSO_4 \cdot 7H_2O$	0.1 g
$MnSO_4 \cdot H_2O$	0.05 g
Sodium succinate $\cdot 6H_2O$	1 g

The latter compound may be replaced in medium C by:

Lactic acid	1 g

The pH of media B and C was 7.3.

Enrichment was accomplished by taking advantage of the very small size of the cells. Sterile cellulose ester filter disks (0.45 µm average pore size) were placed on plates of medium A, B or C. Pond or stream water (0.05 ml) was deposited in the center of the filter disk and the plates were incubated at room temperature for 1.5 to 5 h after which the filter disk was removed. After three days or more of incubation, spreading, semitransparent areas of cells developed entirely within the agar medium where the filter disk had been located. In all cases this characteristic subsurface growth consisted of thin spirilla (0.25 to 0.30 µm in diameter). The organisms were isolated by streaking on medium A, B or C, respectively.

Cody (1968) In 1968 Cody reported on a selective agent for the isolation of freshwater spirilla. He found that 5-fluorouracil at concentrations of 300 µg/ml had little or no inhibitory action on the growth response of *A. serpens* and that pure culture isolates of spirilla could be obtained from natural sources on media containing this chemical substance. Scully and Dondero (1973), however, showed that the use of this agent is generally not as effective as the use of other enrichment procedures.

Strength and Krieg (1971) and Strength et al. (1976) In 1971, Strength and Krieg reported the isolation of an aquatic bacterium from a pond water hay infusion which they later assigned to the genus *Aquaspirillum* as *A. fasciculus* (Strength et al., 1976; Table 2). The two isolation procedures they described at that time (the decreased-oxygen method and the cellulose-powder method) have proven to be unreliable for the isolation of additional strains (Strength et al., 1976) and will therefore not be described here. A more reliable method was described as follows (Strength et al., 1976):

A pond-water hay infusion incubated at 30°C in shallow pans reached maximal numbers of spirilla in the surface scum at three days. Initial enrichment was performed in Pringsheim's soil medium (Rittenberg and Rittenberg, 1962) for four days. A loopful of the surface pellicle of the enrichment culture was suspended in 10 ml sterile water and shaken vigorously in order to disperse the cell flocs that were characteristically formed by this organism. Serial dilutions in sterile water were made from this suspension and each dilution used to seed plates of melted, cooled proline (PR) medium with the following composition (per l distilled water):

L-Proline	0.5 g
K_2HPO_4	0.45 g
$MgSO_4 \cdot 7H_2O$	0.25 g
$MnSO_4 \cdot H_2O$	1 mg

$FeCl_3 \cdot 6H_2O$	1 mg
Agar	7.5 g

pH adjusted to 7.0 with KOH.

After 36 h incubation at 30°C, numerous small (0.1 to 0.4 mm), white, irregularly shaped colonies could easily be detected with a dissecting microscope. The colonies were picked up with a sterile capillary tube and blown out into tubes of PR medium with 0.15% agar. It was better to crush the agar plugs from the capillary tubes after the transfer. Purity was checked by darkfield microscopy: the spirilla were characterized as rods with a diameter of 0.7 to 0.9 µm with bipolar fascicles of flagella responsible for the helical wave propagation.

KUMAR ET AL. (1974) From a freshwater pond in West Bengal, Kumar et al. (1974) isolated a large spirillum with the unique high optimal growth temperature of 41°C (*A. bengal*, Table 2). Later this species was found to be synonymous with *A. serpens* by slot blot hybridization experiments (Boivin et al., 1985) and by DNA-DNA hybridizations (Pot et al., unpublished observations). Enrichment and isolation were accomplished as follows:

In 250 ml Erlenmeyer flasks 10 ml of pond water was added to 90 ml of a sterile 0.5% peptone (Oxoid) solution. Within 24 to 72 h at 30°C the number of spirilla increased 100 to 400 times, but the enrichment was not selective, and incubation for longer periods resulted in a rapid decrease of the number of spirilla. Further enrichment of spirilla was achieved by low-speed centrifugation (1,000 × *g*, for 5 min) which caused the comparatively large spirilla to sediment more rapidly than other, smaller bacteria. The supernatant was carefully decanted and the sediment suspended in 2 ml of sterile tap water.

For isolation the capillary method (Rittenberg and Rittenberg, 1962) was ineffective; the diluting plating method, however, was successful.

From a series of decimal dilutions of the concentrated spirilla, 0.1 ml samples were spread on the surface of tomato-extract agar, containing per l pond water: Evans peptone (0.5%), tomato extract (40 ml, prepared by pressing pieces of fresh, ripe tomatoes through cheesecloth and filtering the juice through paper) and 2% agar. The pH was adjusted to 7.4 with KOH and the medium sterilized at 121°C for 15 min. The dilution plates were incubated at 40°C for 72 h and were then examined with a low-power microscope for colonies. The selected colonies were transferred to slants of the tomato-extract agar and purified by repeated dilution plating.

KROPINSKI (1975) The cultures isolated by Giesberger (1936) were lost but Williams and Rittenberg (1957) reisolated a spirillum which closely resembled *Spirillum serpens* (Giesberger, 1936) and deposited the strain in the American Type Culture Collection (ATCC 11330). Hylemon et al. (1973) renamed this strain *[A.] aquaticum* (Table 2). A chemically defined medium (AAM) containing nicotinic acid was described for this organism in 1975 by Kropinski.

The AAM medium contains per liter:

Na_2HPO_4	7 g
KH_2PO_4	3 g
$(NH_4)_2SO_4$	2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Sodium succinate$6H_2O$	5.6 g
Nicotinic acid	20 mg

pH 6.8.

INOUE AND KOMAGATA (1976) From antarctic soil, an obligate psychrophilic spirillum ("*Spirillum pleomorphum*") was isolated by Inoue (1976) and Inoue and Komagata (1976). The medium they used (PYG) contained per liter:

Peptone	10 g
Yeast extract	5 g
Glucose	3 g
Agar	15 g

pH 7.2.

Inoculated plates were incubated at 0°C for 14 to 24 days. Subsequent subcultures were incubated at the optimum temperature of 9°C.

ARAGNO AND SCHLEGEL (1978) Two strains of a facultatively autotrophic, hydrogen-oxidizing spirillum were isolated from a small eutrophic lake in Switzerland by Schweizer and Aragno (1975). Aragno and Schlegel (1978) classified the strains in the genus *Aquaspirillum* as *[A.] autotrophicum* (Table 2).

Isolation was achieved by filtering the water samples through membrane filters, which were then deposited on mineral agar and incubated under an atmosphere of 60% H_2, 30% air, and 10% CO_2 at 30°C. Pure cultures were obtained by repeated subculturing on mineral agar plates incubated under the same conditions. Purity of the cultures was determined by plating on different organic media and by microscopic observation.

BLAKEMORE ET AL. (1979, 1989) A bipolarly flagellated, microaerophilic, heterotrophic, magnetotactic spirillum containing intracellular chains of magnetite crystals was isolated by Blakemore et al. (1979) by applying a magnetic field to the sediments from a freshwater swamp. The organism was later assigned to the genus *Aquaspirillum* as *A. magnetotacticum* (Maratea and Blakemore, 1981; Table 2). Enrichment, isolation, and growth of strain MS-1 (Table 2) was achieved as follows:

Enriched cultures were obtained by the undisturbed incubation of loosely covered jars filled with water and mud from the sample origin (Table 2) to approximately two thirds of their volume, during one month or more at room temperature (22°C) in dim light. After that time a small amount of mud slurry, containing magnetotactic cells, was diluted 1:100 and dispensed into each of many small vials containing one sixth of their volume of the isolation medium. The vials were sealed and the atmosphere

replaced with an appropriate gas mixture (for routine purposes, the atmosphere was replaced by nitrogen and sufficient air added to provide 0.6 to 1.0% oxygen in the gas phase). After suitable incubation, survival of magnetotactic bacteria was determined from direct microscopic countings of motile, magnetotactic cells. From these cultures, large numbers of magnetotactic cells were separated from the sediment by the application of steady, non-uniform magnetic fields formed by permanent bar magnets. The cells were washed in filtered and sterilized bog water and injected through the stoppers of culture tubes containing prereduced, semisolid isolation medium containing per 90 ml distilled water:

Filtered swamp or bog water	10 ml
Vitamin elixir (Wolin et al., 1963)	1 ml
Mineral elixir (Wolin et al., 1963)	1 ml
Potassium phosphate buffer, pH 6.7	0.5 mM

Add to this mixture:

Vitamin B-12	5 µg
NH_4Cl	25 mg
Sodium acetate (anhydrous)	10 mg
Resazurin	0.2 mg
Ionagar no. 2 (Oxoid)	90 mg

The pH is adjusted to 6.7 with NaOH.

The medium was prereduced under nitrogen, using titanium citrate as reducing agent (Zehnder and Wuhrmann, 1976). The culture tubes were filled in an anaerobic hood and sealed. After inoculation the tubes were incubated at 22°C in the dark until growth became evident as diffuse, spreading, fluffy areas containing the magnetotactic spirilla. A well-isolated area of growth was homogenized, and cells were purified by serial dilution into tubes containing molten, prereduced isolation medium containing 0.85% Ionagar no. 2. Well-isolated colonies, which appeared in these tubes after one week at 30°C, were microscopically homogeneous. However, the cells were subcultured a second and a third time before the cultures were considered pure.

Maintenance of strain MS-1 (Blakemore et al., 1979)

Strain MS-1 was maintained at 30°C with weekly transfers in screw-capped culture tubes with a semisolid growth medium containing per 98 ml of distilled water:

Vitamin elixir (Wolin et al., 1963)	1 ml
Mineral elixir (Wolin et al., 1963)	1 ml
KH_2PO_4	5 mM
Ferric quinate solutions	2.5 ml
Resazurin	0.2 mg

Add to this mixture:

Succinic acid	0.1 g
Sodium acetate (anhydrous)	20 mg
$NaNO_3$	10 mg
Sodium thioglycolate	5 mg
Agar	130 mg

The agar was added after the pH was brought to 6.7 with NaOH. The ferric quinate solution was prepared by adding 2.7 g of $FeCl_3$ and 1.9 g of quinic acid to 1 liter of distilled water. The medium was boiled and 12 ml was added to each screw-capped tube containing approximately 0.1 ml of 5% Na thioglycolate in distilled water. Tubes of semisolid growth medium were autoclaved with caps tightened and allowed to stand overnight for the establishment of O_2 gradients. Inocula consisted of 0.2 ml (about 7×10^7 cells) per 12 ml of medium. Chemically defined growth medium was identical to the semisolid growth medium without agar. The vessels containing the medium were sealed before autoclaving, but after the atmosphere had been replaced by nitrogen and after sufficient air was provided to obtain 0.6 to 1.0% oxygen in the gas phase (Balch and Wolfe, 1976).

A homogeneous population of nonmagnetotactic variants was obtained from cultures of MS-1 grown in isolation medium made with distilled water rather than bog water. Cells grown in this medium, especially with twice as much as the usual amount of nitrate and succinate, grew nonmagnetotactically. To obtain a nonmagnetotactic pure culture from strain MS-1, cultures were transferred five successive times in this medium and were subsequently cloned three successive times as described above. Stocks of the nonmagnetotactic variant were maintained in defined growth medium without ferric quinate.

Terasaki (1980) Terasaki (1973) described five new species and two new subspecies of the genus *Spirillum* (Ehrenberg, 1832), on the basis of an extended morphological, physiological, and biochemical study that he performed. After he transferred these *Spirillum* species to their respective new genera in 1979 (i.e., for *Aquaspirillum*, the transfer to *[A.] psychrophilum*, *[A.] itersonii* subsp. *nipponicum* and *[A.]peregrinum* subsp. *integrum;* Table 2), Terasaki published in 1980 a more detailed description of the methods used for enrichment (boiled shellfish infusion method), selection (glass capillary method) and isolation (streak plate method) of aerobic, mesophilic freshwater and marine chemoheterotrophic spirilla from 20 mud and sand samples collected at various locations in Japan from 1970 to 1980. He concluded that 1) the boiled shellfish infusion method gave a definite increase of numbers of spirilla deriving from mud or sand samples; 2) the capillary method was useful for separating highly motile spirilla from non-motile bacteria, giving highly enriched crude cultures; 3) pure cultures can be isolated by simple streaking on agar plates; and 4) simple modifications could be devised for the isolation of microaerophilic, thermophilic, or psychrophilic spirilla.

Preservation of the Aquaspirilla

The spirilla mentioned in Table 2, except for *A. magnetotacticum*, have been maintained in our laboratory by weekly transfers on peptone succinate-salt medium (MPSS).

MPSS Medium (Krieg, 1984)

Per liter of distilled water add:

Succinic acid (free acid)	1.0 g
$MgSO_4 \cdot 7H_2O$	1.0 g
Bacto peptone (Difco)	5.0 g
$(NH_4)_2SO_4$	1.0 g

$FeCl_3 \cdot 6H_2O$ (0.2% aqueous solution) 1.0 ml
$MnSO_4 \cdot H_{20}$ (0.2% aqueous solution) 1.0 ml

pH is adjusted to 6.8 with KOH.

The medium is dispensed in 10 ml portions before sterilization (121°C for 20 min) into loosely screw-capped tubes. After cooling the tubes are closed and stored at 4°C until use. Inoculum consists of three to five drops from a MPSS culture, delivered from a Pasteur pipette. Inoculated, loosely capped tubes are incubated at 28°C (except for *[A.] psychrophilum* which is incubated at 17°C) until visible turbidity occurs (one to three days, depending on the strain). The tubes of grown culture are then closed and stored at 4°C for one week. Before the next transfer, tubes are brought to room temperature and the new MPSS tubes are inoculated. Purity is regularly checked by plating on MPSS agar.

Giesberger (1936) reported maintenance on ordinary peptone agar slants with three to four monthly transfers. However some strains required the addition of pyruvate and more frequent transfers were necessary for such strains. Terasaki (1972) maintained strains in nutrient agar stabs with monthly transfers. Hylemon et al. (1973) used a semisolid MPSS broth with peptone increased to 1% and containing 0.15% agar with weekly transfers.

Long-term preservation can be accomplished by centrifuging cells from a grown 10 ml MPSS broth culture, suspending them in 2 ml MPSS broth supplemented with 15% glycerol as cryoprotective agent in sterile tubes, and freezing them in liquid nitrogen or at –80°C in a deep freezer. Lyophilization is achieved by resuspending the centrifuged cells of two 10 ml MPSS broth cultures in 3 ml of sterile horse serum (70%) supplemented with glucose (7%) and nutrient broth (0.6%). The suspension is dispensed in 8 to 12 small sterile lyophilization tubes with proper labeling and freeze dried overnight. The lyophilization tubes are sealed by heat and stored at 4°C. Terasaki (1975) has reported the results of freeze-drying freshwater spirilla.

Identification and Taxonomy

The present description of the genera *Spirillum*, *Aquaspirillum*, and *Oceanospirillum* in *Bergey's Manual of Systematic Bacteriology* (Krieg, 1984) is mainly based on morphological, nutritional, and physiological data and on DNA base composition. However, the number of exceptions to the general description of the genera is large. Morphologically, cells are not all helical but can also be vibrioid (*[A.] delicatum*) or straight rods (*A. fasciculus*), and the flagellation can be one flagellum at one (*[A.] delicatum*) or both poles (*[A.] polymorphum* and *A. magnetotacticum*) instead of bipolar tufts. *A. magnetotacticum* is the only member that is obligately microaerophilic, and it is oxidase and catalase negative. Although some species have physiological characteristics which are very typical for the genus, they may show an aberrant morphology; also species with a typical spiral morphology may have physiological properties which do not agree with those described for the genus. Genetic evidence for close intrageneric relationships within *Aquaspirillum* was not available to Krieg (1984). On the contrary, the comparison of the 16S rRNA oligonucleotide catalogs (Woese et al., 1982) of three strains representing *A. serpens*, *[A.] itersonii*, and *[A.] gracile* and DNAr-RNA hybridizations (De Smedt et al., 1980) with *[A.] itersonii* and *[A.] polymorphum* revealed a large heterogeneity, indicating that a thorough genotypical reexamination of the genus was necessary in order to reveal the different subgroups probably present in this genus. This was performed by more DNAr-RNA hybridizations with almost all available strains of all *Aquaspirillum* species, except *A. magnetotacticum* (Pot et al., 1984. Genetic heterogeneity within *Aquaspirillum* and *Oceanospirillum*. Abstract C8 of the FEMS Symposium on "Evolution of Prokaryotes," Munich, FRG). Results of oligonucleotide cataloging of 16S rRNA of representative strains of *A. bengal*, *[A.] dispar* and *[A.] aquaticum* also became available (Woese et al., 1984b). For the species studied with both methods, the results were comparable (Pot et al., manuscript in preparation) and can be summarized as follows (see also The Proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition).

Aquaspirillum Species Belonging to rRNA Superfamily III

All *Aquaspirillum* species except three (*[A.] peregrinum*, *[A.] polymorphum*, and *[A.] itersonii*) belong in rRNA superfamily III, corresponding to the beta group of the Proteobacteria. Within this rRNA superfamily they belong to known rRNA branches (mostly representing a known genus) or they constitute separate rRNA branches or subbranches. This indicates that most of these *Aquaspirillum* species are generically misnamed.

Within rRNA superfamily III, only *A. serpens* and *A. bengal* have very similar rRNA cistrons, supporting the proposal of Boivin et al. (1985) to emend *A. serpens* to include *A. bengal* as a subjective synonym. They constitute a separate rRNA branch which also comprises *A. fasciculus* at a lower $T_{m(e)}$ level (see The Proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition). *A. fasciculus* is the only rod-shaped member of *Aquaspirillum*; it has been classified in this genus

because of its bipolar tufts of flagella and because it possesses some of the typical physiological features of the genus. Because this rRNA branch contains the type species of the genus, *Aquaspirillum* sensu stricto should be restricted solely to members of this rRNA branch. Despite the large difference in GC content (10 mol%), we propose to include also *A. fasciculus* in *Aquaspirillum* sensu stricto. The differentiating features can be found in Table 1.

Eight [*Aquaspirillum*] species (*[A.] anulus*, *[A.] aquaticum*, *[A.] delicatum*, *[A.] giesbergeri*, *[A.] gracile*, *[A.] metamorphum*, *[A.] psychrophilum* and *[A.] sinuosum*) belong in the acidovorans rRNA complex (Willems et al., 1987, 1989). Krieg and Hylemon (1976) have found a resemblance between *[A.] delicatum* and the genera *Comamonas* and *Pseudomonas*. *[A.] aquaticum* is closely related to a group of clinical isolates which also exhibit a characteristic corkscrewlike type of motility; they both belong in the genus *Comamonas* described in The Genus *Comamonas* in the second edition. Among the seven other species only *[A.] giesbergeri* and *[A.] sinuosum* are closely related. All other species occupy a separate position in the acidovorans rRNA complex. In the future, their taxonomic status will be further unravelled by a polyphasic approach.

[A.] dispar and *[A.] putridiconchylium* each constitute a separate rRNA branch in rRNA superfamily III equally far removed from the authentic *Aquaspirillum* rRNA branch and from the genera *Chromobacterium* and *Neisseria*. Woese et al. (1984b) found *[A.] dispar* to be somewhat more related to *A. serpens* than to the other members of the beta group.

[A.] autotrophicum is generically related to *Janthinobacterium*, to *[Pseudomonas] rubrisubalbicans* (a generically misnamed *Pseudomonas* species pathogenic for sugar cane), and to an unnamed group of clinical isolates from different origins, which also show a characteristic corkscrewlike type of motility (Goor et al., 1986).

[*Aquaspirillum*] Species Belonging to rRNA Superfamily IV

Three species are members of rRNA superfamily IV (Stackebrandt et al., 1988; alpha group of the *Proteobacteria*). *[A.] peregrinum* (both subspecies), *[A.] itersonii* subsp. *itersonii*, and *[A.] itersonii* subsp. *vulgatum* are highly related and constitute a separate rRNA subbranch in the *Azospirillum-Rhodospirillum* rRNA complex. This subbranch is comparable with, and equidistantly related to, the subbranches formed by *Azospirillum* and *Rhodospirillum*. This is in agreement with the cataloging data obtained by Woese et al. (1984a, 1984b). Within the alpha group, *[A.] itersonii* was also found to be approximately equally far removed from *Rhodospirillum rubrum* and from *Azospirillum brasilense*. As a consequence, these two [*Aquaspirillum*] species deserve a separate generic rank; the intrageneric relationships within this new genus have not yet been determined completely. *[A.] polymorphum* belongs also in the *Azospirillum-Rhodospirillum* rRNA complex and is equidistantly removed from the former three rRNA subbranches. Therefore this species probably represents another genus. All three [*Aquaspirillum*] species in rRNA superfamily IV share a high GC content and can be differentiated from the other *Aquaspirillum* species by a counterclockwise helix and by the hydrolysis of esculin. They are also characterized by a predominance of coccoid bodies in three to four week-old cultures. Among other aquaspirilla, this feature was only found in *A. fasciculus* and *A. magnetotacticum*. N_2-fixing capacity occurs in *[A.] peregrinum* and *[A.] itersonii*, not in *[A.] polymorphum*. *[A.] polymorphum* and *[A.] magnetotacticum* are the only aquaspirilla having a single polar flagellum at each pole. The genotypic position of *[A.] itersonii* subsp. *nipponicum* is not clear yet; the strain which we have included in our DNA-rRNA hybridizations does not belong in rRNA superfamily IV.

The genotypic heterogeneity within the aquaspirilla has not yet been used to make new nomenclatural propositions because all the data needed are not yet available. Therefore we propose to base identification on the results of Krieg (1984), Boivin et al. (1985) and Blakemore et al. (1989; Table 1). In this table the different species are grouped according to their genotypic relationships. *Spirillum volutans* is not included in this scheme, because according to its rRNA catalogs (Woese et al., 1984b), it belongs in the beta group where it constitutes a separate branch together with *Nitrosovibrio*, *Nitrosospira*, *Nitrosolobus*, *Nitrosococcus mobile*, and *Nitrosomonas europaea*. It is clearly different from the other aquaspirilla by its low GC content and its large cell dimension.

From these results it is obvious that future identification must rely heavily on the phenotypic reinvestigation of the present *Aquaspirillum* species in order to 1) describe the species in view of their new generical position; and 2) detect differentiating characteristics with their closest neighbors, needed for an appropriate identification key. This may drastically change some of the present definitions but should allow a more reliable identification. The present definition of the genus *Aquaspirillum* will also drastically change. The fact that a straight rod, capable of fixing nitrogen, is the only species

besides the type species that can be maintained in the genus *Aquaspirillum* (except for *A. magnetotacticum*, which was not investigated) raises more questions on the taxonomical value of the helical structure. Moreover, it is generally known that the ability to form spirals tends to disappear after prolonged transfer in culture media, indicating that the spiral form is not a very stable feature and hence not reliable for identification purposes. The fact that (rigid) spiral forms are distributed all over the Gram-negative bacteria (see also The Genus Oceanospirillum in the second edition.) makes it questionable if the spiral form is an ancestral form rather than a feature that can arise by mutation.

Acknowledgements. B. P. is indebted to the Instituut tot aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw (Belgium) for a scholarship; M. G. and J. D. L. to the National Fund for Scientific Research (Belgium) and to the Fund for Medical Scientific Research (Belgium) for research and personnel grants.

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Prokaryotes (2006) 5:723–736
DOI: 10.1007/0-387-30745-1_31

CHAPTER 3.2.7

Comamonas

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Introduction

The genus *Comamonas* contains species of Gram-negative, aerobic, nonpigmented, rod-shaped bacteria, which are motile by means of at least one polar tuft of flagella and have a non-fermentative chemoorganotrophic metabolism. They are quite ubiquitous in the environment and have been isolated from soil, mud and water. *Comamonas* strains have also been isolated from denitrifying activated sludge as well as from various clinical samples and from the hospital environment, but they are not seen as pathogenic to healthy humans. The genus currently comprises four species, *Comamonas terrigena*, *Comamonas testosteroni*, *Comamonas denitrificans* and *Comamonas nitrativorans*.

Phylogeny

Phylogenetically, the genus *Comamonas* belongs to the Comamonadaceae lineage in the β-subclass of the Proteobacteria together with other genera including *Acidovorax*, *Brachymonas*, *Hydrogenophaga*, *Polaromonas*, *Rhodoferax*, *Variovorax*, *Xylophilus* and various species inappropriately assigned to the genus *Aquaspirillum*. This position was first elucidated by DNA:rRNA hybridizations (De Vos et al., 1985; Willems et al., 1991) and later by 16S rDNA sequence analysis (Wen et al., 1999). A dendrogram based on 16S rDNA sequences showing the positions of the four *Comamonas* species that presently comprise the genus is given in Fig. 1. Whereas all *Comamonas* species are grouped together, the former *Comamonas acidovorans* is further removed and consequently, and in view of additional differences, was transferred to a new genus, *Delftia*, as *Delftia acidovorans* (Wen et al., 1999). None of the other members of the Comamonadaceae appears especially closely related to *Comamonas*, with all genera having approximately 92–94% 16S rDNA similarity with *Comamonas*.

Taxonomy

Before its revival in 1985 (De Vos et al., 1985), the taxonomic position of the *Comamonas* strains had been obscure because of incomplete descriptions and lack of original cultures for comparison. The genus has had a rather turbulent nomenclatural history (Table 1).

The name *Comamonas* was proposed in 1962 to replace the name *Lophomonas* which appeared to be a later homonym of a protozoan taxon (Davis and Park, 1962). The genus *Lophomonas* had earlier been created for a group of Gram-negative rod-shaped bacteria which, like the peritrichous genus *Alcaligenes*, attack only few carbohydrates but bear two to four lophotrichous flagella. They are common in the human intestine as well as in water and mud (Galarneault and Leifson, 1956). The monotype strain was *Lophomonas alcaligenes*, and one of its strains was reported to mutate into a stable peritrichously flagellated organism, leading Galarneault and Leifson to decribe *Lophomonas alcaligenes* as identical to the peritrichously flagellated *Vibrio alcaligenes*. This last name had been proposed to replace *Bacillus faecalis alcaligenes* (Lehmann and Neumann, 1927), a species of Gram-negative rods that do not attack carbohydrates and were isolated from human feces (Petruschky, 1896).

When replacing the name *Lophomonas* with *Comamonas*, the former type species *Lophomonas alcaligenes* was considered inappropriate because of differences between the original descriptions of *Vibrio alcaligenes* (syn. *Lophomonas alcaligenes*) and those of the new genus *Comamonas* (Davis and Park, 1962). Therefore *Vibrio percolans*, isolated from the filtrate of a hay infusion, was designated as the new type species "*Comamonas percolans*." *Vibio cyclosites* and *Vibrio neocistes* were also assigned to *Comamonas* but their species allocation was not specified (Davis and Park, 1962).

Comamonas percolans was renamed "*Comamonas terrigena*" by Hugh (1962) because he

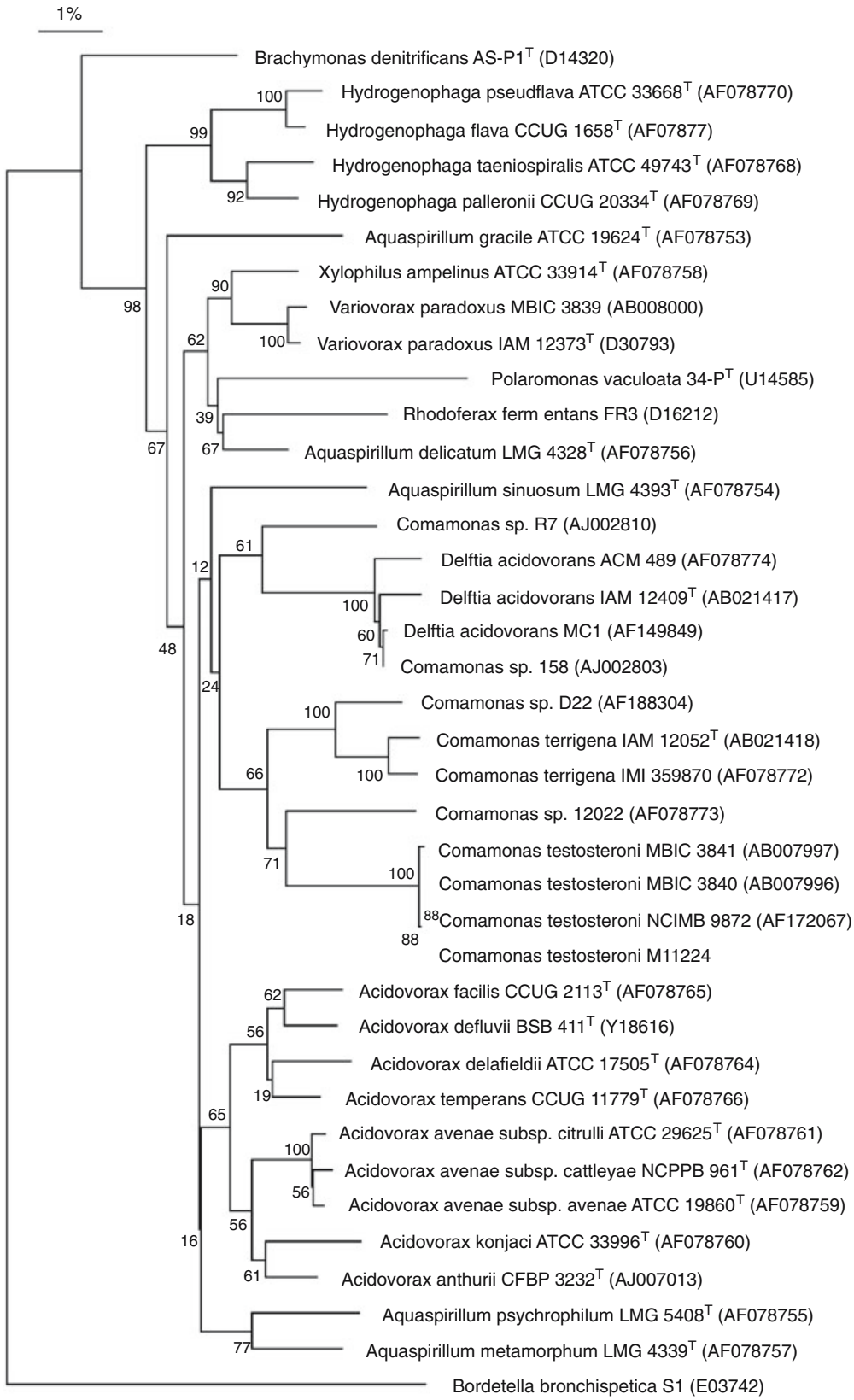

Fig. 1. Dendrogram showing the phylogenetic position of *Comamonas* in the family Comamonadaceae. The neighbor-joining tree was derived from a distance matrix of 16S rDNA sequences. European Molecular Biology Laboratory (EMBL) accession numbers are given at the branch tips and bootstrap values (% of 500 replications) are given at the branching points.

Table 1. The history of *Comamonas*.

Year	Author	Observation
1894	Gunther	*Vibrio terrigenus* is isolated from soil
1896	Petruschky	*Bacillus faecalis alcaligenes* is isolated from human stools
1923	Mudd and Warren	*Vibrio percolans* is isolated from the filtrate of a hay infusion
1926	Den Dooren de Jong	*Pseudomonas acidovorans* sp. nov. is isolated from soil
1927	Lehmann and Neumann	*Bacillus faecalis alcaligenes* is replaced by *Vibrio alcaligenes*
1928	Gray and Thornton	*Vibrio cyclosites* sp. nov. and *Vibrio neocistes* sp. nov.
1956	Galarneault and Leifson	*Lophomonas alcaligenes* gen. nov. is synonym of *Vibrio alcaligenes*
1962	Davis and Park	*Lophomonas* is renamed *Comamonas* gen. nov. with type species *Comamonas percolans*. *Vibrio alcaligenes*, *Vibrio cyclosites* and *Vibrio neocistes* are also assigned to *Comamonas*
1962	Hugh	*Comamonas percolans* is renamed *Comamonas terrigena*
1965	Hugh	*Comamonas terrigena* and *Pseudomonas testosteroni* united as *Pseudomonas terrigena*
1974	Nozhevnikova and Zavarzin	*Comamonas compransoris* sp. nov.
1980	Söder	*Comamonas compransoris* is renamed *Pseudomonas compransoris*
1980	Skerman et al.	*Comamonas* and *Lophomonas* not on the *Approved Lists of Bacterial Names*
1985	De Vos et al.	*Comamonas terrigena* is revived
1987	Tamaoka et al.	*Pseudomonas acidovorans* and *Pseudomonas testosteroni* are renamed *Comamonas acidovorans* and *Comamonas testosteroni*
1991	Willems et al.	*Aquaspirillum aquaticum* and clinical group E. Falsen (EF) 10 incorporated into *Comamonas terrigena*
1991	Willems et al.	Family Comamonadaceae is created
1993	Meyer et al.	*Comamonas comparansoris* transferred to *Zavarzinia compransoris* gen. nov.
1999	Wen et al.	*Comamonas acidovorans* transferred to *Delftia acidovorans* gen. nov.
2001	Gumaelius et al.	Description of new species: *Comamonas denitrificans*
2001	Etchebehere et al.	Description of new species: *Comamonas nitrativorans*

considered it to be a later subjective synonym of *Vibrio terrigenus*, an organism isolated from surface soil and motile by means of bipolar tufts of flagella (Gunther, 1894). While the original isolate was no longer available, Hugh (1962) showed that Gunther's description matched that of *Comamonas percolans*. Later, he reported that *Comamonas terrigena* ATCC 8461^T and *Pseudomonas testosteroni* ATCC 11996^T are highly similar and suggested that both species be united as *Pseudomonas terrigena* (Hugh, 1965).

The new species *Comamonas compransoris* was proposed for a facultatively lithotrophic CO- or H_2-oxidizing organism (Nozhevnikova and Zavarzin, 1974). In *Bergey's Manual of Systematic Bacteriology*, it was placed in *Pseudomonas* section V as "*Pseudomonas compransoris*" (Palleroni, 1984). It has since been shown to belong to the α subclass of the Proteobacteria and has been transferred to a new genus as *Zavarzinia compransoris* (Meyer et al., 1993).

Comamonas was not included in the *Approved Lists of Bacterial Names* (Skerman et al., 1980), nor in *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt, 1984).

On the basis of DNA:rRNA hybridization, phenotypic, serological and protein electrophoresis data, the genus *Comamonas* was revived and *Vibrio cyclosites* and *Vibrio neocistes* were included in *Comamonas terrigena*, which was designated a monotype species (De Vos et al., 1985). Using DNA:rRNA hybridizations, it was shown that *Comamonas terrigena* is a member of rRNA superfamily III, now called "the βsubclass of the Proteobacteria," where it belongs to the *Pseudomonas acidovorans* rRNA branch together with *Pseudomonas testosteroni* and several other *Pseudomonas* species (De Vos et al., 1985). Later *Pseudomonas acidovorans* and *Pseudomonas testosteroni* were renamed "*Comamonas acidovorans*" and "*Comamonas testosteroni*," respectively, on the basis of phenotypic, chemotaxonomic and DNA homology data (Tamaoka et al., 1987).

In a polyphasic study of the genus *Comamonas*, five genotypic subgroups were found on the basis of DNA:rRNA and DNA:DNA hybridizations, immunotyping and protein gel electrophoresis. Three of these subgroups corresponded to the three species (*C. terrigena*, *C. testosteroni* and *C. acidovorans*, now *Delftia*), whereas the fourth subgroup contained *Aquaspirillum aquaticum*, a freshwater spirillum, and clinical isolates of E. Falsen (EF) group 10 and the fifth subgroup contained other EF group 10 strains and misnamed *Pseudomonas alcaligenes* and *Pseudomonas pseudoalcaligenes* subsp. *pseudoalcaligenes* strains. Phenotypic analysis, however, revealed three large phena, corresponding to the three named species, with the phenon of *Comamonas terrigena* also comprising the strains of the fourth and fifth genotypic subgroups. Therefore these

latter two subgroups were included in *Comamonas terrigena* together with the type strain of *Aquaspirillum aquaticum* (Willems et al., 1991).

Later, as conclusion from a 16S rDNA phylogenetic study of the family Comamonadaceae, *Comamonas acidovorans* was removed from *Comamonas* and transferred to a new genus as *Delftia acidovorans* (Wen et al., 1999).

Finally, denitrifying members of the genus have been isolated from activated sludge in South America and Europe and were nearly simultaneously described as *Comamonas nitrativorans* (Etchebehere et al., 2001) and *Comamonas denitrificans* (Gumaelius et al., 2001), respectively. The close phylogenetic relationship (more than 97% rDNA sequence similarity) of both species certainly supports the need for a more detailed taxonomic study including DNA relatedness. Furthermore, the phenotypic data as reported for the two species do not allow a clear phenotypic differentiation (see below).

Habitat

Comamonas commonly occurs in soil, mud and water, natural as well as polluted environments, various clinical samples, the hospital environment, and horse or rabbit blood (Willems et al., 1991). *Comamonas testosteroni* has also been reported in bulk tank milk (Jayarao and Wang, 1999), metal working fluids (Laitinen et al., 1999), and activated sludge (Boon et al., 2000). *Comamonas denitrificans* (Gumaelius et al., 2001) and *Comamonas nitrativorans* (Etchebehere et al., 2001) have so far only been reported once as a member of the microbial denitrifying component in activated sludge. Clinically isolated *Comamonas* strains are regarded as rare opportunistic pathogens (Gilardi, 1985).

Isolation

No specific selective isolation procedures yielding only *Comamonas* isolates have been described, but *Comamonas* strains can generally be isolated from water by plating on nutrient agar. The original *C. terrigena* type strain was isolated on a medium of hay infusion filtrate (Mudd and Warren, 1923). The capacity of *Comamonas* strains to degrade particular aromatic compounds, hydrocarbons and higher dicarboxylic acids can be exploited for selective enrichment by using these compounds as sole carbon source and nitrogen (in the case of nitrogen-containing aromatic compounds) source in a mineral medium. For some strains of *C. terrigena*, methionine and nicotinamide act as growth factors and should be added to mineral media (Tamaoka et al., 1987). Compounds used to isolate or enrich for *Comamonas* strains include phenol and *m*-cresol (Gray and Thornton, 1928), abietic and dehydroabietic acid (Morgan and Wyndham, 1996), and poly-3-hydroxybutyrate (Jendrossek et al., 1993). *Comamonas testosteroni* strains have been isolated using the following compounds: testosterone (Talalay et al., 1952), imidazolyl-propionate and imidazolyl-lactate (Coote and Hassal, 1973), *p*-cresol (Dagley and Patel, 1957), fumarate, bromosuccinate, anthranilate, kynurenate and poly-3-hydroxybutyrate (Stanier et al., 1966), naphthalene (García-Valdés et al., 1988), phenanthrene (Goyal and Zylstra, 1996), chloro- and methyl-phenol (Hollender et al., 1997), polychlorinated biphenyls (Joshi and Walia, 1995), nitrophenols and nitrobenzene (Zhao and Ward, 1999), and 3-chloroaniline (Boon et al., 2000). *Comamonas testosteroni* strains have also been isolated by selecting for cadmium resistance (Kanazawa and Mori, 1996).

Furthermore, *Comamonas* members were also isolated from the clinical environment (Gilardi, 1971; Gilardi, 1985; Ben-Tovim et al., 1974; De Vos et al., 1985; Willems et al., 1991). Sources include blood, pus, urine, pharyngeal mucosae, kidneys, feces, burst appendix, intravenous tubing, and urinary catheters. The isolation procedures from such clinical samples are those generally used for the isolation of Gram-negative glucose-nonfermenters. They include the use of a blood agar medium such as trypticase soy agar (TSA) plus defibrinated blood and a selective enteric medium such as MacConkey agar. The commonly used incubation regime for primary isolation media of 24 h at 35°C should be extended with 24 h at 30°C to permit growth of glucose-nonfermenters that grow slowly at 35°C and may be masked by other bacteria (Rubin et al., 1985).

Identification

Comamonas cells are straight to slightly curved rods, 0.5–2 by 1–6 μm. They are motile by means of polar or bipolar tufts of flagella. On nutrient agar, colonies of *C. terrigena* and *C. testosteroni* are round with a smooth to wavy margin, convex, smooth to granular, and nonpigmented. After three days at 28°C, colony diameters of 0.4–3 mm are attained. Colonies of *C. nitrativorans* are reported as cream-colored on TSA (Etchebehere et al., 2001), while those of *C. denitrificans* are described as yellow-white on nutrient agar by Gumaelius et al. (2001). Some *Comamonas* strains may produce a brown diffusible pigment.

Reliable and relatively fast identification at genus level is possible by determining the 16S

rDNA sequence of a strain thought to belong to *Comamonas*, since sequences from reference strains are available for comparison. An oligonucleotide probe targeting the 16S rRNA has been used to identify strains of *Comamonas*, then still including *Comamonas acidovorans* (Amann et al., 1996), but not the two newest species. Amplified 16S rDNA restriction analysis using two restriction enzymes permits differentiation of the different genospecies (Vaneechoutte et al., 1992). It will be necessary to include at least the type strains of all species in a DNA fingerprint study to verify the identification level of these techniques.

Identification is also possible using polyacrylamide gel electrophoresis of cellular proteins and immunofusion (De Vos et al., 1985; Willems et al., 1991), zymogram and fatty acid analyses (Tamaoka et al., 1987), and DNA:DNA hybridizations (Tamaoka et al., 1987; Willems et al., 1991).

Phenotypic identification on its own can be more complicated because many bacterial taxa share general phenotypic properties with *Comamonas*. The taxa to be compared with *Comamonas* will be determined largely by the isolation source (e.g., clinical or environmental) of the strains.

Comamonas strains from the clinical environment will have to be compared with other Gram-negative taxa, such as Enterobacteriaceae, some *Pseudomonas* species, and *Stenotrophomonas maltophilia*. Enterobacteriaceae are easily recognized by their fermentative carbohydrate metabolism and negative oxidase reaction, but differentiation from *Pseudomonas* species is more complex. The following characters were proposed as minimal requirements for the identification of clinically isolated *Comamonas* strains: motility by means of bipolar tufts of at least three flagella; no acid produced from glucose; acid produced from fructose and mannitol by *C. acidovorans*, but not by *C. testosteroni*; indophenoloxidase positive; no H_2S production in Kligler iron agar; and accumulation of poly-3-hydroxybutyrate (Gilardi, 1985). In this scheme, differentiation from *Pseudomonas alcaligenes* and *Pseudomonas pseudoalcaligenes* subsp. *pseudoalcaligenes*, species that are easily confused with *Comamonas* (Pickett and Greenwood, 1986), is based only on a different flagellation (less than three polar flagella in the *Pseudomonas* species). The following additional tests can be used: growth on L-arginine and diaminobutane (regarded as absent in *Comamonas*, present in both *Pseudomonas* species) and growth on adipate, pimelate, suberate, azelate, and sebacate (present in *Comamonas*, but absent in both *Pseudomonas* species). Some of these characters are present in at least one of the denitrifying *Comamonas* species (e.g., growth on L-arginine in *C. denitrificans*) or were not reported for them.

Comamonas strains from soil and water have to be differentiated from a larger variety of taxa. Table 2 presents features for the differentiation of *Comamonas* from most other Gram-negative taxa that may be isolated from soil and water. It

Table 2. Differentiating phenotypic features between *Comamonas* and other Gram-negative taxa occurring in soil and water.

Taxon	Not present in *Comamonas*	Present only in *Comamonas*
Vibrionaceae	Fermentative carbohydrate metabolism	
Enterobacteriaceae	Fermentative carbohydrate metabolism	Oxidase
Delftia	Growth on D-fructose and D-mannitol	
Hydrogenophaga and *Flavobacterium*	Insoluble yellow pigment	
Acidovorax	Polar monotrichous flagellation, growth on D-glucose	Bipolar tufts of flagella
Brachymonas	Nonmotile coccobacilli, denitrification	
Stenotrophomonas		Poly-3-hydroxybutyrate inclusions
Rhizobium and *Agrobacterium*	Exopolysaccharide production on carbohydrate-containing media, symbiotic nitrogen-fixation with legumes, or plant tumor induction	
Rhodospirillaceae	Facultatively photo- or chemi-autotrophic growth, photosynthetic pigments (brown-red to purple or green)	
Pseudomonas	Use of D-glucose as sole carbon source	
Alcaligenes	Peritrichous flagellation	
Aquaspirillum	Helical cell shape	
Acinetobacter		Oxidase
Sphaerotilus	Cells forming long chains, sheet formation	
Xanthobacter	Insoluble yellow pigment, microaerophilic, and nitrogen fixation	
Azospirillum	Microaerophilic, nitrogen fixation	

Table 3. Features differentiating *Comamonas terrigena*, *Comamonas testosteroni*, *Comamonas nitrativorans* and *Comamonas denitrificans*.

	C. terrigena	*C. testosteroni*	*C. nitrativorans*[a]	*C. denitrificans*[b]
Denitrification with formation of N_2	–	–	+	+
Use of carbon sources				
Glycolate	–[c]	+[c]	nd	+
L-Histidine	–[c]	+[c]	nd	–
2-Aminobenzoate	–[c]	+[c]	nd	nd
Citrate	–[c]	+[c]	–	D
Benzoate	–[c]	+[c]	+	nd
Gluconate	D[c]	+[c]	–	–
Pyruvate	D[c]	D[c]	–	+
Malonate, tartrate	–[c]	–[c]	–	+
L-Arginine, L-lysine and salicin	–[c]	–[c]	nd	+
Assimilation of testosterone[d]	–	+	nd	nd
Occurrence of 2-OH-14:0[e]	–	+	nd	nd
Occurrence of 2-OH-16:0, 2-OH-18:0[e]	–	+	nd	nd
Mol% G+C	64.0–66.0	62.5–64.5	nd	60.4–60.8

Symbols: +, >90% of strains positive; –, >90% of strains negative; D, result varies among strains; and nd, no data available.
[a]From Etchebehere et al. (2001).
[b]From Gumaelius et al. (2001).
[c]As tested with auxanographic techniques using API systems.
[d]From Tamaoka et al. (1987).
[e]From Willems et al. (1989).

can be added here that the presence of hydroxy putrescine is characteristic for the genera of the β-Proteobacteria. Table 3 gives features for the differentiation of *Comamonas* species.

Cultivation

Comamonas strains can be grown on nutrient agar and many other commonly used media such as tryptic soy agar and Columbia agar with or without blood. They are able to use many organic acids and amino acids, but few sugars (De Vos et al., 1985). They can degrade a variety of complex aromatic compounds, steroids and many man-made complex organic compounds. Optimal growth temperature is 30°C.

Preservation

Comamonas cultures can be maintained on nutrient agar slants at 4°C for up to 2 months. Lyophilization or storing in liquid nitrogen can be used for long-term preservation of strains on the condition that cryoprotectants are added. Generally, the use of standard procedures is recommended.

Physiology

Comamonas strains have an aerobic respiratory type of metabolism. Most strains of *C. terrigena* and *C. testosteroni* are capable of nitrate reduction, but cannot reduce nitrites (Willems et al., 1991), while all strains of *C. nitrativorans* and *C. denitrificans* can reduce nitrate all the way to N_2. Although *Comamonas* strains were identified in a study of denitrifying biofilms at a water treatment plant, they were thought not to be true members of the biofilm community, but to have washed in from previous stages (Lemmer et al., 1997). Other data, however, confirm the denitrification capacity of some members of the genus (Vanbrabant et al., 1993). In particular, it has been shown that *Comamonas* strain SGLY2 is capable of aerobic denitrification and nitrogen production without nitrite building up. This strain has been studied for use in mixed-culture aerated reactors (Patureau et al., 1997).

Comamonas strains are able to degrade a wide variety of aromatic compounds. *Comamonas* strain JS765 has a 1,2-dioxygenase able to break down nitrobenzene to nitrohydrodiol which spontaneously decomposes to catechol and nitrite. Catechol is then broken down via the meta-cleavage pathway, the genes of which have been sequenced (Parales et al., 1997; Genetics). This pathway has been studied in comparison with similar pathways in *Pseudomonas* strains (He and Spain, 1999). *Comamonas testosteroni* strains that degrade polycyclic aromatic compounds such as phenanthrene, naphthalene and anthracene have been described and the genes involved Genetics have been characterized (Goyal and Zylstra, 1996). The mechanism for phenol tolerance was studied in the phenol-degrading *C. tesosteroni* strains P15 and E23 (Yap et al., 1999).

The meta-cleavage pathway is used by *C. testosteroni* for the degradation of 4-chlorophenol and related compounds (Hollender et al., 1997). *Comamonas testosteroni* can degrade 4-toluene sulfonate, which is thought to be taken up by an inducible secondary proton symport system (Locher et al., 1993). The degradation of 4-toluene sulfonate and 4-toluene carboxylate involves oxygenation of the side chain to 4-sulfobenzoate and terephthalate, respectively, which are then oxidized to protocatechuate. Genes involved in these pathways have been sequenced and characterized (Junker et al., 1997b; Genetics). Terephthalate is broken down by a terephthalate dioxygenase system to (1R, 2S)-dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylic acid which is further degraded to protocatechuate by (1R,2S)-dihydroxy-1,4-dicarboxy-3,5-cyclohexadiene dehydrogenase (Oppenberg et al., 1995). The dioxygenase system is a Rieske [2Fe-2S] protein with two subunits α and β which are thought to form an $\alpha_2\beta_2$ structure (Schlaefi et al., 1994). The sulfonate and sulfate ester degradation as a source of sulfur for growth of Gram-negative bacteria has recently been reviewed (Kertesz, 2000).

Comamonas testosteroni I2 was shown to degrade recalcitrant components such as aniline and chloroaniline (Boon et al., 2001). Both components are among the most widely produced industrial amines commonly used for the production of polyurethanes, rubber, azo dyes, drugs, pesticides, etc. The presence of these toxic amines in natural environments is strictly regulated by the environmental protection agencies of the European Union (EU) and the United States.

Testosterone can be used as sole carbon source by *C. testosteroni* and induces the expression of enzymes involved in catabolism of steroids and aromatic hydrocarbons and the repression of at least one amino acid degrading enzyme. It was suggested that steroids may play a regulatory role in the synthesis of catabolic enzymes during adaptive growth (Moebus et al., 1997). Several of the enzymes involved in the conversion and break down of steroids have been studied and their genes sequenced (Genetics). The structure and function of δ^5-3-ketosteroid isomerase and 3-oxo-δ^5-steroid isomerase, which catalyze the the conversion of δ^5- to δ^4-3-ketosteroids by intramolecular proton transfer, have been studied (Brothers et al., 1995; Zao et al., 1996, 1997). A bile acid 3-α-sulfate sulfohydrolase of *C. testosteroni* was purified and characterized (Tazuke et al., 1994).

Comamonas strains enriched from wastewater from an aerated stabilization basin of a bleached kraft pulp mill were reported to degrade terpenes such as abietane and pimarane-type resin acids (Morgan and Wyndham, 1996).

Some *Comamonas* strains are able to degrade poly-3-hydroxybutyrate using an extracellular poly-3-hydroxybutyrate depolymerase, which can also hydrolyze poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly-3-hydroxyvalerate. The enzyme differs from other poly-3-hydroxybutyrate depolymerases in that it is insensitive to phenylmethylsulfonyl fluoride and hydrolyzes poly-3-hydroxybutyrate to 3-hydroxybutyrate monomers (Jendrossek et al., 1993). The encoding gene of a *Comamonas* sp. strain was cloned and partly sequenced, and the resulting partial protein was analyzed (Jendrossek et al., 1995). A very similar sequence was obtained for *C. testosteroni* strain YM1004 (Shinomiya et al., 1997). *Comamonas* sp. strain P37C, isolated from compost, was able to degrade a range of poly-3-hydroxyalkanoates (Quinteros et al., 1999).

The enzymes involved in the first steps of quinoline and 3-methyl quinoline degradation by *C. testosteroni* strain 63, quinoline 2-oxidoreductase and 2-oxo-1,2-dihydroquinoline 5,6-dioxygenase, have been characterized. Quinoline 2-oxidoreductase is a molybdo-iron flavoprotein and 2-oxo-1,2-dihydroquinoline 5,6-dioxygenase is a single component protein (Schach et al., 1995).

Comamonas testosteroni produces 5-aminolevulinic acid, a precursor to tetrapyrroles, from aminoacylated tRNA-Glu via a two-step pathway involving glutamyl-tRNA reductase and glutamine-1-semialdehyde-2,1-aminomutase (Hungerer et al., 1995).

The biosynthesis of aromatic amino acids via several pathways has been extensively studied among pseudomonad bacteria. In *C. testosteroni* prephenate dehydrogenase and arogenate dehydrogenase are reactive with either nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP). No arogenate dehydratase activity is present; prephenate dehydratase is present and its activity is stimulated by L-tyrosine (Byng et al., 1983).

Genetics

The genes of *C. testosteroni* involved in the degradation of naphthalene and phenanthrene have been cloned and characterized. At least two different sets of phenanthrene degradation genes may be present among different *Comamonas testosteroni* strains. They are different from the naphthalene degradation genes (*nha*) of *Pseudomonas putida* (Goyal and Zylstra, 1996).

The gene *phtD* for 4,5-dihydroxyphthalate decarboxylase, an enzyme involved in phthalate metabolism in *C. testosteroni*, has been identified and sequenced. The deduced amino acid sequence shows similarity to the *pth5* genes of *P.*

putida, and the sequence upstream of *pthD* shows a high similarity to *pth1* in *P. putida*, where it is thought to be a positive regulator of the other *pth* genes. These findings suggest a common origin for the genes involved in the phthalate metabolism (Lee et al., 1994).

The *bphD* gene of *C. testosteroni* strain B-356, encoding for 2-hydroxy-6-oxo (phenyl/chlorophenyl) hexa-2,4-dienoic acid hydrolase, was sequenced, and the gene product has a mechanism of action similar to that of classical lipases and hydrolases (Ahmad et al., 1995). In the same strain, biphenyl/chlorobiphenyl dioxygenase, the enzyme system involved in the first step of the degradation of biphenyl or chlorobiphenyl compounds, was characterized. The genes for the three components of this enzyme system have been sequenced: *bphA* and *bphE* encode the two subunits of a terminal Fe-S oxygenase, *bphF* encodes a ferredoxin, and *bphG* encodes a ferredoxin reductase and is not located near the *bphAEF* genes (Sylvestre et al., 1996b). Many of these enzymes have been characterized (Hurtubise et al., 1995; Hurtubise et al., 1996) and some have been used to produce enzyme chimeras by combination with oxygenase components from other bacteria (Chebrou et al., 1999). Genes for subsequent degradation steps have also been characterized: *bphB* encodes 2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase (Sylvestre et al., 1996a) and *bphC* encodes 2,3-dihydroxybiphenyl-1,2-dioxygenase which catalyzes meta-1,2 fission of the aromatic ring (Bergeron et al., 1994). Finally, it has been demonstrated (Hein et al., 1998) that in this strain two meta-cleavage dioxygenases coexist with different degrees of sensitivity towards intermediates of the biphenyl catabolic pathway and suggesting a complex regulation system.

Genes involved in the degradation of catechol via the meta-cleavage pathway have been sequenced and amino acid sequences deduced for strain *Comamonas* sp. JS765: *cdoE* encodes catechol 2,3-dioxygenase, *cdoT* encodes a ferredoxin, and *cdoR* is a regulatory gene (Parales et al., 1997).

A gene cluster encoding for the degradation of 3-(3-hydroxyphenyl)-propionate from *C. testosteroni* strain TA441 was cloned and sequenced. It contains a regulatory gene, *mhpR*, and the following six genes for catabolic enzymes: *mhpA* for a flavin-type hydroxylase, *mhpB* for an extradiol dioxygenase, *mhpD* for 2-keto-4-pentenoate hydratase, *mhpF* for an acylating acetaldehyde dehydrogenase, *mhpE* for 4-hydroxy-2-ketovalerate aldolase, and *mhpC* for a meta-cleavage hydrolase (Arai et al., 1999b). The same research group (Arai et al., 2000) studied the arrangement and the regulation of the meta pathway in the breakdown of phenol.

The genes involved in the degradation of 4-toluene sulfonate and 4-toluene carboxylate of *C. testosteroni* have been extensively studied. The first step of oxygenation of the side chain to 4-sulfobenzoate and terephthalate, respectively, requires three enzymes: a 4-toluenesulfonate methyl-monooxygenase system consisting of a reductase B and oxygenase M encoded by the *tsaBM* genes, a 4-sulfobenzyl alcohol dehydrogenase encoded by *tsaC* and a 4-sulfobenzaldehyde encoded by *tsaD*. These genes are coexpressed under the regulation of the product of *tsaR*. Some of the enzymes involved have been purified and partially characterized. The tsaMB oxygenase system is a class IA mononuclear iron oxidase with tsaM having a Rieske [2Fe-2S] center, and tsaC is a short-chain zinc-dependent dehydrogenase (Junker et al., 1996). The conversion of 4-sulfobenzoate to protocatechuate is catalyzed by a 4-sulfobenzate-3,4-dioxygenase system consisting of a reductase C and an oxygenase A, again with a Rieske [2Fe-2S] center, encoded by the genes *psbAC* (Junker et al., 1996). The genes *tsaMBCD*, *tsaR*, and *psbAC* are located on conjugative plasmids in some *C. testosteroni* strains (Junker and Cook, 1997a). Terephthalate is also broken down to protocatechuate, but genes involved have not yet been characterized. Protocatechuate is broken down via a meta-cleavage pathway by enzymes thought to be encoded on the chromosome (Junker and Cook, 1997a).

The gene cluster encoding a phenol hydroxylase in the phenol-degrading *C. testosteroni* strain R5 (*phcKLMNOP*) and the regulatory gene *phcR* have been cloned and expressed in *Pseudomonas aeruginosa*, conferring the use of phenol as sole carbon source on this bacterium (Teramoto et al., 1999). A second gene cluster which can confer the ability to use phenol (*aphKLMNOPQB* and the corresponding regulatory genes *aphR* and *aphS*) was studied in *C. testosteroni* strain TA441 (Arai et al., 1999a).

In *C. testosteroni*, the genes for several enzymes involved in the breakdown of steroid compounds have been studied. The gene for a 3-α-hydroxysteroid dehydrogenase has been sequenced and expressed through cloning, and the enzyme was characterized (Abalain et al., 1995; Oppermann and Maser, 1996a). Because of its similarity to ribosomal proteins L10 and L7/12, it was suggested that this enzyme may be formed by fusion of two ribosomal proteins (Baker, 1996). The gene for 3-ketosteroid-δ^4-5-α-dehydrogenase was sequenced and located downstream of a gene for 3-ketosteroid-δ^1-dehydrogenase. Although both enzymes are functionally similar, their genes are probably not derived from a common ancestor (Florin et al., 1996). A 3β/17β hydroxysteroid dehydrogenase

was studied by mutagenic replacement within the active site to identify important residues (Oppermann et al., 1997). The steroid-inducible gene *stdC* of *C. testosteroni* was cloned and sequenced. It was homologous to open reading frames (ORFs) of unknown function in the polyhydroxyalkanoic acid cluster of several other bacteria and is transcribed as a monocistronic mRNA that is abundant in cells grown on steroid carbon sources (Cabrera et al., 1997). Recently, in a model for the regulation of the 3-α-hydroxysteroid hydrogenase/carbonyl reductase (Xiong et al., 2001), it was proposed that the hydrogenase is under the control of two repressor proteins of which the binding to the promotor and the messenger RNA is de-repressed by an inducer such as testosterone.

The gene for an aliphatic nitrilase from *C. testosteroni* was sequenced and overexpressed, and the enzyme was purified and its primary structure determined. A Cys^{163} residue is thought be essential in the active site (Levy-Schil et al., 1995).

Several plasmids have been isolated from or transferred to *Comamonas* strains, and they may contribute to the catabolic versatility of these organisms. *Comamonas testosteroni* strains T-2 and PSB-4 and the type strain were reported to contain plasmids pTSA and pT2T, plasmid pPSB, and no plasmids, respectively (Junker and Cook, 1997a). Plasmid pTSA (85 kb) is a conjugative plasmid of the IncP1 group and carries the genes *tsaMBCD*, *tsaR* and *psbAC*, involved in the degradation of 4-toluene sulfonate to protocatechuate, and two copies of insertion sequence IS*1071*. Plasmid *pPSB* (85 kb) also is a conjugative plasmid; it carries only the *psbAC* gene as well as two copies of IS*1071*. Conjugative experiments with the type strain have suggested that the psb genes are located in a composite transposon (Junker and Cook, 1997a).

Plasmid RP4::Tn*4371* with genes for biphenyl and 4-chlorobiphenyl degradation was transferred from *Enterobacter agglomerans* to indigenous soil bacteria, including *Comamonas* strains, where these catabolic enzymes were successfully expressed (De Rore et al., 1994). Plasmid PR4::Mu3A, carrying chromosomal DNA fragments encoding for the use of biphenyl as a sole carbon source, were transferred to *C. testosteroni* and the enzymes were effectively expressed (Springael et al., 1996). Plasmids pACK5 and pACT72, including a replicon of the cryptic *Acetobacter pasteurianus* plasmid pAC1, were transferred to and successfully expressed in *C. terrigena* (Grones and Turna, 1995). Plasmid pE43 from *Pseudomonas aeruginosa* and plasmid pPC3 from *Arthrobacter globiformis*, carrying genes for dehalogenation of chlorobenzoates, permitted *C. testosteroni* strain VP44 to grow on ortho- or para-biphenyls as sole carbon source (Hrywna et al., 1999).

Plasmid PNB2 (Boon et al., 2001) has been isolated from *Comamonas testosteroni* I2, an aniline- and chloroaniline-degrading strain. So far, the chloroaniline capacity could not be demonstrated on the plasmid by conjugative transformation with a *Ralstonia* strain, in contrast to the aniline capacity. Additional data suggest that oxidative deamination of chlorinated and non-chlorinated aromatics may be completed by a different set of genes (Boon et al., 2001).

Ecology

Comamonas strains have been recovered from sites heavily contaminated with complex organic compounds and heavy metals, and resistance to heavy metals such as cadmium and nickel has been reported (Stoppel and Schlegel, 1995; Kanazawa and Mori, 1996). A strain tentatively identified as *C. testosteroni* was among the strongest chromate-reducing bacteria found in the cooling water of an electricity plant and may therefore be implicated in the blockage of pipes through precipitation of chromium (III) oxide (Cooke et al., 1995). Most of the isolates from a polychlorinated biphenyl (PCB)-polluted site were *Comamonas testosteroni* strains capable of degrading biphenyl and a variety of polychlorinated biphenyls (Joshi and Walia, 1995).

Many *Comamonas* strains from various environments are capable of degrading poly-3-hydroxybutyrate (PHB; Mergaert and Swings, 1996), and the extracellular PHB depolymerase of one strain has been purified and characterized (Jendrossek et al., 1993).

The broad substrate specificity of its 3-α-hydroxysteroid dehydrogenases and its occurrence in the intestinal tract of vertebrates have led to suggestions that *C. testosteroni* and related bacterial strains may contribute to the bioactivation or inactivation of hormones, bile acids and xenobiotics (Oppermann and Maser, 1996a). *Comamonas testosteroni* possesses steroid-inducible hydroxysteroid dehydrogenases and carbonyl reductases which may provide protection against natural and synthetic toxic compounds in soil and the intestinal tract of mammals (Oppermann et al., 1996b).

Pathogenicity

Although many *Comamonas* strains have been isolated from clinical samples and from the hospital environment (Willems et al., 1991), they are regarded as very occasional opportunistic pathogens (Gilardi, 1985), and no evidence of

pathogenic effect on healthy people has been reported.

Applications

Because they can break down a wide variety of complex organic compounds (including carboxylic and dicarboxylic aliphatic or unsaturated carboxylic acids, aromatic compounds, and sterols), *Comamonas* strains contribute to bioremediation processes. *Comamonas testosteroni* has been used for the degradation of 4-toluenesulfonic acid in a continuously operated fixed-bed biofilm reactor (Khlebnikov and Peringer, 1996). Analysis of the bacterial communities in activated sludge revealed the presence of *Comamonas* strains (Kaempfer et al., 1996). *Comamonas terrigena* strain N3H, immobilized in an alginate gel, has been used for the degradation of the anion-active surfactant dihexyl-sulfosuccinate (Huska et al., 1996; Toth et al., 1996; Proksova et al., 1999). *Comamonas* strains JS46 and JS 47 have been studied in mixed and non-mixed culture immobilized cell reactors for the degradation of *m*- and *p*-nitrobenzoate (Goodall et al., 1998b). The experimental data were used for process modeling (Goodall and Peretti, 1998a).

A quinohemoprotein ethanol dehydrogenase, which catalyzes the NAD-independent oxidation of a broad range of alcohols to their aldehydes and on to the carboxylic acid, has been purified from ethanol-grown *C. testosteroni* cells. The enzyme was studied for use in the production of (S)-solketal (2,2-dimethyl-1,3-dioxolane-4-methanol) by enantioselective oxidation of racemic solketal (Geerlof et al., 1994; Machado et al., 1999). The active holoenzyme contains pyrroquinoline-quinone, calcium ions, and heme *c* (De Jong et al., 1995). The encoding gene (*qhedh*) was cloned, sequenced and expressed in *E. coli* (Stoorvogel et al., 1996). The enzyme has been applied in electrodes through immobilization in a redox polymer network (Stigter et al., 1997) and can be used for the enantioselective oxidation of secondary alcohols (Godde et al., 1999).

The capacity of *C. testosteroni* BS1310 (pBS1010) to degrade sulfoaromatic compounds resulted in the design of a Clark-type electrode to measure the *p*-toluene concentration (Makarenko et al., 1999).

The ability of *C. testosteroni* to selectively degrade L-lysine from racemized lysine crystals has been applied in the large-scale production of D-lysine (Takahashi et al., 1997).

Finally, a number of studies point to other potential applications of *Comamonas* strains. *Comamonas testosteroni* strain CMI 2848 had the highest activity for hydroxylating 3-cyanopyridine to 3-cyano-6-hydroxypyridine among 4600 isolates screened in a study contributing to the synthesis of new agricultural chemicals (Yasuda et al., 1995). Because hydroxylated *N*-hetero-aromatic compounds are difficult to produce, the characteristic of a quinoline-oxidoreductase of *C. testosteroni* 63 to mediate the hydroxylation of *N*-hetero-aromatic compounds such as pyridine to hydroxy-substituted pyrimidines may be of pharmaceutical importance (Fetzner, 1999).

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Prokaryotes (2006) 5:737–746
DOI: 10.1007/0-387-30745-1_32

CHAPTER 3.2.8

The Genera *Chromobacterium* and *Janthinobacterium*

MONIQUE GILLIS AND JOZEF DE LEY

Purple-pigmented bacteria have been described since the end of the 19th century; they were reported as discoloring a variety of natural materials and occasionally as the causative agent of septicemia in humans and animals. Bacteria producing purple and violet colonies due to the production of a nondiffusible pigment, violacein, were classified in a redefined genus *Chromobacterium* by Buchanan (1918). The structure of violacein, an indole derivative produced via the oxidation of tryptophan, is shown in Fig. 1. It can be easily identified (Johnson and Beer, 1971) spectrophotometrically from the following properties: 1) in ethanolic solution it has an absorption maximum at 579 nm and a minimum at 430 nm; 2) by adding 10% (v/v) H_2SO_4 the solution turns green with an absorption maximum at 700 nm; and 3) when NaOH is added to an ethanolic solution, the solution turns green and afterwards reddish brown. Recently, violacein has also been characterized by nuclear magnetic resonance spectroscopy and mass spectrometry (Riveros et al., 1988). The pigment is only abundantly produced when tryptophan is available in the culture medium.

Although violacein is produced by only a few groups of bacteria, its presence does not necessarily indicate a close relationship between these organisms. An extensive phenotypic study (Sneath, 1956, 1960, 1974) within *Chromobacterium*, as defined by Buchanan (1918), provided evidence of two groups within this genus: a fermentative and mesophilic one (growth at 37°C but not at 4°C) and a nonfermentative and psychrophilic one (growth at 4°C but not at 37°C), for which he proposed two species, *Chromobacterium violaceum* and [*Chromobacterium*] *lividum*, respectively. However, these two species have been reported as being not closely related (Moffet and Colwell, 1968; Sneath, 1974) and since the latter was misnamed, in this chapter its epithet and all other misnamed epithets shall be enclosed in square brackets.

Violacein is not a good taxonomic marker as shown by the fact that not only have nonpigmented *Chromobacterium violaceum* and [*Chromobacterium*] *lividum* strains been isolated, but also since marine, violacein-producing strains were isolated, which were assigned on phenotypic grounds to the genus *Alteromonas* (Gauthier, 1976, 1982). Later, Van Landschoot and De Ley (1983) confirmed that these marine strains were genuine *Alteromonas*.

The relationships between the violacein-producing bacteria classified in *Chromobacterium* (Sneath, 1974) could finally be elucidated by De Ley et al. (1978). DNA-rRNA hybridizations within and between the *violaceum* and the *lividum* taxons and with different other Gram-negative genera demonstrated clearly that these purple-pigmented bacteria represent two tight but separate groups within rRNA superfamily III (see also De Ley's introduction to the Proteobacteria in The Proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition) which are less related to each other than to other genera from this rRNA superfamily. In consequence, a new genus *Janthinobacterium* with one species *J. lividum* was created for the *lividum* taxon and the genus name *Chromobacterium* was restricted to the mesophilic, fermentative *violaceum* taxon (De Ley et al., 1978). *Chromobacterium* constitutes a separate rRNA branch on which all *violaceum* strains cluster with a $T_{m(e)}$ of 76.5 to 80°C. *Janthinobacterium* constitutes a separate rRNA subbranch which is closely related to the rRNA subbranch containing [*Pseudomonas*] *rubrisubalbicans* and a yet unnamed group of bacteria isolated from clinical material (see Fig. 6 in The Proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition). The *Janthinobacterium*-[*Pseudomonas*] *rubrisubalbicans* rRNA branch is more closely related to the solanacearum rRNA branch than to *Chromobacterium*, emphasizing the deep phylogenetic gap between *Chromobacterium* and *Janthinobacterium*. Cataloging results (Woese et al., 1984) confirm the separate position of both genera in the beta group (Stackebrandt et al., 1988). In this chapter, *Chromobacterium* and *Janthinobacterium* are treated together merely on historical grounds.

Moss et al. 1978 described a new group of violacein-containing, fermentative, psychrophilic, fresh water bacteria and assigned them to a new species *fluviatile* in the genus *Chromobacterium*. It differs from *C. violaceum* by a thin flat, spreading colony type, some other phenotypic features (see below) and % GC values of 50 to 52. Within *C. violaceum* the GC content varies from 65 to 68 mol%, indicating at once that these new organisms cannot be members of *Chromobacterium*. DNA-rRNA hybridizations (Moss and Bryant, 1982) demonstrated that [*Chromobacterium*]* *fluviatile* is not closely related to the *C. violaceum* rRNA cluster. With a $T_{m(e)}$ of 70 to 71°C vs. the *C. violaceum* rRNA probe, they occupy a separate position and are further removed from *C. violaceum* than the latter species is from the Neisseriaceae (Rossau et al., 1989). Comparison of their 16S rRNA sequences corroborates this conclusion (Dewhirst et al., 1989). Therefore, these organisms cannot belong in *Chromobacterium*; they have thus been misnamed and shall not be discussed here further. The differentiation of these three groups of violacein-producing organisms is given in Table 1.

This chapter was taken unchanged from the second edition.

*Since this manuscript was submitted, Logan (1989) proposed a monospecific genus *Iodobacter* to accomodate the [*Chromobacterium*] *fluviatile* group. Their phenotypic results on *Iodobacter* and the "atypical, *Janthinobacterium lividum* strains" are integrated in Table 1.

Chromobacterium

The following description of the genus is based on that of *Chromobacterium violaceum* by Sneath (1974, 1984). The genus *Chromobacterium* consists of Gram-negative, oxidase-positive, catalase-positive rods with rounded ends which are usually motile by both a single polar flagellum and by one to four lateral flagella. The two types of flagella differ in their wave types, staining capacity, and antigenic characteristics. Chromobacteria are facultative anaerobes and produce violet colonies on solid media; a violet ring is formed in liquid media at the surface with a fragile pellicle. Growth is best at 30 to 35°C; the minimum growth temperature is 10 to 15°C and the maximum is 40°C (20% of the strains can grow at 44°C). They are chemoorganotrophs using mainly carbohydrates fermentatively. Nitrate and usually nitrite are reduced, mostly with a variable gas production. They grow on ordinary media and are resistant to benzylpenicillin (10 µg/ml) and to vibriostatic agent 0/129 [2,4-diamino-6,7-diisopropylpteridine (30 µg/disc)]. The GC content of the DNA is 65 to 68 mol%. Other characteristics are as described by Sneath (1984) for *C. violaceum*. The type and sole species is *C. violaceum*. Between representative strains of this species, more than 83% DNA binding was found (De Ley et al., 1978).

C. violaceum is a saprophyte from soil and water and is normally considered as nonpathogenic for humans. Occasionally, however, it is an opportunistic pathogen of extreme virulence for humans and animals (see below).

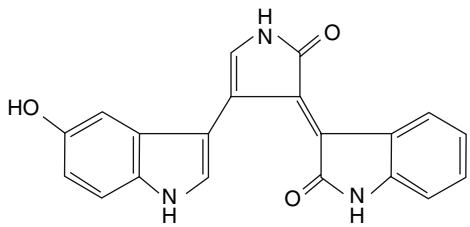

Fig. 1. Structure of violacein.

Habitats

C. violaceum is mainly found in soil and water (Sneath, 1956), where it usually constitutes only a minor component of the total microflora. It is found in temperate but more frequently in tropical and subtropical regions. It was suggested that it can be isolated more frequently from soil than from water (Corpe, 1951; Moss and Ryall, 1981). Its role in the rhizosphere of plants is not clear, although Hussain and Vančura (1970) isolated *C. violaceum* strains from rhizosphere soil of maize and demonstrated that the inoculation of maize

Table 1. Characteristics differentiating the groups of violacein-producing organisms.

Character	*Chromobacterium*	*Janthinobacterium*	*Iodobacter* [*Chromobacterium*] *fluviatilis*
Growth at 4°C	–	+ (96%)[a]	+
Growth at 37°C	+	–	–
Fermentation of glucose (O/F test)	F[b]	0[c] (90%)	F
Anaerobic growth	+	– (96%)	+
Cyanide produced	+	–	–
Turbid zone on egg yolk agar (lecithinase)	+	–	(+)[d]
Acid from			
Trehalose	+	– (82%)	+
L-arabinose	–	+	–
D-cellobiose	–	+ (99%)	–
D-galactose	–	+ (96%)	–
Gluconate	+	– (99%)	+ (96%)
D-maltose	–	+ (96%)	+
N-acetylglucosamine	+	– (99%)	+
Lactate utilization	+	+ (99%)	–
Casein hydrolysis	+	– or (+)	+
Esculin hydrolysis	–	+ (80%)	–
Arginine decarboxylase	+	–	–
GC content (mol%)	65–68	65–66	50–52

[a]Numbers in parentheses indicate the % of strains giving the indicated reaction.
[b]F, fermented.
[c]O, oxidized.
[d](+) weakly positive.
The phenotypic data are from Sneath (1984), Moss et al. (1978) and Logan (1989). The GC values are from De Ley et al. (1978) and from Moss et al. (1978) for *Iodobacter* [*Chromobacterium*] *fluviatilis*.

seeds with such *C. violaceum* strains significantly increased the yield of dry matter of the plant.

C. violaceum could not be recovered from food products (Koburger and May, 1982).

C. violaceum has been isolated from severe infections in humans and animals; water or soil contaminated with *C. violaceum* is mostly the causal agent. Although a *C. violaceum* infection in humans can result in mild diarrhea with a subsequent recovery (Sneath et al., 1953), it is mostly fatal for both humans and animals. A review of the earlier literature concerning chromobacteriosis was given by Sneath (1960). Later rare fatal cases of infection with *C. violaceum* were reported in different countries, i.e., the United States (Johnson et al., 1971), Vietnam (Ognibene and Thomas, 1970), Cuba (Machin Villafranca et al., 1986), Taiwan (Wu et al., 1986), Argentina (Kaufman et al., 1986), Nigeria (Onile et al., 1984), Brazil (Petrillo et al., 1984), and Australia (Wilkey and McDonald, 1983). The symptoms of the infection can vary considerably but lead mostly to septicemia, causing a rapid death. In the different case reports the symptoms were liver and lung abscesses (Sneath, 1960), meningitis (Shetty et al., 1987), adenitis (Sorensen et al., 1985), periorbital cellulitis (Simo et al., 1984; Feldman et al., 1984), toxemia, and multiple skin abscesses (Petrillo et al., 1984). Only exceptionally nonfatal chromobacterial sepsis has been reported as, i.e., a case of multiple liver abscesses (Suarez et al., 1986) or a systemic chromobacteriosis (Victorica et al., 1974). The disease can be cured by tetracyclines if treated at an early stage (Moss and Ryall, 1981). A recent comparison of the in vitro activity of 25 antimicrobial agents against clinical strains of *C. violaceum* showed that ciprofloxacin, norfloxacin, and perfloxacin are also highly active (Aldridge et al., 1988). For some eye infections, early recognition and aggressive treatment (with tetracycline, chloramphenicol, and cotrinoxazole) may result in a higher cure rate (Feldman et al., 1984).

Infections in animals were also described and studied intensively, i.e., in swine (Laws and Hall, 1964; Liu et al., 1988), Malayan gibbons, a Malayan sun bear (Groves et al., 1969), an Assam macaque (McClure and Chang, 1976), a dog (Gogolewski, 1983), and monkeys (Collins et al., 1985).

Virulence for mice and the pathology of infections appear to be similar for both nonpigmented and pigmented *C. violaceum* strains (Sivendra and Tan, 1977). Virulent *C. violaceum* strains produce an endotoxin which is more reactive than that from avirulent strains and it is suggested that the virulent strains are protected from phagocytic attack by elevated levels of superoxide dismutase and catalase (Miller et al., 1988). Experimental infections with *C. violaceum* or with its endotoxin were studied in mice and pigs (Zwang et al., 1987; Liu et al., 1988).

Enrichment and Isolation

The medium and conditions used to isolate and enumerate *C. violaceum* can strongly influence their recovery (Ryall and Moss, 1975; Koburger and May, 1982). Chromobacteria can grow easily on common laboratory media such as nutrient agar or GYCA (1% glucose, 0.5% yeast extract, 3% $CaCO_3$, and 2% agar in distilled water), and violacein is produced in the presence of oxygen. Sneath (1960) concluded that blood agar base was more suitable for the isolation of small numbers of *C. violaceum* from medical sources, because they can be inhibited by the presence of peroxide which can be present in nutrient agar.

For isolation from soil and water, the common media are not so suitable because chromobacteria represent only a minor part of the present microflora, so that large numbers of other bacteria can overgrow or prevent chromobacteria from growing well and producing violacein, which is an important feature in screening. Therefore, an enrichment procedure or a selective medium is recommended. In most cases, the selective media enrich for *Chromobacterium* as well as for *Janthinobacterium* and [*Chromobacterium*] *fluviatile*. The latter two taxa can be differentiated from *C. violaceum* afterwards by their phenotypic features (see Table 1).

Corpe (1951) described the following simple enrichment technique: "Five grams samples of soil were placed in sterile Petri dishes and soaked with sterile distilled water. Sterile polished or precooked rice grains were sprinkled over the surface of the soil and the plates incubated at 23–25°C for five days. At the end of the incubation period, the rice grains were partially covered with purple membranous growth indicating the presence of *Chromobacterium* sp."

Ryall and Moss (1975) used a selective medium based on quarter strength nutrient agar to count chromobacteria and janthinobacteria in water samples:

Composition of the Medium of Ryall and Moss (1975).

Beef extract (Oxoid)	0.25 g
Yeast extract	0.5 g
Peptone	1.25 g
NaCl	1.25 g
Agar	20 g
Distilled water	1 liter

To the molten agar medium (45°C), filter-sterilized solutions of colistin and sodium deoxycholate were added to give final concentrations of 15 µg/ml and 0.3 mg/ml, respectively. For counting chromobacteria and janthinobacteria in soil samples, cycloheximide was also added to

give a final concentration of 30 µg/ml to prevent overgrowth by fungi.

Composition of the Medium of Keeble and Cross (1977).

An improved selective medium (Keeble and Cross, 1977) is based on Bennett's agar:

Yeast extract (Difco)	1 g
Beef extract (Oxoid)	1 g
Bacto-casitone (Difco)	2 g
Glucose	10 g
Agar (Lab. M no. 2)	18 g
Distilled water	1 liter

To the molten agar medium were added filter-sterilized solutions of neomycin hydrochloride, cycloheximide, and nystatin to give a final concentration of 50 µg/ml of each. This medium is described as improving the pigmentation.

After comparing several media, Koburger and May (1982) reported that Bennett's agar was best for the isolation of both *C. violaceum* and *Janthinobacterium lividum* when incubated at 25°C but that at 35°C *Aeromonas* membrane agar gave the highest recoveries of *C. violaceum*. *Aeromonas* membrane agar (Rippey and Cabelli, 1979) is prepared as follows:

Tryptose	0.5 g
Trehalose	0.5 g
Yeast extract	0.2 g
NaCl	0.3 g
KCl	0.2 g
$MgSO_4 \cdot 7H_2O$	0.02 g
$FeCl_3 \cdot 6H_2O$	0.01 g
Bromothymol blue	0.004 g
Deionized water	100 ml

The ingredients are dissolved at room temperature, the pH is adjusted to 8.0 with 10 N NaOH, 1.5 g agar is added, and the mixture is autoclaved at 121°C for 15 min. After the addition of 1.0 ml ethanol, the mixture is cooled to 50°C, and 2 mg ampicillin and 10 mg desoxycholate are added. It is recommended that the poured plates be stored in the dark at 4°C and used within 6 weeks.

For inoculation, 0.1-ml samples are pipetted onto the surface and spread. They are allowed to dry for 30 min and incubated at 25 and 35°C for 7 and 5 days, respectively. All purple colonies developing on the plate were isolated and identified. Koburger and May (1982) suggested that the failure in other studies (Ryall and Moss, 1975; Keeble and Cross, 1977) to recover *C. violaceum* from soil and water and their own failure to isolate it from a limited number of food products can be related to the temperature sensitivity of these organisms. *C. violaceum* dies rapidly at 4°C.

Chromobacterium can also be isolated on a citrate-ammonium salts medium (Moss and Ryall, 1981) as described for *Janthinobacterium* further in this chapter.

Preservation of Strains The organisms can survive for several years in dilute peptone water (0.1% peptone) at room temperature. On GYCA or nutrient agar slants they can be kept for some months. Lyophilization and L-drying can be used to preserve them for longer periods. Freezing in nutrient broth containing 15% glycerol with storing at –80°C or in liquid N_2 is also recommended.

Identification

When definitely pigmented, *Chromobacterium* has only to be differentiated from other violacein-producing nonmarine strains belonging in the genus *Janthinobacterium* or to [*Chromobacterium*] *fluviatile*. The differentiating features are given in Table 1. *Chromobacterium* differs from *Alteromonas luteoviolacea* in its higher GC content and its ability to use citrate, glucose, fructose, and mannose (Gauthier, 1976, 1982).

Nonpigmented *C. violaceum* strains can be confused with members of *Vibrio* and *Aeromonas*. However, *C. violaceum* differs from *Aeromonas* in its flagellar morphology, its negative reaction for indole and for methyl red, and its production of HCN. Moreover, *C. violaceum* differs from *Vibrio* in its GC content and by a negative response to the lysine and ornithine decarboxylase tests but by a positive reaction in the arginine decarboxylase test. Unlike *C. violaceum*, most strains of the genera *Aeromonas* and *Vibrio* are able to use D-mannitol as a carbon source (Sneath, 1984; Sivendra and Tan, 1977). Singh et al. (1988) used a DNA dot blot technique and a DNA probe from their nonpigmented isolate to differentiate the latter strain from *Aeromonas* and to identify it as *C. violaceum*.

Members of *Chromobacterium* can easily be identified by hybridization with a ^{3}H-labelled 23S rRNA probe from *C. violaceum* NCTC 9757: only members of *C. violaceum* have $T_{m(e)}$ values between 76.5 and 80°C. [*Chromobacterium*] *fluviatile* strains have $T_{m(e)}$ values of 70 to 71°C. Members of *Janthinobacterium* have $T_{m(e)}$ values of 67 to 68°C and can further be identified by hybridization with a ^{3}H-labelled 23S rRNA probe of *J. lividum* NCTC 9796. All other taxa are below 72°C $T_{m(e)}$.

Special Features and Applications

In this part, we call attention to products or metabolic pathways that have been intensively studied in *C. violaceum* and their possible applications.

First of all, the biosynthesis of violacein (Riveros et al., 1988) and the different effects of this pigment have been studied. Violacein has antibiotic activity (DeMoss, 1967; Hoshino et al., 1987) and protects against predation by protozoa. In the older literature (Burbanck, 1942; Singh,

1942, 1945), *C. violaceum* and violacein itself are described as lethal to some ciliated protozoa. Amoebae and flagellates can also be killed by these bacteria (Singh, 1942). Later Groscop and Brent (1964) showed that violacein is toxic to soil amoebae while *C. violaceum* is toxic to *Vorticella microstoma* (Curds and Vandyke, 1966). Violacein has also a trypanocide effect (Riveros et al., 1988).

The production of violacein can be used as a microbiological assay for L-tryptophan (Sebek, 1965).

Apart from violacein, *C. violaceum* produces other antibiotics. Several were isolated and characterized in the last years: aerocyanidin, bearing an isonitrile group and being active against Gram-positive bacteria (Parker et al., 1988); aerocavin, exhibiting in vitro activity against both Gram-negative and Gram-positive bacteria (Singh et al., 1988); 3,6-dihydroxyindoxazene, inhibiting growth of Gram-negative bacteria (Hamada et al., 1983); and factor Y-T0678H (6-hydroxy-3-oxo-1,2-benzisoxazolin), exhibiting a selective activity against Gram-negative bacteria (Imai et al., 1983).

Other important compounds are produced by *C. violaceum*: arphamenines A and B, two specific inhibitors of aminopeptidase B that enhance immune responses (Umezawa et al., 1983).

The production and function of *C. violaceum* aminopeptidases involved in enkephalin hydrolysis was studied by Weiss et al. (1983).

Cooper et al. (1985) studied novel potentiators of β-lactam antibiotics and isolated two new compounds, SQ 28,504 and SQ 28,546, from a *C. violaceum* strain. These novel bacterial metabolites are glycopeptides which act synergistically with β-lactam antibiotics against Gram-negative bacteria.

Earlier β-lactamase, primarily active against cephalosporins, was shown in a *C. violaceum* strain (Farrar and O'Dell, 1976).

C. violaceum has also been used in studies on the production of HCN (Brysk et al., 1969; Niven et al., 1975), of unusual sugar compounds (Stevens et al., 1963), and of extracellular polysaccharides (Corpe, 1964). The tough membranous aspect of some *C. violaceum* colonies is the result of the latter polysaccharides (Corpe, 1964). Their role in the formation of soil aggregates has been suggested (Martin and Richards, 1963).

The Copper-containing phenylalanine monooxygenase (Benkovic et al., 1986; Pember et al., 1987a, 1987b; Fujisawa and Nakata, 1987) and the denitrification processes (Bazylinski et al., 1986) were intensively studied. It was shown that denitrification terminates with the production of N_2O and that NO_2^- reduction to N_2O is not coupled to growth but may serve as a detoxification mechanism.

A *C. violaceum* strain has been isolated that can grow on and use acetonitrile as sole carbon source (K. D. Chapatwale et al., 1988)

Another *C. violaceum* strain solubilized a considerable amount of pure gold after 40 days incubation in nutrient medium because the cyanide produced during growth and stationary phase formed the complex anion $[Au(CN)_2]^-$ with gold (Smith and Hunt, 1985).

Janthinobacterium

The basic description of this genus was given by Sneath in 1984. *Janthinobacterium* consists of Gram-negative rods with rounded ends, sometimes slightly curved. No resting stages are formed. They are usually motile by means of both a single polar flagellum and by one to four lateral flagella. They are strict aerobes. They produce violet colonies on solid media; in nutrient broth a violet ring is formed at the junction of the liquid surface and the container wall. They are resistant to benzylpenicillin (10 μg/ml) and to vibriostatic agent 0/129 (30 μg/disc). The most striking features are: 1) janthinobacteria are aerobic chemoorganotrophs with a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. 2) They have an optimum growth temperature of 25°C, a maximum growth temperature of 32°C and a minimum growth temperature of 2°C. 3) Only a few strains produce HCN. 4) They can grow rapidly on a medium containing citrate and ammonia as sole carbon and nitrogen source. 5) Nitrate and nitrite are reduced mostly with visible gas production. 6) The GC content is 61 to 67 mol%.

They are soil and water organisms (Moss et al., 1978; Koburger and May, 1982) common in temperate climates. They are sometimes the causative agent of food spoilage. The type and sole species is *J. lividum*. Within this species, percentages of DNA binding of at least 48% were encountered between representative strains (De Ley et al., 1978). The membrane-forming strains, formerly named *C. amethystinum* or *C. membranaceum* and sometimes referred to as "atypical *C. lividum* strains" (Moss and Ryall, 1981), and an unusual strain (ATCC 14487) which preferentially grows at 4°C all belong in *J. lividum* (De Ley et al., 1978; Sneath, 1984). The typical features in which *Janthinobacterium* differs from *Chromobacterium* can be found in Table 1.

Habitats

J. lividum strains have been isolated from water and soil from rivers, creeks, lakes, and springs (Ryall and Moss, 1975; Koburger and May, 1982; Williams and Albright, 1984; Quevedo-

Sarmiento et al., 1986; Gonzalez et al., 1987). They mostly comprise only a small percentage of the total microflora, e.g., a mean of 5% of the total aerobic heterotrophic bacterial population in natural spring water (Quevedo-Sarmiento et al., 1986); a mean of 915 *J. lividum* isolates in 1 g water or soil (Koburger and May, 1982); and less than 1 to 3% of the total population sampled in lake water (Williams and Albright, 1984). According to Moss et al. (1978) they constitute approximately 75% of the total amount of purple-pigmented bacteria isolated from water and soil samples in a lowland river (river Wey in the UK).

They are frequently isolated from a variety of foods, i.e., processed poultry (Cox, 1975); turbot (Mudarris and Austin, 1988); various vegetables (Koburger and May, 1982); and Japanese noodles (Naito et al., 1986a). In turbot gills (Mudarris and Austin, 1988), *J. lividum* represented approximately one-third of the aerobic heterotrophic bacteria (7×10^5/g wet weight of gill tissue). In vegetables, they are present in quantities varying from 130 to over 6000/g (Koburger and May, 1982). Isolates from slaughterhouse effluents (Etherington et al., 1976) were colorless, could grow on meat, and exhibited a high proteolytic activity (Dainty et al., 1978), which is exceptional for members of this genus (Sneath, 1984).

Bettelheim et al. (1968) isolated bacterial strains from germinating seeds of *Psychotria nairobiensis* (Rubiaceae) and *Ardisia crispa* (Myrsinaceae), which they identified then as *C. lividum*, thus now *J. lividum*. By an immunofluorescence technique, Bettelheim et al. (1968) detected these organisms in tissues of several leaf-nodulated Rubiaceae and Myrsinaceae, and presented the hypothesis that some *J. lividum* strains are bacterial endophytes. We shall not review the discussions and disagreements in the literature on this issue (Lersten and Horner, 1976; see also The Genera Chromobacterium and Janthinobacterium in Volume 5) because it was shown that Bettelheim's isolates are not genuine janthinobacteria (De Ley et al., 1978) since they belong in rRNA superfamily IV, where they are a member of the [*Flavobacterium*] *capsulatum* rRNA branch (The Proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition).

The role, if any, of *J. lividum* strains in the induction of sporangia in *Phytophthora cinnamomi* has been discussed (Zentmeyer, 1965; Broadbent et al., 1974).

Enrichment and Isolation

As mentioned before, *Janthinobacterium* can be enriched and isolated on the same media as given in detail for *Chromobacterium* above.

According to Moss and Ryall (1981), they grow rapidly and can be selected on a citrate ammonium salts agar medium:

Citric acid	2 g
NaCl	1 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$NH_4H_2PO_4$	1 g
K_2HPO_4	1 g
Ionoagar nr. 2 (Oxoid)	15 g
Distilled water	1 liter

Koburger and May (1982) recovered *J. lividum* from a variety of food samples only on Bennett's agar (see above) at 25°C. On this medium they found higher counts in water and soil samples than on the medium of Ryall and Moss (1975) or on *Aeromonas* membrane agar (Rippey and Cabelli, 1979).

Maximal numbers of *J. lividum* were isolated at 25°C from turbot gills (Mudarris and Austin, 1988) on the following medium (%w/v or v/v):

$CaCl_2$	0.1
Yeast extract	0.1
Beef extraxt	0.5
Casein	0.6
Tryptone	0.2
Agar no. 1	1.5
Aged sea water	75
pH	7.2

Identification

Characteristics differentiating *J. lividum* from *C. violaceum* and *Iodobacter fluviatilis** have been given in Table 1. Differences with *Alteromonas luteoviolacea* have been reported above. Although colorless *J. lividum* strains could be confused with *Pseudomonas fragi* (Moss and Ryall, 1981), Dainty et al. (1978) were able to identify correctly their nonpigmented isolates as *J. lividum*.

Because *J. lividum* strains constitute a narrow rRNA cluster with $T_{m(e)}$ values between 77 and 79°C vs. ^{3}H labelled 23S rRNA from *J. lividum* NCTC 9796, they can easily be identified by hybridizing with this rRNA probe. [*Pseudomonas*] *rubrisubalbicans*, [*Aquaspirillum*] *autotrophicum*, and an as yet unnamed group of strains isolated from clinical material and described by E. Falsen (Culture Collection, University of Göteborg, Sweden) have $T_{m(e)}$ values of approximately 76°C and are the closest neighbors of *J. lividum* (see The Proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition). Phenotypically (M. Goor et al. 1986; see The Genus *Aquaspirillum* in Volume 5) it is possible to differentiate *J.*

*The latter species was originally named *Iodobacter fluviatile,* but since *Iodobacter* is masculine, it obviously has to be *Iodobacter fluviatilis.*

Table 2. Characteristics differentiating *Janthinobacterium* from [*Pseudomonas*] *rubrisubalbicans* and similar unnamed clinical isolates.

Character	*Janthinobacterium*	[*Pseudomonas*] *rubrisubalbicans* and unnamed clinical isolates[a]
Urease	–	+
Hydrolysis of esculin	+	–
Growth on D-fucose, 5-keto-gluconate, acetate, caprate, amylamine, and tryptamine	–	+
Growth on L-histidine	+	–

Isolates of Falsen (1989).

lividum from the group formed by [*Pseudomonas*] *rubrisubalbicans* and the unnamed clinical isolates (Table 2) and from [*Aquaspirillum*] *autotrophicum* (see Table 1 in The Genus *Aquaspirillum* in Volume 5).

Results from M. Goor et al. (1986).

Preservation of Strains *J. lividum* strains survive for several years at 4°C in 0.1% peptone or at –80°C in nutrient broth with 15% glycerol as a cryoprotectant. Lyophilization and storage at 4°C is also applied although the viability may be low (Sneath, 1984).

Special Features and Applications

For the different effects of violacein, we refer to the discussion under "*Chromobacterium*" above. The antimicrobial activity of violacein isolated from *J. lividum* has recently been described (Naito et al., 1986b). The location of the pigment was investigated by Lin and Kester (1988), and in broth cultures, both intracellular and extracellular pigment was found. Intracellular pigment is located in the cell membrane. Extracellular pigment is water soluble and shows a blue shift when compared to solvent-extracted pigment. It was suggested that extracellular pigment is possibly non-covalently bound to a small protein.

J. lividum produces 2,5-diketogluconate (Bernaerts and De Ley, 1971) via 2-ketogluconate.

Kwok et al. (1987) described a *J. lividum* strain as a bacterial antagonist inducing suppression of *Rhizoctonia* damping-off in container media amended with composted hardwood tree bark.

Strains from food produce sometimes extracellular metallo-proteinases (Etherington et al., 1976; Dainty et al., 1978) which could play a role in tenderizing meat.

A *J. lividum* strain was used to study the effects of the insecticide acephate on bacterial growth and nutrient uptake (Williams and Albright, 1984).

Acknowledgments. We are indebted to the National Fund for Scientific Research (Belgium) and the Fund for Medical Scientific Research (Belgium) for research and personnel grants.

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Prokaryotes (2006) 5:747–750
DOI: 10.1007/0-387-30745-1_33

CHAPTER 3.2.9

The Genera *Phyllobacterium* and *Ochrobactrum*

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The study of leaf nodulation in several tropical plant families dates back to the beginning of the 20th century. Various genera of bacteria, e.g., *Phyllobacterium*, were thought to be associated with leaf nodules (see below). *Phyllobacterium* was described by Knösel (1962) but the genus only found wide taxonomic recognition with the publication of *Bergey's Manual of Systematic Bacteriology* (Knösel, 1984). DNA-rRNA hybridizations revealed a close relationship between *Phyllobacterium* and bacteria from the Centers for Disease Control (CDC) group Vd within the "alpha" subclass (rRNA superfamily IV) of the proteobacteria together with those throughout of the genera *Brucella*, *Rhizobium*, *Mycoplana*, and *Agrobacterium* (De Ley et al., 1987; Stackebrandt et al., 1988). Holmes et al. 1988 proposed the name *Ochrobactrum anthropi* for CDC group Vd. They also demonstrated that *Phyllobacterium* showed closest phenotypic similarity to *Ochrobactrum anthropi*. In the present chapter, the genera *Phyllobacterium* and *Ochrobactrum* are treated together because of their close phylogenetic relatedness. Nevertheless, the ecological niches from which these bacteria have been isolated are quite different: *Phyllobacterium* strains occur in leaf nodules and in the rhizosphere, whereas *Ochrobactrum* strains have been isolated almost exclusively from human clinical material. In clinical microbiology, *Ochrobactrum*, more recently described as *Achromobacter* group Vd until formally named, was usually treated together with *Achromobacter xylosoxidans* (more recently known as *Alcaligenes xylosoxidans* subsp. *xylosoxidans*) (Gilardi, 1978; Rubin et al., 1985). It has now become apparent that *Ochrobactrum* and *Phyllobacterium* are not related phylogenetically to *Achromobacter xylosoxidans*, which belongs to the *Alcaligenaceae* in the "beta" subclass (rRNA superfamily III) of the proteobacteria (De Ley et al., 1986).

Habitats

The Occurrence of *Phyllobacterium* in Leaf Nodules

Plants of over 400 species of the families Rubiaceae and Myrsinaceae are reported to produce leaf nodules. Short-lived bacterial colonies become established intercellularly and die before full leaf expansion (Lersten and Horner, 1976). Bacterial leaf nodules were defined by Horner and Lersten (1972) as:

Internal cavities in the leaf lamina, open to the exterior by way of stomatal pores only in early stages of nodule development. The bacteria inhabiting such nodules are part of a population maintained by the host plant in the vegetative and floral buds and passed to the succeeding generations through the seeds.

This definition rules out all casual bacteria-plant associations.

Despite more than a century of research on leaf nodulation (for a review, see Lersten and Horner, 1976) it is not yet clear which bacteria are involved and how they participate in the nodulation process. In the past, the bacterial symbionts in the nodules were recognized as belonging to the genera *Chromobacterium*, *Xanthomonas*, *Klebsiella* and *Phyllobacterium* (Horner and Lersten, 1972). Knösel (1962) isolated and described *Phyllobacterium* and stated that it is able to induce characteristic nodules on leaves (Knösel, 1984), basing his conclusions on earlier investigations by Miehe (1911), von Faber (1912), and de Jongh (1938). However, it is not clear whether the bacteria previously studied by these workers are identical to those described by Knösel. There is no proof for the leaf nodulation capacity of *Phyllobacterium*.

The Occurrence of *Phyllobacterium* in the Rhizosphere

Pseudomonas fluorescens and *Phyllobacterium* were found to be the two most frequently occurring bacteria on root surfaces during a large-scale assessment of the rhizobacterial communities of young sugar beet plants (between the second and 10th leaf stage) (Lambert et al., 1990). These bacteria were found in 198 out of 1,100 plants investigated in Belgium and Spain at densities up to 2×10^8 per gram of root. This was the first record of the occurrence of *Phyllobacterium* in the rhizosphere. Extensive analyses of the microflora from the rhizoplane of other crop plants have not revealed the presence of *Phyllobacterium*.

This chapter was taken unchanged from the second edition.

Occurrence of *Ochrobactrum* (CDC group Vd) in Clinical Samples and the Environment

Ochrobactrum anthropi strains have been isolated predominantly from human blood, urogenital tracts, urine, respiratory tracts, ears, and wounds (Tatum et al., 1974; Holmes et al., 1988). Only a few strains were isolated from feces, spinal fluid, eye, hospital apparatus, or the environment (arsenical cattle-dipping fluid, soil, sewage, and from thin-layer Sephadex plates) (Holmes et al., 1988). The clinical significance of *O. anthropi* remains largely unknown; strains of this species are considered to be opportunistic pathogens. One strain has been reported in association with a pancreatic abscess (Appelbaum and Campbell, 1980) and another has been associated with a puncture wound leading to osteochondritis of a foot (Barson et al., 1987).

Isolation

Isolation of *Phyllobacterium* from the Sugar Beet Rhizosphere (Lambert et al., 1990)

> The entire root system of a plant is carefully washed to remove adhering soil, then vigorously shaken for 15 min using a flask shaker in a phosphate buffered saline solution containing 0.025% Tween 20. Serial dilutions of the resulting suspensions are plated on 10% trypticase soy broth plus 2% agar (TSBA). After incubation at 28°C for 2 days, *Phyllobacterium* colonies are beige-colored, large (±5 mm), and convex with an entire edge. They are very mucoid and glistening. Several subcultures are necessary in order to obtain pure isolates. *Phyllobacterium* readily grows on other nonselective media for aerobic, heterotrophic bacteria (nutrient agar, trypticase soy agar, Luria-Bertani agar, etc.).

Isolation of *Phyllobacterium* from Leaf Nodules (Knösel, 1984)

> Washed leaf pieces containing nodules are macerated by rubbing and placed in saline. After shaking, serial dilutions of the suspension are plated onto carrot juice agar containing yeast extract. After incubation at 28°C, a variety of pigmented and nonpigmented colonies develops. Typical colonies are transferred into liquid carrot juice medium which is then used to inoculate the tests used for identification. The cultures should be observed after 24 to 48 h to confirm the presence of star clusters.

Isolation of *Ochrobactrum anthropi* from Clinical Material

> Samples from human blood, urine and urogenital tract, sputum, wound, throat, stool, rectum, and other material are plated on blood agar or on MacConkey agar (Rubin et al., 1985; Holmes et al., 1988). Two colony types were recognized on blood agar at 35°C after 48 h: 1) an extremely mucoid type, 0.1 to 4 mm in diameter, opaque and gray-white, showing beta-hemolysis; and 2) a pinpoint type, opaque and semiglossy, gray or white, showing beta-hemolysis after prolonged incubation (Chester and Cooper, 1979). On MacConkey agar after continued incubation, colonies from 3 to 5 mm diameter were formed, opaque and pink to purple. Half the strains produced colonies with such notably gummy consistency that it was difficult to remove them from the agar surface (Chester and Cooper, 1979). On MacConkey agar, *Ochrobactrum* can be easily overlooked due to the small size of the colonies after overnight incubation (Chester and Cooper, 1979).

Preservation of Cultures

Well-grown cultures may be kept at 4°C for 2 to 3 months on nutrient agar slants in screw-capped bottles. Isolates can be stored freeze-dried or as a suspension in 25% glycerol at –70°C.

Identification

Phyllobacterium and *Ochrobactrum* are both Gram-negative, strictly aerobic bacteria with a respiratory type of metabolism. They show oxidase and catalase activity and both grow on nutrient agar. Indole is not produced. They oxidize glucose and xylose but not lactose (Clark et al., 1984; G. L. Gilardi, personal communication, Lambert et al., 1990). They do not produce extracellular enzymes for the hydrolysis of Tween 80, DNA, gelatin, starch, or casein (Gilardi, 1978). The API 20NE system will identify them as *Achromobacter* sp., which is still a "dumping ground" (Kersters and De Ley, 1984) for various aerobic peritrichously flagellated rods. Recent work on *Phyllobacterium* and *Ochrobactrum* has led to the differentiation scheme given in Table 1. An extensive description of *Phyllobacterium* and *Ochrobactrum* can be found in Lambert et al. (1990) and Holmes et al. (1988), respectively. A comment should be made on the flagellar arrangement of strains of both genera. *Phyllobacterium* and *Ochrobactrum* strains both show 1 to 3 polar, subpolar, or

Table 1. Features that differentiate *Phyllobacterium* and *Ochrobactrum* (CDC group Vd).

Property	*Phyllobacterium*[c]	*Ochrobactrum*[d]
Utilization[a] of:		
L-Citrulline	–	+
Glutarate	–	+
Erythritol	–	+
Glycine	–	+
L-Tryptophan	+	–
Hydrolysis[b] of paranitrophenol-α-maltoside	+	–
Hydrolysis[b] of paranitrophenol-α-xylopyranoside	+	–

Using API 50CH and 50AO, 50AA strips (API System, La Balme-les-Grottes, Montalieu-Vercieu, France).
Using API ZYM strips.
15 strains total. From Lambert et al. (1990).
56 strains total. From Holmes et al. (1988).

lateral flagella (Lambert et al., 1990). According to Holmes et al. 1988 and Gilardi (1978) *Ochrobactrum* strains are characterized by peritrichous flagella, but other workers have found only polar, subpolar, or lateral flagella but no definitely peritrichous flagella (Clark et al., 1984; Chester and Cooper, 1979).

An unambigous identification of *Phyllobacterium* or *Ochrobactrum* is possible by application of carbon assimilation tests (API 50CH, 50AO, or 50AA) or various conventional tests (Tatum et al., 1974; Rubin et al., 1985; Holmes et al., 1988; Lambert et al., 1990) or by protein electrophoretic fingerprinting. Variability in Gram reaction, the presence of curved cells with swollen-ends, and the formation of extremely small colonies after overnight incubation, which develop into mucoid colonies upon continued incubation, may lead to incorrect identification as *Corynebacterium* or *Klebsiella* (Chester and Cooper, 1979).

Knösel (1984) used the criterium of nitrate reduction to differentiate *Phyllobacterium myrsinacearum* (+) from *P. rubiacearum* (–). Lambert et al. (1990), however, found that *P. rubiacearum* LMG $1t1^T$ reduced nitrates. Using the API 2ONE system, they found that all *Phyllobacterium* strains showed a slight NO_3^- reduction. These observations cast serious doubt on the usefulness of this criterion for species differentiation within the genus *Phyllobacterium*. All *Ochrobactrum* strains are capable of nitrate reduction (Gilardi, 1978; Clark et al., 1984; Holmes et al., 1988).

Physiological Properties

Cellular fatty acids of *Ochrobactrum anthropi* (Dees and Moss, 1978) as well as antimicrobial susceptibilities have been determined (von Graevenitz, 1985; Gilardi, 1989). Growth of *Phyllobacterium* strains was inhibited by doxycycline, novobiocin, framycetin, and tetracycline (Lambert et al., 1990). The most intriguing question that remains unanswered is whether *Phyllobacterium* has a real symbiotic cyclical relationship with its hosts. Reinfection studies did not allow unambiguous conclusions to be made. It has also been suggested that leaf nodule bacteria produce plant growth hormones, particularly cytokinins, which are necessary for the normal functioning of the plant (Fletcher and Rhodes-Roberts, 1976; reference is not an exact matchRodrigues-Pereira et al., 1972). Other authors claimed that leaf nodule bacteria can fix nitrogen, but this was contested by Van Hove (1976) and Lersten and Horner (1976). The lack of ability to fix nitrogen is also indicated by the absence of the *nif* HDK-like genes (Lambert et al., 1990), which occur in all the classical nitrogen-fixing bacteria.

Phyllobacterium is able to interact with plant tissues, as demonstrated by the tumor induction by Ti-plasmid-carrying *Phyllobacterium* strains on *Kalanchoe* plants (Lambert et al., 1990). Tumor induction involves the attachment of bacterial cell wall sites to plant cell wall sites prior to the induction of T-DNA transfer. The chromosomal genes *chv*A, *chv*B, *exo*C, and *att* are the only ones found to be involved in attachment but could not be detected in *Phyllobacterium* strains, suggesting that other genes are present with similar functions. There are no data indicating that *Phyllobacterium* is pathogenic or deleterious to plants.

A striking feature, common to both *Phyllobacterium* and *Ochrobactrum*, is nutritional versatility, which makes their rapid growth and proliferation possible in rich environments such as the root surface and clinical sites.

A number of *Phyllobacterium* isolates showed antifungal and antibacterial activities (Lambert et al., 1990).

The oxidation of lactose to 3-ketolactose is a unique feature of many *Agrobacterium* strains (Kersters and De Ley, 1984) and is negative for all *Phyllobacterium* and *Ochrobactrum* strains.

Applications

No applications of the genera *Phyllobacterium* or *Ochrobactrum* are yet known. However, the fact that *Phyllobacterium* is a predominant bacterium on the root surface of sugar beet plants, its capacity to "communicate" with plant tissues, and its non-pathogenic status make this bacterium an interesting new candidate for use in plant growth promotion or biological control of soil-borne diseases. Indeed, some of the sugar beet isolates exert a broad-spectrum antifungal activity against major phytopathogenic fungi.

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Prokaryotes (2006) 5:751–757
DOI: 10.1007/0-387-30745-1_34

CHAPTER 3.2.10

The Genus *Derxia*

JAN HENDRIK BECKING

In the genus *Derxia* only a single species is recognized, *D. gummosa*. This species was originally described by Jensen et al. (1960) from an isolate obtained from Indian soil, but was later found to be widely distributed in soils of South America (Brazil), Southern Africa, Indonesia, and China, but not in temperate regions.

Although Roy and Sen (1962) described a second species of *Derxia* (*D. indica* sp. nov.) from a sample of partially retted jute plant (*Corchorus olitorius* L.) from Uttar Pradesh, India, they did not present cultural and physiological data showing that their isolate was sufficiently different from *Derxia gummosa* to warrant the designation of a new species.

The genus is named for H. G. Derx (1894–1953), a Dutch microbiologist.

Using rRNA cistron similarity as a criterion for genetic relatedness, De Smedt et al. (1980) found that *D. gummosa* is quite different from species of the genera *Azotobacter*, *Azomonas,* and *Beijerinckia*. From hybridization studies with DNAs from three *D. gummosa* strains using the ^{14}C-labeled rRNAs from a great variety of other organisms, it was quite obvious that the *Derxia* rRNA cistrons most closely resemble those of *Pseudomonas acidovorans*, *P. solanacearum*, *Chromobacterium violaceum*, *Janthinobacterium lividum*, and *Alcaligenes faecalis.* These taxa, together with a few others, constitute the third rRNA superfamily of De Ley (see The Proteobacteria: Ribosomal RNA Cistron Similarities and Bacterial Taxonomy in the second edition). Members of these taxa have in common that they consist of Gram-negative rods, usually 1.0–4.0 µm in length and 0.5–1.0 µm in diameter, do not possess resting stages, have a GC content of the DNA of 57–72 mol%, are chemoheterotrophic, exhibit respiratory (oxidative) metabolism, and occur in soil and water habitats. The third rRNA superfamily of De Ley (De Smedt et al., 1980) is identical with the beta subclass of the Proteobacteria, according to the phylogenetic taxon nomenclature of Stackebrandt et al. 1988.

Based on numerical analysis of a large number of attributes, Thompson and Skerman (1979) showed fusion of their five *Derxia* strains tested with "*Azotomonas*" ("*Azotomonas insolita*" Stapp, 1940, sometimes termed "*Pseudomonas insolita*" [Stapp] Brisou, 1961) at group 1296 in their dendrograms of hierarchical interrelations (see also Krieg and Holt, 1984). This indicates that *Derxia* is not related to the genera *Azotobacter* or *Azomonas* and thus confirms the conclusions of De Smedt et al. (1980).

Habitats

Derxia is primarily a soil organism occurring in tropical soils. There is no plausible explanation for why this organism is exclusively found in tropical environments and is apparently absent from temperate soils. It has not yet been recorded from aquatic habitats, although it is likely that it also occurs there. Such habitats in these regions have, however, not yet been thoroughly investigated using conditions selective for this organism.

Isolation

The original isolate was obtained from a West Bengal (Adisaptagram, India) soil of pH 6.5 by the sieved-soil plate method (see "General Enrichment Procedures" in The Family Azotobacteraceae in the second edition) using plates with a nitrogen-free, mineral mannitol (2%) agar, incubated at room temperature (about 25°C). Yellowish colonies appeared after 5 days around some of the soil particles; these colonies gradually increased in size and finally assumed a rust-brown color. Isolation and further purification were performed by repeated transfer and plating on the same nitrogen-free mannitol agar.

Campêlo and Döbereiner (1970) obtained this organism from Brazilian soils also with the sieved-soil plate method, utilizing a nitrogen-free, mineral starch (2%) medium. This medium was a modification of a medium used by Lipman (1903), supplemented with $NaHCO_3$ (0.1 or 0.01%).

Isolation Medium for *Derxia gummosa* (Campêlo and Döbereiner, 1970; Modified from Lipman, 1903)

Distilled water	1 liter
Starch	20.0 g
K_2HPO_4	0.05 g
KH_2PO_4	0.15 g
$MgSO_4 \cdot 7H_2O$	0.20 g
$CaCl_2$	0.02 g
$NaHCO_3$	0.1 g
$FeCl_3$ (10%, aqueous solution)	1 drop
$Na_2MoO_4 \cdot 2H_2O$	0.002 g
Bromothymol blue (0.5%, ethanol solution)	5 ml
Agar	20.0 g

In a survey of 100 samples of Brazilian soil from the states of São Paulo, Rio de Janeiro,

This chapter was taken unchanged from the second edition.

Pernambuco, and Para, Campelo and Döbereiner (1970) found that 36% of the soils tested were positive for this organism. Soil humidity seems to favor the presence of *Derxia*, since 77% of the flooded soils, 36% of the humid soils, and only 13% of the drier soils tested contained this organism. In a comparative study with agar plates seeded with soil or with root pieces of various plants (mostly Gramineae) of the same locality, *Derxia* appeared more frequently on the plates inoculated with roots (33%) compared to those inoculated with soil (26%). *D. gummosa* was found to be present in soils ranging in pH from 4.5 to 6.5, but was observed to be most frequent in soils of pH 5.1–5.5.

This species was isolated by the author from various soils of Indonesia, China, Brazil, tropical Africa, and South Africa with the sieved-soil plate method using nitrogen-free, mineral, glucose (1–2%) agar (J. H. Becking, unpublished observations).

Derxia strains are relatively acid tolerant and were therefore also isolated by the author from cultures designed to enrich for *Beijerinckia* strains using a liquid, acidic, nitrogen-free, glucose medium. This liquid medium has the following composition:

Enrichment Medium for *Derxia gummosa* (J. H. Becking, unpublished observations)

Distilled water	1 liter
Glucose	20.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g

Adjust to pH 5.0.

The medium is dispensed into petri dishes so as to form a thin layer 2–3 mm deep in order to maintain aerobic conditions. The liquid medium is inoculated with soil particles and incubation is at 25° or 30°C.

In the sieved-soil plate method mentioned for the isolation, soil particles are evenly distributed over the surface of nitrogen-free mineral agar containing mannitol (Jensen et al., 1960), glucose (J. H. Becking, unpublished observations), or starch (Campelo and Döbereiner, 1970). It should be noted that mannitol is not utilized by all *Derxia gummosa* strains. Of the six strains tested by Thompson and Skerman (1979), three did not use mannitol. Moreover, some strains, including the type strain, produced only very scant growth on starch. All strains did, however, utilize glucose as sole source of carbon.

Therefore for general practice of the sieved-soil plate method, the following glucose mineral agar medium is recommended:

Nitrogen-free, Mineral Glucose Agar for the Isolation of *Derxia gummosa* with the Sieved-Soil Plate Method (g/liter of distilled water):

Glucose	20.0
K_2HPO_4	0.8
KH_2PO_4	0.2
$MgSO_4 \cdot 7H_2O$	0.5
$FeCl_3 \cdot 6H_2O$	0.025 (or 0.05)
$Na_2MoO_4 \cdot 2H_2O$	0.005
$CaCl_2$	0.05
Agar	15.0

Adjust to pH 6.9.

On the agar plates seeded with the soil particles, yellowish colonies develop around the soil particles. These colonies eventually become larger and acquire a rust-brown color. Isolates should be further purified by repeated streaking on new agar plates. For agar plates obtained from liquid enrichment cultures, one should look for slimy, yellowish colonies with a rather plicated surface (see Fig. 5).

Cultivation

For routine maintenance, the following nitrogen-free, mineral glucose medium can be used (g/liter of distilled water):

Nitrogen-free, Mineral Glucose Agar Medium

Glucose	10.0 (or 20.0)
K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.25 (or 0.2)
NaCl	0.25
$FeSO_4 \cdot 7H_2O$	0.1
$CaCl_2$ or $CaCO_3$	0.1
$Na_2MoO_4 \cdot 2H_2O$	0.005
Agar	15.0

Adjust to pH 6.9.

Jensen et al. (1960) used a nitrogen-free, mineral mannitol agar of the following composition (g/liter of distilled water):

Nitrogen-free, Mineral Mannitol Agar Medium

Mannitol	10.0
K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2$	0.1
$CaCO_3$	5.0
$Na_2WO_4 \cdot 2H_2O$	0.0005
$FeCl_3$ and $Na_2MoO_4 \cdot 2H_2O$	trace
Agar	15.0.

The medium recommended by Campelo and Döbereiner (1970) contained starch as carbon source (g/liter of distilled water):

Nitrogen-free, Mineral Starch Agar Medium

Starch	20.0 g
K_2HPO_4	0.05 g
KH_2PO_4	0.15 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2$	0.02 g
$NaHCO_3$	0.1 g
$FeCl_3$ solution (10%, aqueous solution)	1 drop

$Na_2MoO_4 \cdot 2H_2O$	0.002 g
Bromthymol blue solution (0.5%, ethanol solution)	5.0 ml
Agar	20.0.

As already stated before, not all *Derxia gummosa* strains can utilize mannitol as sole source of carbon, while with starch as the carbon source, some strains only produce scant growth.

Preservation of Cultures

The maintenance procedures of *Derxia gummosa* are the same as those described for the genus *Beijerinckia* (see The Genus *Beijerinckia* in Volume 5).

Identification

Derxia can be distinguished from other genera of N_2-fixing bacteria by its very slimy (gummy) growth, both on agar plates and in liquid media, combined with the very pleomorphic appearance of the cells (Figs. 1 to 2, 3, 4) depending on age and type of medium. On agar media *Azotobacter* and *Azomonas* species never produce such slimy colonies as those of *Derxia* (Fig. 5). Confusion with *Beijerinckia* is unlikely, since *Beijerinckia* cells usually show very characteristic cells with only two polar lipoid bodies, whereas *Derxia* cells contain numerous lipoid bodies. Moreover, in contrast to *Beijerinckia* colonies, the colonies of *Derxia* acquire on aging a typical dark rust-brown or mahogany-brown color. In addition, in contrast to *Azotobacter*, *Azomonas* and *Beijerinckia*, *Derxia* cells are catalase negative.

In contrast to the spiral-shaped or vibrioid cells of *Azospirillum* (see The Genera Azospir-

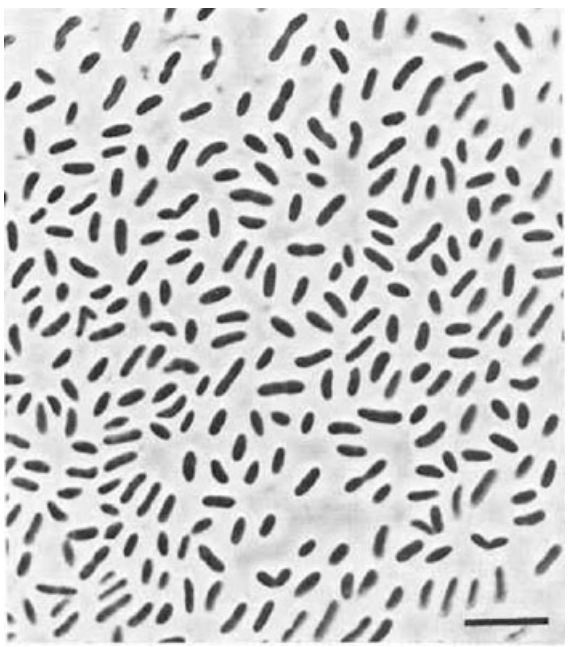

Fig. 1. Seven-day-old cells of *Derxia gummosa* on nitrogen-free agar containing 2% glucose. Phase contrast microscopy. Bar = 10 µm.

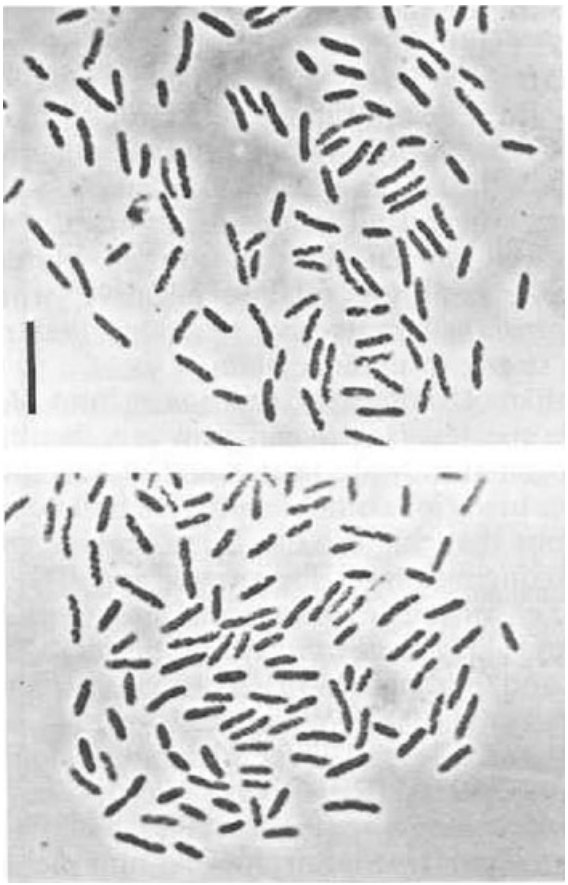

Fig. 2. Three-week-old cells of *Derxia gummosa* on nitrogen-free glucose agar, showing shrinkage of the cells. Phase contrast microscopy. Bar = 10 µm.

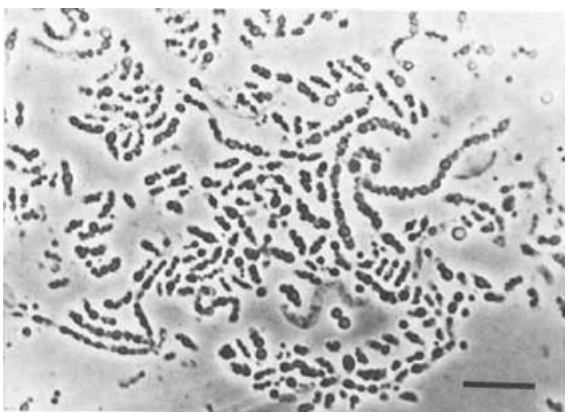

Fig. 3. Ten-day-old cells of *Derxia gummosa* on peptone agar with 2% glucose. Phase contrast microscopy. Bar = 10 µm.

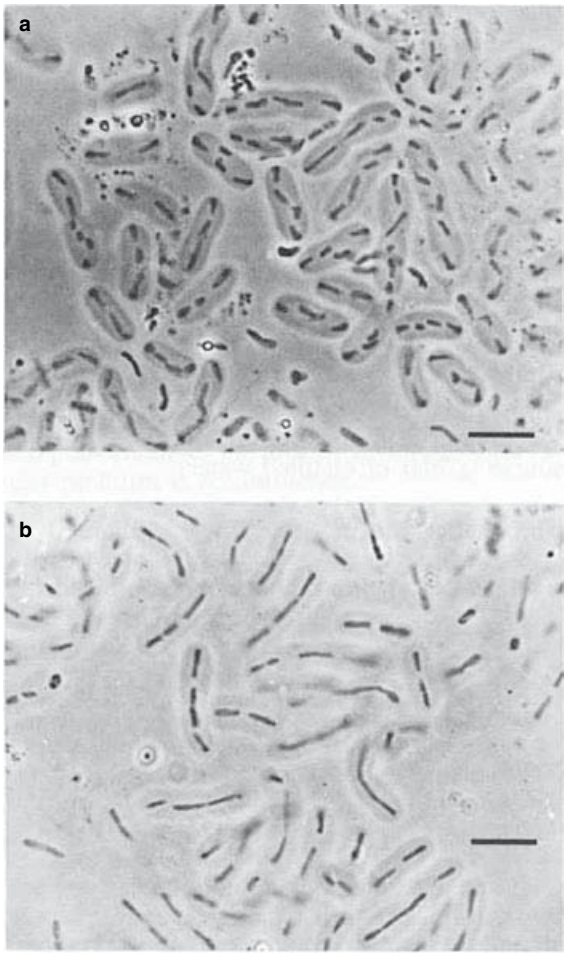

Fig. 4a. Three-month-old cells of *Derxia gummosa* on nitrogen-free glucose agar. The cells show shrinkage and are enclosed by a thick slime envelope. Phase contrast microscopy. Bar = 10 μm. Fig. 4b. More pronounced shrinkage of cells of a four-month-old *Derxia gummosa* culture on nitrogen-free glucose agar. Note the thick slime envelopes enclosing the rows of cells. Bar = 10 μm.

illum and Herbaspirillum in the second edition), *Derxia* cells are mainly straight rods and the lipoid bodies are more numerous. *Derxia* colonies are far more slimy than those of *Azospirillum*, and the mahogany-brown color they eventually acquire is in contrast to the pink or whitish color of *Azospirillum* colonies. Moreover, there are some minor physiological differences. *Derxia* strains can exhibit nitrogen-dependent growth on glucose, whereas only some strains of *Azospirillum* (i.e., only those belonging to the species *A. lipoferum*) can do so. On the other hand, *Derxia* strains generally do not grow well on malate, whereas all strains of *Azospirillum* can grow well on this carbon source. Moreover, *Derxia* strains are catalase negative, whereas *Azospirillum* strains give reactions that range from strong to undetectable.

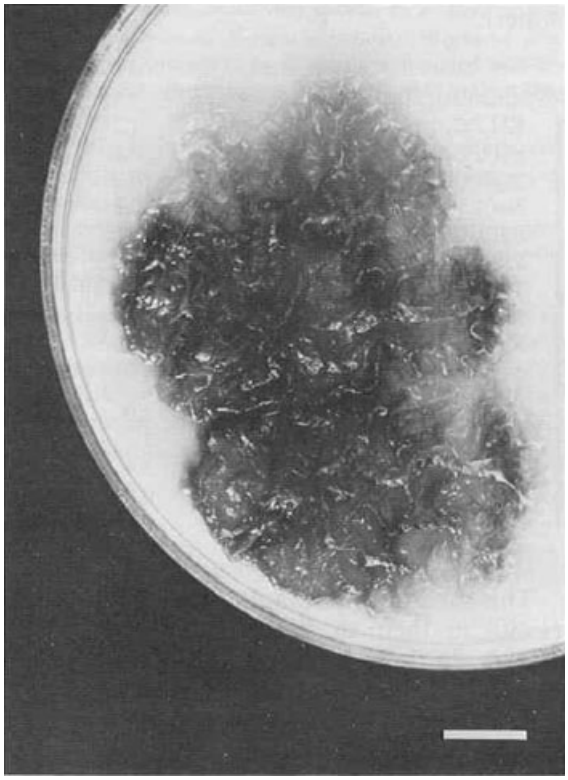

Fig. 5. Colony of *Derxia gummosa* on nitrogen-free glucose agar with calcium carbonate showing the coarse and wrinkled colony surface. Bar = 1 cm.

Unlike *Azotobacter*, *Azomonas*, and *Beijerinckia* species, *Derxia* species can grow as a facultative hydrogen autotroph. In this ability, they show affinities to various other facultative hydrogen autotrophs that can also use H_2 to provide energy and reducing power for growth and CO_2 fixation, i.e., various *Pseudomonas* and *Alcaligenes* species ("*Hydrogenomonas*" species) (Buchanan and Gibbons, 1974), *Xanthobacter* species (Wiegel et al., 1978; Wiegel and Schlegel, 1984; Malik and Claus, 1979), nitrate-reducing *Paracoccus* species (Rittenberg, 1969), *Bradyrhizobium japonicum* (Hanus et al., 1979), and *Azospirillum lipoferum* (Malik and Schlegel, 1981; Sampaio et al., 1981) (see also The Mesophilic Hydrogen-Oxidizing (Knallgas) Bacteria in the second edition). For further details on facultative hydrogen autotrophy of *Derxia*, see section "Physiological and Biochemical Properties" in this Chapter.

Species Description

Derxia gummosa Jensen, Petersen, De and Bhattacharya, 1960.

Cells are Gram-negative, rod-shaped with rounded ends, 3.0–6.0 μm in length, and 1.0–

1.2 µm wide, occurring singly or in short chains. Cells are rather pleomorphic, depending on age and the medium. In aging cultures, cells often remain together, forming long filaments of sometimes locally swollen or distorted cells. Some cells may assume enormous sizes (up to 30 µm). Young cells have a homogeneous cytoplasm; older cells typically show large refractile bodies throughout the whole cell. Resting stages are not known. Cells are motile by a short polar flagellum; motile cells are numerous in liquid glucose media containing combined nitrogen, but are rare on nitrogen-deficient solid media. The organism is aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Molecular nitrogen is fixed under aerobic conditions and also under decreased oxygen pressures (microaerophilic conditions). Optimum temperature is 25–35°C; growth is slow at 15°C, feeble at 40°C; no growth occurs at 50°C. Growth occurs from pH 5.5 to about 9.0; no growth occurs at pH 4.4. Liquid cultures turn into a gelatinous mass, but growth near the surface is more luxuriant and forms a thick, tough pellicle. Colonies on agar media are at first slimy and semitransparent, later massive and opaque, highly raised with a wrinkled surface. Older colonies develop a rust-brown to dark mahogany-brown color. They are catalase negative. A wide range of sugars, alcohols, and organic acids are oxidized, mostly to CO_2, but a small amount of acid, probably acetic acid, is produced during growth in an alkaline medium. They can grow as a facultative hydrogen autotroph. The GC content of the DNA in three strains of *Derxia gummosa* ranges from 69.2–72.6 mol% (T_m) (De Smedt et al., 1980). In the type strain, it is 70.4 ± 1.7 mol% (T_m) (De Ley and Park, 1966).

The appearance of cells from young cultures is depicted in Fig. 1. Older cells on sugar-rich media contain large refractile bodies. Especially on glucose-peptone agar, very elongated cells are produced containing many refractile bodies (Fig. 3). The refractile material is probably poly-β-hydroxybutyrate, since it stains with Sudan III and Sudan black, but some vacuoles which do not stain may also be involved. On nitrogen-free glucose agar, older cells undergo shrinkage (Fig. 2) and are finally enclosed by a slime envelope (Fig. 4).

Motile cells may become numerous in liquid glucose media containing ammonia or glutamate as the nitrogen source. The cells usually have a single polar flagellum, but some may have a flagellum at each pole. According to Thompson and Skerman (1979), the single polar flagellum is less than a full wave and is less than 3 µm in length.

Growth in liquid media usually starts as a ring at the glass-liquid interface and develops into a thick, wrinkled, tough pellicle. Shallow layers of medium change into a firm gelatinous mass after a couple of weeks. The color gradually becomes a dark red-brown.

Growth on nitrogen-deficient agar media begins as thin, whitish, or semitransparent scattered colonies. Later, more massive, highly raised, or dome-shaped colonies emerge which rapidly assume a diameter of 1 cm or more (giant colonies) (Fig. 5). These colonies are very reminiscent of those of *Beijerinckia* species. Colonies are at first whitish or dull yellow with a smooth surface, but the surface soon becomes coarse and wrinkled, and the color deepens to a dark mahogany brown. The slime of these colonies is very tenacious and gumlike, but in the other developmental stages, it is softer and smeary.

Physiological and Biochemical Properties

In the original description, Jensen et al. 1960 mentioned the appearance of *Derxia gummosa* on nitrogen-free, mineral glucose agar as a few "massive" colonies among many "thin," whitish colonies. The type of colony used as inoculum affected neither the relative numbers nor the distribution of the two colony types. They reported further that the "massive" colonies fixed nitrogen, whereas the "thin" colonies did not. Tchan and Jensen (1963) observed that the formation of "massive" colonies was stimulated by the addition of small quantities of combined nitrogen to the medium and that dinitrogen fixation could not be initiated in a medium completely free of combined nitrogen.

Hill and Postgate (1969) found that, with a small quantity of combined nitrogen, batch cultures of *Derxia gummosa* in otherwise nitrogen-free media could be established with agitation in air. However, this supplement was unnecessary if the cultures were left stagnant or if the atmospheric oxygen concentration was lowered to 0.1 atm or less. Once established, continuous cultures could be maintained in air, provided vigorous stirring was avoided. Hill (1971) demonstrated in more detail that *D. gummosa* is very sensitive to oxygen and that the presence of oxygen markedly affects its colony morphology. At an oxygen concentration of less than 0.2 atm, only the "massive"-colony type is produced on nitrogen-free, mineral agar media; the "thin"-colony type which predominates in air was absent. Only the "massive" colonies reduced acetylene. Apparently, the copious production of a viscous and tenacious slime inhibits the penetration of oxygen and therefore stimulates nitrogen fixation. The colonial dimorphism on agar probably arises because only when the local oxygen concentrations on the agar surface are

depressed can dinitrogen fixation and subsequent growth to the "massive" colony type take place.

The efficiency of nitrogen fixation by *D. gummosa* varies between 9 and 25 mg N/g of glucose consumed, but in most strains it is distinctly lower than in *Azotobacter* or *Beijerinckia*.

Growth on nitrogen-deficient media or under nitrogen-fixing conditions is stimulated by small amounts of combined nitrogen (nitrate, ammonia), particularly at the start (Tchan and Jensen, 1963). There is no requirement for amino acids under nitrogen-fixing conditions. However, small amounts of yeast extract are stimulatory for growth under nitrogen-fixing conditions (J. H. Becking, unpublished observations), and although there is no apparent requirement for vitamins or growth factors, it is possible that small amounts of biotin are stimulatory for growth of some strains (J. H. Becking, unpublished observations).

As sole source of carbon, growth is good to excellent on glucose, fructose, ethanol, glycerol, mannitol (only utilized by some strains), and sorbitol; growth on lactate is scant. No growth or only a trace of growth occurs on lactose, galactose, maltose, sucrose, formate, acetate, propionate, pyruvate, succinate, malate, fumarate, dulcitol, and sometimes starch. Butyrate, citrate, benzoate, and xylose suppress growth. Growth on methane or methanol as the sole carbon source has also been demonstrated for *D. gummosa* (Sampaio et al., 1981).

Growth with combined nitrogen sources is much faster than with molecular nitrogen and is completely uniform, in contrast to the uneven growth on nitrogen-free agar. Colonies change from a pale yellow through rust-brown to almost black (darkest if nitrate is present), and sometimes a light brown, water-soluble pigment is produced. Growth with glutamic acid, ammonium acetate, alanine, sodium nitrate, and urea decreases from abundant to good in approximately the same sequence. Aspartic acid, asparagine, and peptone give a much slower growth, which is uneven and mostly confined to scattered colonies. Glycine seems to be toxic.

Nitrate is not reduced to nitrite or N_2 in a glucose-nitrate medium. Indole is not produced from tryptophan.

Trace elements, particularly molybdenum, are required for dinitrogen fixation, but vanadium cannot replace molybdenum (Jensen et al., 1960). Thus, *Derxia* apparently does not have an alternative vanadium-activated nitrogenase.

D. gummosa has been shown to grow as a hydrogen autotroph in an atmosphere containing $H_2 + CO_2 + O_2$, with either N_2 or NH_4^+ as the nitrogen source (Pedrosa et al., 1980). Indeed, it appears to grow nearly as well autotrophically as it does heterotrophically. Ribulose-1,5-bisphosphate carboxylase activity, which mediates CO_2 fixation, occurs in autotrophically-grown cells but not in cells grown heterotrophically.

A medium for testing the autotrophy has the following composition (g/liter of distilled water):

Medium for Testing Autotrophy

KH_2PO_4	1.2
K_2HPO_4	0.8
$MgSO_4 \cdot 7H_2O$	0.2
NaCl	0.2
$CaCl_2 \cdot 2H_2O$	0.02
$FeSO_4 \cdot 7H_2O$	0.002
Trace element solution (see below)	2.0 ml
Biotin	10 µg
Agar	15.0

Trace element solution (g/liter)

$Na_2MoO_4 \cdot 2H_2O$	1.0
$MnSO_4 \cdot H_2O$	1.75
H_3BO_3	1.4
$CuSO_4 \cdot 5H_2O$	0.04
$ZnSO_4 \cdot 7H_2O$	0.12

This medium was employed by Pedrosa et al. (1980) in their studies on the autotrophy of *Derxia*. In these studies, a very low concentration of potassium malate (0.1 g/liter) was sometimes added to the medium. It should be noted that most strains of *Derxia* (including the type strain) give only scant growth with malate. Thompson and Skerman (1979) reported no growth on DL-malate in all of the six strains that they tested.

Applications

No studies have yet been done on the occurrence and significance of this organism in the rhizosphere of crop plants. However, Campêlo and Döbereiner (1970) found *Derxia* more frequently on agar plates inoculated with roots (33% positive for *Derxia*) compared to those inoculated with soil (26%).

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Prokaryotes (2006) 5:758–777
DOI: 10.1007/0-387-30745-1_35

CHAPTER 3.2.11

The Genera *Leptothrix* and *Sphaerotilus*

STEFAN SPRING

Introduction

Representatives of the genera *Leptothrix* and *Sphaerotilus* were among the first microorganisms to be recognized in the environment and described in detail by scientists. The type species of the genus *Leptothrix*, *L. ochracea*, was already observed in the late eighteenth century and described by Roth (1797) under the synonym "*Conferva ochracea*." Later, Kützing (1843) proposed to place this species within the genus *Leptothrix*. Ten years earlier, the same author had published a description of the species *Sphaerotilus natans* (Kützing, 1833), which is today still known under this name. These early publications were probably evoked by the observation of ocherous deposits (clearly visible to the naked eye) in ponds or slowly running water. A microscopic examination of these suspicious aggregates led then to the discovery of filamentous microorganisms, which were obviously responsible for the deposition of iron or ferromanganese oxides in a slimy matrix, resulting in the typical color of the formed amorphous flocs or surface films.

Further investigations of the *Sphaerotilus-Leptothrix* group were induced by their potential economic importance. The massive growth of these microorganisms at industrial sites can lead to technological problems like clogging of water distribution systems or the bulking of activated sludge (Dondero, 1975). More recently, the significance of *Leptothrix* species in the corrosion of steel could be demonstrated (Olesen et al., 2000; Rao et al., 2000). On the other hand, a potential application of these microorganisms in the biological clearance of heavy metals from contaminated water supplies is being discussed (Nelson et al., 1999; Solisio et al., 2000).

Both genera, *Leptothrix* and *Sphaerotilus*, have been always considered as closely related because of the conformity of some suspicious morphologic traits (Mulder and Van Veen, 1963). Later, their close relationship was confirmed by phylogenetic and phenotypic data, thereby justifying their treatment as a group. Characteristic traits, which distinguish members of this group from other phylogenetically related species, are the capability to form tubular sheaths and the precipitation of copious amounts of oxidized iron or manganese.

Phylogeny and Taxonomy

Phylogeny and Related Genera

Members of the genera *Leptothrix* and *Sphaerotilus* comprise a phylogenetically coherent cluster within the β1-subgroup of the Proteobacteria. The similarity values among 16S rRNA gene sequences representing strains of the *Sphaerotilus-Leptothrix* group are in the range between 96.3 and 99.8% (Pellegrin et al., 1999). It has to be noted, however, that to date no 16S rRNA sequence of *L. ochracea* (the type species of the genus *Leptothrix*) is available. In addition, the physiology of this species is largely unknown, because it could not be isolated in pure culture. Hence, it cannot be excluded that the type species of *Leptothrix* is phylogenetically only distantly related to the other species of this genus.

Representatives of the genera *Aquabacterium*, *Ideonella*, *Rubrivivax*, *Roseateles* and two misclassified species, *[Alcaligenes] latus* and *[Pseudomonas] saccharophila*, are phylogenetically closely related to the *Sphaerotilus-Leptothrix* group. Together they form the *Rubrivivax* line of descent, which is tightly associated with the Comamonadaceae (Wen et al., 1999). The relative branching order of most species within the *Rubrivivax* group is difficult to determine, mainly because of the restricted number of varying positions available for the estimation of phylogenetic distance values. Depending on the method and database used for tree reconstruction, it may happen that representatives from other genera get intermixed with species from the *Sphaerotilus-Leptothrix* group. Therefore, it is difficult to decide if the capability of sheath-formation within this group evolved from a common ancestor, i.e., if it is a monophyletic trait. Despite the close phylogenetic relationship of members of the *Rubrivivax* branch, a differentiation is easily possible based on phenotypic

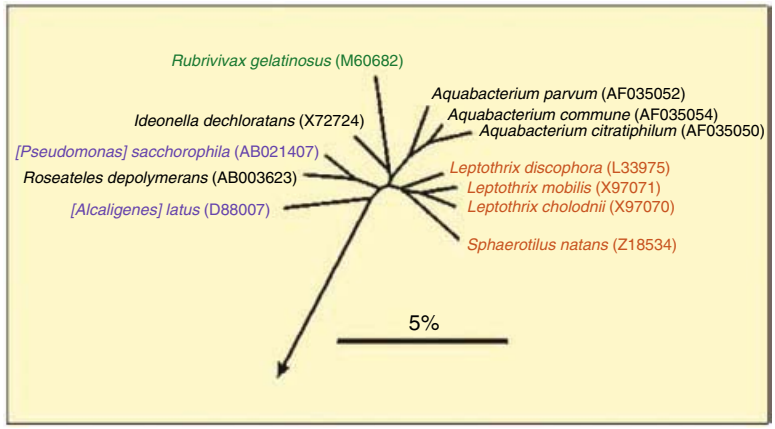

Fig. 1. Phylogenetic tree showing the position of members of the *Sphaerotilus-Leptothrix* group among representatives of the *Rubrivivax*-branch of the β-Proteobacteria. Sheath-forming species are labeled in red, photoautotrophic species in green, and facultatively chemolithoautotrophic species in blue. The GenBank/EMBL accession number for each sequence is shown in parentheses. With the exception of *Sphaerotilus natans* (DSM 565) and *Leptothrix cholodnii* (CCM 1827), sequences of type strains were used. This tree was reconstructed using the ARB program package (Ludwig and Strunk, 1997). It is derived from a distance matrix on a selection of 16S rRNA sequences using the neighbor-joining method of Saitou and Nei (1987). Phylogenetic distances were calculated as described by Felsenstein (1982). The sequence of *Escherichia coli* was used as an outgroup (not shown). The bar indicates 5% estimated sequence divergence.

characteristics. It is noteworthy that the genera in this tight assemblage represent three basically different metabolic types, namely photoautotrophs (*Rubrivivax*), facultative chemolithoautotrophs (*[Alcaligenes] latus* and *[Pseudomonas] saccharophila*) and respiratory chemoorganotrophs (e.g., *Ideonella* and *Sphaerotilus*). In Figure 1, a representative phylogenetic tree based on 16S rRNA gene sequence data has been selected which adequately reflects the phenotypic classification within this group.

Important phenotypic characteristics useful for distinguishing members of the genera *Leptothrix* and *Sphaerotilus* from other species belonging to the *Rubrivivax*-lineage are listed in Table 1.

Phenotypic Characteristics

Common Traits Some important phenotypic traits are shared by all members of the genera *Leptothrix* and *Sphaerotilus*: Cells are straight rods, Gram-negative and motile by flagella. Growth is filamentous under natural conditions, owing to the formation of tubular sheaths that surround single and linear chains of cells. Sheaths are produced by excretion of fibrillar polymeric substances that are crosslinked to form a mesh-like fabric closely fitting to the cells. In contrast to slimes or capsules, this matrix is not in intimate contact with the cells. In aquatic habitats rich in iron and manganese, sheaths get incrusted with ferric hydroxide (*Sphaerotilus*) or ferromanganese oxides (*Leptothrix*). Without these incrustations, they appear as very thin, almost transparent structures and are therefore not easily recognized by phase contrast microscopy. Occasionally, cells move out of the sheath or lyse and leave behind an empty space or gap within the filament, thereby facilitating the observation of the sheath. Treatment of slide preparations with ethanol may improve the visibility of sheaths under phase contrast.

Further conspicuous morphological features of members of this group include the formation of slime, holdfasts, and false branching of filaments. These traits are frequently recognized in the genus *Sphaerotilus*, but are less common among representatives of the genus *Leptothrix*. The sheaths of *S. natans* and several *Leptothrix* species are surrounded by a slime layer that may be involved in the accumulation of iron hydroxide and in the oxidation of Mn(II). Holdfasts are sticky, disc-like evaginations or blebs formed at one cell pole opposite to the flagellum by which organisms attach to walls of containers, submerged plants, stones and other surfaces. Holdfast formation can be easily observed in pure cultures of *Sphaerotilus natans* and *Leptothrix lopholea*, but is absent or variable in other cultured *Leptothrix* species. False branching is characteristic for *Sphaerotilus natans* and several *Leptothrix* strains. It develops if a cell attaches to an existing filament and forms a new filament, thereby forming a branch.

Metabolism is strictly aerobic, respiratory and chemoorganoheterotrophic. The possibility of an autotrophic or mixotrophic growth of these bacteria with ferrous iron as electron acceptor has been discussed for many years, but up to now no

Table 1. Differential characteristics of genera and misclassified species belonging to the *Rubrivivax* group of the β-Proteobacteria.

Characteristic	*[Alcaligenes] latus*	*[Pseudomonas] saccharophila*	*Roseateles*	*Rubrivivax*	*Ideonella*	*Aquabacterium*	*Leptothrix*[a]	*Sphaerotilus*
Number of species	1	1	1	1	1	3	5	1
Flagellation	Peritrichous	One polar	Several polar	One polar	Several, polar or subpolar	One polar	One polar; subpolar tuft	Subpolar tuft
Formation of sheaths	–	–	–	–	–	–	+	+
Carotenoid pigments	–	–	+	+	–	–	–	–
Photoautotrophic growth	–	–	–	+	–	–	–	–
Autotrophic growth with H_2	+	+	–	+	NR	NR	NR	NR
Anaerobic growth	–	–	–	+	+	+	–	–
Oxidation of Mn^{2+}	NR	NR	NR	NR	NR	–	+	–
G + C content (mol%)	69–71	69	66	70–72	68	65–66	68–71	69
Isolation source	Soil	Mud	River water	Mud	Activated sludge	Drinking water	Freshwater, sediment	Freshwater, activated sludge

Symbols: +, present in all species; –, absent in all species; and NR, not reported.
[a]Physiological data for *Leptothrix lopholea* and *L. ochracea* are not available.
From Kersters and De Ley (1984); Palleroni (1984); Mulder and Deinema (1992); Malmqvist et al. (1994); Spring et al. (1996); Kalmbach et al. (1999); and Suyama et al. (1999).

definite evidence could be presented which would support this assumption. Representatives of both genera, *Leptothrix* and *Sphaerotilus*, require vitamin B_{12} as an essential growth factor in mineral media. A number of *Leptothrix* strains have been found to require in addition thiamine and biotin as growth factors (Rouf and Stokes, 1964). In broth culture, a flocculent growth is typical. Poly-β-hydroxybutyrate is stored as reserve material. The major quinone type detected is ubiquinone Q8 (>90% of total quinones). The four major components of the cellular fatty acids are *cis*-9 hexadecanoic acid (*cis*-9 16:1), hexadecanoic acid (16:0), *cis*-9,11 octadecanoic acid (*cis*-9,11 18:1) and decanoic acid (12:0). In all strains tested, the hydroxylated fatty acid 3-hydroxydecanoic acid (3-OH 10:0) can be detected, however in some cases, only in low amounts (Kämpfer, 1998). The G +C content of DNA is similar in both genera and ranges between 68 and 71 mol%. In general, chemotaxonomic features characteristic for the *Sphaerotilus-Leptothrix* group are very similar in other organisms belonging to the *Rubrivivax* group and Comamonadaceae, so that a clear differentiation of species or even genera within this phylogenetic group is hardly possible based solely on chemotaxonomic traits.

Distinguishing Traits Albeit closely related, several phenotypic characteristics allow the differentiation of the genera *Leptothrix* and *Sphaerotilus*. The ability to oxidize soluble manganese (Mn^{2+}) compounds to solid manganic (Mn^{4+}) oxides is restricted to *Leptothrix* species. This trait has been originally used for differentiation because it can be easily observed in Mn^{2+}-containing media or habitats. Further distinguishing traits, however, were only revealed by a detailed taxonomic study of cultured strains of both genera. They include the storage of polysaccharides as reserve material only by *Sphaerotilus* strains, size of cells, structure of the sheath surface (*Leptothrix* sheaths show a rough surface when viewed by electron microscopy, in contrast to the sheaths of *Sphaerotilus* strains which have a smooth surface), utilization of carbon sources, and the pronounced response of *S. natans* to an increase of organic nutrient concentration in contrast to a poor response of most *Leptothrix* species.

A summary of phenotypic characteristics useful for the classification of both genera is presented in Table 2.

Table 2. Main morphological and physiological characteristics of the genera *Leptothrix* and *Sphaerotilus*.

Characteristic	*Leptothrix*[a]	*Sphaerotilus*
Cell dimensions		
Width (μm)	0.6–1.5	1.2–2.5
Length (μm)	1.5–14	1–10
Flagella		
Monotrichous, polar	+	–
Polytrichous, subpolar	+	+
Structure of sheath surface[b]	Rough	Smooth
Reserve material		
Poly-β-hydroxybutyrate	+	+
Polysaccharide	–	+
Major fatty acids[c]	*cis*-9 16:1, 16:0, *cis*-9,11 18:1	*cis*-9 16:1, 16:0, *cis*-9,11 18:1
Hydroxylated fatty acid	3-OH 10:0	3-OH 10:0
Major quinone type	Q-8	Q-8
Oxidation of Mn^{2+}	+	–
Growth stimulation by increase of nutrient concentration	–/+[d]	+
Need for vitamin B_{12}	+	+
Carbon sources used for growth[e]		
L-Alanine	–	+
L-Asparagine	–	+
L-Aspartate	–	+
Butyrate	–	+
D-Fructose	D	+
D-Glucose	D	+
D-Gluconate	–	+
L-Ornithine	–	+

Symbols: +, 90% or more strains are positive; –, 90% or more strains are negative; D, 11–89% of the strains are positive.
[a]Results are based on strains available from culture collections.
[b]Electron microscopic observation of unstained preparations.
[c]Number of carbon atoms:number of double bonds.
[d]Most freshly isolated strains show no pronounced response, with the exception of *Leptothrix cholodnii* strains.
[e]Utilization of carbon sources was tested in GMBN medium (Richard et al., 1985; Kämpfer, 1998).
From Van Veen et al. (1978); Spring et al. (1996); and Kämpfer (1998).

Problems in the Taxonomy of Members of the *Sphaerotilus-Leptothrix* Group

The Proposal of Pringsheim Members belonging to the *Sphaerotilus-Leptothrix* group share some important phenotypic characteristics which are easily recognized. On the other hand, distinguishing traits are sometimes difficult to determine and require the investigation of defined pure cultures. Certain strains of this group show a considerable morphological variability, depending on the respective environmental conditions, leading to the effect that virtually identical or similar strains were described under different species names. These circumstances probably led Pringsheim (Pringsheim, 1949a; Pringsheim, 1949b) to believe that all strains of this group should be placed in the genus *Sphaerotilus*. He also concluded that *L. ochracea* would be a morphological variant of *S. natans*

because both species lack visible deposits of manganese oxides on their sheaths. The investigations which led to these conclusions were however inadequate because they were based on only a few partly described strains or on the observation of uncultured bacteria in natural environments. Several studies in the following years clearly revealed the mistakes in Pringsheim's nomenclature. Nevertheless, his assumptions produced a lot of confusion in the taxonomic literature on the *Sphaerotilus-Leptothrix* group between the 1950s and 70s. In that time, for instance, a variety of manganese-oxidizing strains, which probably belong to different *Leptothrix* species, were described under the name *Sphaerotilus discophorus* (e.g., Rouf and Stokes, 1964).

Instability of Phenotypic Characteristics Several difficulties regarding the taxonomy of this group are due to the loss of important phenotypic traits upon cultivation (Van Veen et al., 1978). The reversible or nonreversible loss of physiological and morphological characteristics of strains in pure culture caused divergent descriptions of species, which were originally described on the basis of observations in the natural environment (e.g., *L. discophora*, see below). The ability to form sheaths has been irreversibly lost by several strains of *Sphaerotilus natans* and by most *Leptothrix* strains available from public culture collections, with the exception of *L. cholodnii* (formerly "*L. discophora*") SP-6 (= LMG 8142). Gaudy and Wolfe (1961) reported that sheath formation in *Spaerotilus natans* is affected by the composition of the culture medium and inhibited at high concentrations of peptone. The colony morphology of freshly isolated strains corresponds to the ability of sheath formation and can be smooth (sheathless cells) or rough and filamentous (sheath-forming cells). Sometimes even various colony morphologies of the same strain can be observed on one agar plate. The manganese-oxidizing activity in *Leptothrix* strains is independent from the formation of sheaths and more stable in most strains, but loss of this trait has also been reported.

A report on the disc- or holdfast-formation of an uncultured sheathed bacterium, which was tentatively identified as *L. discophora* (Carlile and Dudeney, 2001), illustrates the taxonomic problems within this group. Interestingly, the formation of basal discs or holdfasts at one end of the filament (*discophora* means disc-bearing) was mentioned in the original description of this species by Schwers (1912), which was based on photomicrographs of cells in natural environments. In later studies, however, disc formation was never again observed in pure cultures of this morphotype, so that it was concluded that the presence of this trait within the genus *Leptothrix* was confined to the species *L. lopholea* (Mulder, 1989a). If the authors of the abovementioned studies have in fact described the same species, the ability to form discs would not distinguish between *L. discophora* and *L. lopholea*, this trait being more stable in the latter. To avoid this kind of confusion, descriptions of new strains should be based on freshly isolated pure cultures that were studied, if possible, under conditions most similar to natural habitat conditions.

Lack of Reference Strains A major problem in the taxonomy of the genus *Leptothrix* is the availability of reference strains. The type species of the genus *Leptothrix*, *L. ochracea*, is not cultured and the description is based only on morphological observations. Although the description of "*L. pseudo-ochracea*" was based on pure cultures, no type strain was designated. On the other hand, the type strains of *L. lopholea*, LVMW 124, and *L. cholodnii*, LVMW 99, are not available from public culture collections and have apparently been lost. The strain LMG 7171 can be used as reference strain for *L. cholodnii* until a neotype is designated, but for *L. lopholea*, no other strains are available, preventing detailed taxonomic studies on this species.

Ecology

Ecology of Sheath Formation

The production of a sheath takes place at the expense of energy and requires synthesis and excretion of a large amount of cellular material. Therefore, it can be assumed that sheath formation was developed by microorganisms under a selective pressure and offers several ecological and nutritional advantages in the environment. A linear arrangement of single cells within a tubular sheath enables bacteria to form filaments, without actual enlargement of cell size. It has been shown that filamentous growth is an effective strategy of bacteria to exceed the size limit of particles edible by protozoa, thereby allowing them to escape from grazing (Sommaruga and Psenner, 1995). In addition, sheaths provide cells with physical protection from infection by bacteriophages (Winston and Thompson, 1979), bacterial predators (Venosa, 1975), and bacterivorous metazoa. Obviously tough sheaths impregnated with ferric oxides cannot be consumed by metazoa and are not penetrated by bdellovibrios or phages.

Rigid sheaths in combination with extracellular slime represent an ideal matrix for the rapid

build-up of biofilms in the form of floating aggregates or dense layers on solid surfaces. The ecological advantages of biofilm formation for bacteria are well known and include reduction of flow rates in running waters, adsorption of nutrients, and establishment of heterogenous microniches characterized by various chemical gradients. The reversible inhibition of sheath formation by high nutrient concentrations in some *Sphaerotilus* strains may be an indication for the important role played by the sheath surface in the accumulation of nutrients (Gaudy and Wolfe, 1961).

The sheath provides cells with an additional compartment close to their outer surface. Enzymes and proteins which are secreted by the cell can be incorporated in the sheath matrix, thereby allowing the extracellular production or degradation of compounds. It is possible that members of both genera use the sheath or the space between sheath and outer cell surface for the oxidation of iron and/or manganese to avoid toxic concentrations of metal compounds within the cell.

Methods for Studying Distribution and Abundance

Members of the *Sphaerotilus-Leptothrix* group are not only abundant in natural environments, but also may play a role in industrial processes leading to several technical problems. They were associated with bulking of activated sludge (Wagner et al., 1994), corrosion of stainless steel (Olesen et al., 2000), clogging of water distribution systems (Dondero, 1975), and slime formation in paper mill factories (Pellegrin et al., 1999). To prevent damage caused by the prolific growth of these organisms, it could be advantageous to monitor them in environmental samples without time-consuming enrichment and cultivation steps. Although in some samples the affiliation of bacteria to the *Sphaerotilus-Leptothrix* group may be assigned on the basis of phase-contrast microscopic appearance, distinction between genera or single species is hardly possible. Consequently, culture-independent methods for the identification and detection of these species were developed. Two approaches could be promising: whole cell hybridization with fluorescently labeled oligonucleotide probes and polymerase chain reaction (PCR) detection of *Leptothrix* species using primers targeting *mofA*, an enzyme gene involved in manganese oxidation.

Whole Cell Hybridization Fluorescence in situ hybridization (FISH) using oligonucleotide probes is a well established technique for the identification and in situ detection of microorganisms based on signature regions of their 16S rRNA genes (Amann et al., 1995). Several probes were developed targeting 16S rRNA sequences of members of the *Sphaerotilus-Leptothrix* group. A check of published probe sequences against a current database of 16S rRNA gene sequences (Ludwig and Strunk, 1997) revealed, however, that some probes which were originally intended to be specific for a single species are in fact targeting a variety of different species, partly not even belonging to the *Sphaerotilus-Leptothrix* group. To minimize the risk of mistaken identification of uncultured bacteria by crossreactivity of probes, it is advisable to use simultaneously at least two different specific probes, distinguishable by fluorescence label. Suitable combinations of probes for the *Sphaerotilus-Leptothrix* group can be found in Table 3.

Table 3. Specificity of oligonucleotide probes targeting 16S rRNA sequences of members of the *Sphaerotilus-Leptothrix* group.

Sequence of:			Hybridization with probe[a]			
Species	Strain	Accession no.	SNA[b]	PSP-6[c]	LDI[b]	PS-1[c]
Leptothrix cholodnii	CCM 1827	X97070	+	+	–	+
	SP-6	L33974	+	+	–	+
L. discophora	SS-1^T	L33975	–	–	+	+
L. mobilis	Feox-1^T	X97071	+	+	–	–
Sphaerotilus sp.	IF4	AF072914	+	–	–	–
S. natans	6^T	L33980	+	–	–	–
Target region of probe (*E. coli* positions)			656–673	138–155	649–666	66–82
Sequences with complementary target region, not affiliated to the *Sphaerotilus-Leptothrix* group[d]			13	9	4	0

Symbols: +, hybridization of probe; –, no hybridization of probe under stringent conditions; and ^{T}, type strain.
[a]Reactivity of the probe was either checked experimentally or determined by sequence comparison.
[b]Data from Wagner et al. (1994).
[c]Data from Siering and Ghiorse (1997).
[d]Only sequences of cultured strains available in public data bases were counted.

The identification of distinct species by FISH is still difficult, even by using a combination of specific probes, but the combined observation of morphological features (e.g., sheath formation) and hybridization signals should at least enable the identification of uncultured bacteria at the genus level. In Figure 2, the in situ detection of uncultured *Leptothrix* bacteria in an environmental wetland sample is shown.

Low signal intensities of labeled cells and samples with low numbers of target cells represent frequently encountered problems of the FISH method. In habitats with low numbers of sheathed bacteria, e.g., water distribution systems or sediments, the application of microscope slides can be a promising approach to obtain suitable samples for hybridization experiments. Microscope slides were successfully used for the enrichment of sheathed bacteria by employing to advantage their ability to adhere efficiently to smooth surfaces (Spring et al., 1996; Carlile and Dudeney, 2001). After removal and cleaning, the overgrown side of these slides can be used for FISH experiments. To improve signal intensities of labeled cells, it may help to treat samples prior to hybridization with diluted hydrochloric or oxalic acid, to remove iron and manganese oxides from sheaths, which could otherwise prevent efficient penetration of oligonucleotide probes into cells.

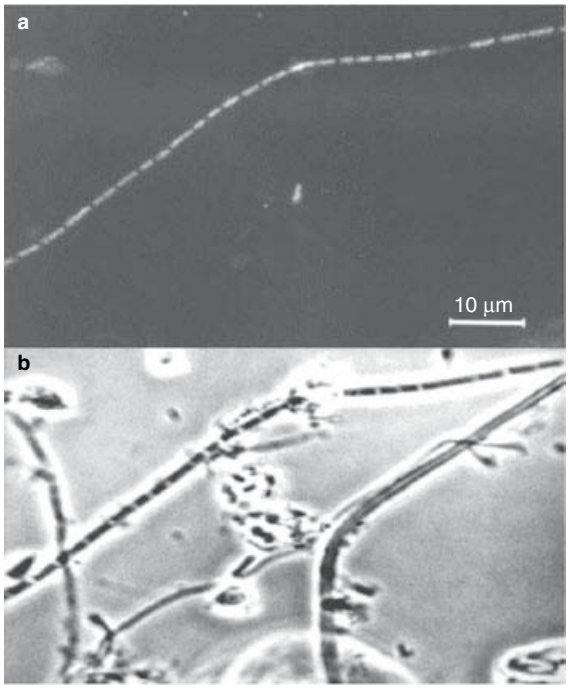

Fig. 2. Laser scanned epifluorescence (a) and matching laser scanned phase contrast image (b) of previously unidentified filamentous bacteria labeled by the PS-1 oligonucleotide probe in a sample of particulate material from the Sapsucker Woods wetland, Ithaca, NY. From Ghiorse et al. (1996), with permission.

Diagnostic PCR An alternative approach for the detection of *Leptothrix* bacteria was developed by Siering and Ghiorse (1997b). They used specific PCR primers for the amplification of the gene *mofA*, encoding a putative multi-copper oxidase involved in manganese oxidation by *Leptothrix* species (see section "Oxidation of Fe^{2+} and Mn^{2+}" in this Chapter).

The gene *mofA* was originally retrieved by cloning a DNA fragment of *Leptothrix discophora* SS-1, but homologous genes were identified also in several other *Leptothrix* species (Siering and Ghiorse, 1997b). The application of nondegenerate PCR primers enabled the in vitro amplification of a 706-bp portion of the gene *mofA* from a pure culture of *L. discophora* SS-1 and extracted nucleic acids from a water sample of a wetland iron seep, but not from nucleic acids extracted from a sediment sample of the same site. The obtained results were in agreement with previous observations which indicated that in this habitat, the growth of *Leptothrix* bacteria is restricted to ferromanganese surface films and the root zone of *Lemna* species in the water column. Potential advantages of this protocol are an increased specificity and sensitivity compared to the FISH method. However, in contrast to the latter method, no information on the morphology of the detected cells can be obtained.

The Genus *Leptothrix*

Habitats

Leptothrix species are widely distributed in the environment and can be easily found at sites which are characterized by a circumneutral pH, an oxygen gradient and a source of reduced iron and manganese minerals. Typical habitats include iron seeps of freshwater wetland areas, forest ponds, iron springs and the upper layers of sediments (Ghiorse and Ehrlich, 1992). At suitable sites, the massive growth of these organisms can be easily observed with the naked eye as ocherous masses emerging as surface films, solid mats or fluffy, dispersed material. Depending on the amount of oxidized manganese, the color can vary from yellowish-orange to dark brown. A wetland iron seep, shown in Fig. 3, is characterized by a pronounced ocherous color originating from a prolific growth of *Leptothrix* bacteria.

An iron-oxidizing microbial mat that developed at an iron seep where anoxic groundwater (rich in ferrous iron) flows over a stone wall was described in detail by Emerson and Revsbech (1994). They could demonstrate that *Leptothrix*

Fig. 3. Ellis Hollow iron seep (near Ithaca, NY). A wetland site with massive growth of *Leptothrix* species (mainly *L. ochracea*) resulting in typically colored surface films. From Ghiorse and Ehrlich (1992), with permission.

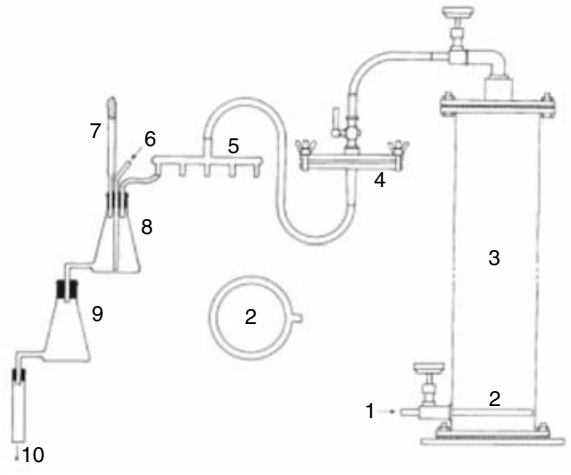

Fig. 4. Apparatus for growing *Leptothrix* species in running artificial iron-containing ditch water. (1) Inlet for tap water. (2) Distributor for the incoming tap water. (3) Cylinder containing iron-stone soil. (4) Seitz filter. (5) Manifold for distributing the sterile iron-containing water. (6) Inlet for the gas mixture (1% O_2, 5% CO_2, and 94% N_2). (7) Inoculation tube. (8) Erlenmeyer flask at a high level. (9) Erlenmeyer flask at a low level fitted with outlets (10). Reproduced with permission from Mulder and Deinema (1992).

species represent a significant fraction of the active biomass in the upper few millimeters of this dense microbial mat.

Further studies presented indirect evidence for the association of *Leptothrix* bacteria with lacustrine ferromanganous micronodules (Stein et al., 2001) or microbial mats in areas of possible deep-lake hydrothermal venting (Dymond et al., 1989).

Most *Leptothrix* species are restricted to natural, unpolluted environments with low concentrations of easily degradable organic nutrients. A prominent exception is *L. cholodnii*, which is frequently detected in sewage sludge (Kämpfer, 1997).

Isolation

Several methods for the enrichment of *Leptothrix* species from the environment are based on the tendency of these filamentous organisms to attach to surfaces. Mulder and Van Veen (1963) used continuous flow devices to imitate the natural growth conditions of these organisms. A schematic of such an apparatus is shown in Fig. 4.

For producing iron-containing ditch water, an iron-cylinder is filled with iron-stone soil supplemented with 1 to 2 g of ferric carbonate/kg of soil. After an incubation period of about 3 weeks, during which the soil is kept saturated with water, enough ferric iron is reduced to ferrous iron to start a continuous flow of tap water percolating through the soil. The soil extract running off the iron cylinder from the upper outlet is sterilized by filtration and supplied dropwise to the upper Erlenmeyer flasks, which can be inoculated with various samples containing *Leptothrix* bacteria. To avoid rapid nonbiological oxidation of ferrous iron, the upper Erlenmeyer flasks can be aerated with a gas mixture of low oxygen content. Comparable to the situation in unpolluted slowly running water, bacteria which are able to attach to solid surfaces have a selective advantage within this device because they obtain more nutrients than bacteria moving with the medium. The described apparatus can be also applied for the observation of pure cultures of *Leptothrix* bacteria under conditions similar to their natural environment.

A less laborious enrichment method was introduced by Rouf and Stokes (1964), who filled glass cylinders with water taken from the environment and added extracted alfalfa straw as nutrient source, $MnCO_3$, and freshly precipitated $Fe(OH)_3$. After several days, the flocculent growth of sheathed bacteria adhering to the walls of the cylinder indicated the enrichment of *Leptothrix* bacteria.

An alternative method, which is also based on the capability of *Leptothrix* species to attach to smooth surfaces, was applied by Spring et al. (1996). Microscope slides were put into a sample of freshwater sediment, and after several weeks, sheaths of filamentous bacteria encrusted with ferric oxides covered parts of the slides. The slides can be removed from the sediment and used for isolation after washing in sterile tap water to remove loosely attached sediment bacteria.

Isolation of pure cultures can be achieved by streaking material from enrichment cultures on previously dried agar plates containing low levels of nitrogen and carbon sources. Enrichment cul-

tures may not be necessary if natural environments are studied in which *Leptothrix* bacteria can be detected by their flocculent growth. Flocculent cell material from these sites can be washed several times with sterile tap water and streaked directly on solid media.

The isolation medium used by Rouf and Stokes (1964) has the following composition (per liter of tap water): Peptone, 5.00 g; ferric ammonium citrate, 0.15 g; $MgSO_4 \cdot 7\ H_2O$, 0.20 g; $CaCl_2$, 0.05 g; $MnSO_4 \cdot H_2O$, 0.05 g; $FeCl_3 \cdot 6\ H_2O$, 0.01 g; and agar, 12.00 g. The plates are incubated at 25°C for 5–14 days in the dark. Colonies of *Leptothrix* strains can be easily distinguished from most contaminating bacteria on this medium by their dark-brown color.

Identification

So far six different species of *Leptothrix* can be distinguished on the basis of phenotypic characteristics. Reference strains for taxonomic studies are only available for three species, viz., *L. cholodnii, L. discophora* and *L. mobilis*. Descriptions of the remaining species are incomplete because they are based solely on microscopic observations (*L. ochracea*) or poorly characterized isolates (*L. lopholea* and "*L. pseudo-ochracea*"), hence preventing an accurate identification of newly isolated strains using culture-dependent methods. The name of "*Leptothrix pseudo-ochracea*" was not included in the *Approved Lists of Bacterial Names* (Skerman et al., 1980) and has therefore no formal taxonomic status. Differential characteristics of the known *Leptothrix* species are listed in Table 4. Detailed morphological descriptions follow below.

L. OCHRACEA *Leptothrix ochracea* is the most common iron-precipitating ensheathed bacterium that probably occurs all over the world in slowly running ferrous iron-containing waters, poor in readily decomposable organic matter. Low oxygen tensions seem to be required for prolific growth of this species (Emerson and Revsbech, 1994). The pronounced development and activity of *L. ochracea* in iron- and manganese-containing waters give rise to the accumulation and deposition of large masses of ferric oxide and, probably, manganese dioxide (MnO_2), which are thought to be responsible for the formation of bog ore (see for instance, Ghiorse and Chapnick, 1983).

Descriptions of this species are based on observations in the natural environment or in the laboratory on enrichment cultures of slowly running soil extract. The most typical characteristic of *L. ochracea* is the formation of large numbers of almost empty sheaths within a relatively short time. The mechanism of this procedure can be followed in a slide culture of the organism, in an iron-containing soil extract medium, under a phase-contrast microscope. In this way, the behavior of *L. ochracea* in crude culture can be observed continuously. It will be seen that chains of cells leave their sheath at the rate of 1–2 μm/min, continuously producing a new smooth and hyaline sheath connected with the old envelope (Fig. 5a). Impregnation and covering of the sheaths with iron probably take place after the

Table 4. Differential characteristics of *Leptothrix* species.

Characteristic	*L. ochracea*	*"L. pseudo-ochracea"*	*L. lopholea*	*L. cholodnii*	*L. discophora*	*L. mobilis*
Cell dimensions						
Width (μm)	1.0	0.8–1.3	1.0–1.4	0.7–1.5	0.6–0.8	0.6–0.8
Length (μm)	2–4	5–12	3–7	2.5–15	2.5–12	1.5–12
Flagella						
Monotrichous polar	+	+	−	+	+	+
Polytrichous subpolar	−	−	+	−	−	−
Holdfasts	−	−	+	−	V	−
False branching	−	−	+	−	+	−
Deposition of MnO_2 on sheath surface	−	+	+	+	+	ND
Growth at						
35°C	ND	ND	ND	+	−	+
pH 8.5	ND	ND	ND	+	−	+
Growth on[a]						
D-Fucose	ND	ND	ND	−	+	−
Fumarate	ND	ND	ND	−	−	+
DL-Lactate	ND	ND	ND	+	−	−
G+C content (mol%)	ND	ND	ND	68–70	71	68

Symbols: +, present in all strains; −, absent in all strains; ND, not determined; and V, variable (depending on growth conditions).

[a]Growth was determined in GMBN medium supplemented with the respective carbon source (Kämpfer et al., 1995).

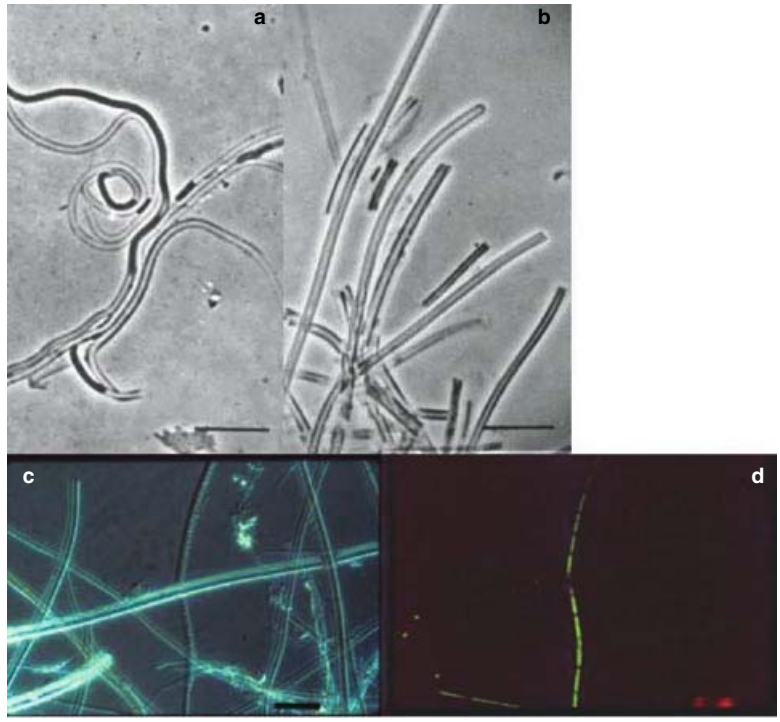

Fig. 5. Morphological characteristics of *L. ochracea*. Bar = 10 µm. (a) Phase contrast micrograph of cells moving out of sheaths and subsequently forming new sheaths. From Van Veen et al. (1978), with permission. (b) Broken old sheaths covered and impregnated with ferric hydroxide in slowly running iron(II)-containing soil extract. From Mulder and Van Veen, 1963, with permission. (c, d) Acridine orange staining of cells partly covered by a hyaline sheath. Same field viewed by differential interference contrast (c) and epifluorescence microscopy (d). Intact bacterial cells stained with acridine orange fluoresce bright green under violet illumination. Note that the chain of single cells appears as continuous filament when viewed by interference contrast. Courtesy of W. C. Ghiorse.

cells have left the envelopes. Aged golden-brown and highly refractile sheaths are brittle, so that they are easily broken into relatively short fragments. Viewed under phase contrast, such fragments are very characteristic and appear similar to broken glass capillaries (Fig. 5b, c). In Mn(II)-containing environments or enrichment cultures, sheaths of this species can be easily distinguished from sheaths of the other *Leptothrix* species which are characterized by irregular encrustations of granular MnO_2. In contrast, the sheaths of *L. ochracea* are only impregnated with ferric oxides and lack MnO_2 deposits, leading to a smooth and slender appearance of aged sheaths. The lack of MnO_2 encrustations in sheaths of *L. ochracea* can be detected either by using an electron microscope equipped with an X-ray energy dispersive microanalysis system (Ghiorse and Ehrlich, 1992) or by treatment with oxalic acid (Carlile and Dudeney, 2001). A diluted solution (1%) of oxalic acid, a solvent for hydrated ferric oxide, can make the sheaths of *L. ochracea* transparent and almost disappear, whereas sheaths of other *Leptothrix* species are unaffected owing to the impregnation with manganese oxides.

Investigators who have studied and described *L. ochracea* under natural conditions were unable to obtain pure cultures (Cholodny, 1926; Charlet and Schwartz, 1954). Others who thought they had isolated *L. ochracea* had, in fact, described one of the other species of this genus (Winogradsky, 1888; Winogradsky, 1922; Molisch, 1910; Lieske, 1919; Cataldi, 1939; Prüße, 1957). In some instances, an organism resembling *L. ochracea* has been isolated, viz., "*L. pseudo-ochracea*." A potential lithoautotrophic metabolism of *L. ochracea* with Fe(II) as electron donor could, therefore, never be proved with pure cultures and is uncertain, since the organism normally grows at a pH value of 6–7, at which Fe(II) is readily oxidized nonbiologically. In addition, its Mn (II)-oxidizing capacity, which probably occurs under natural conditions, has never been confirmed. It is, however, possible that in this species, the Mn(II) oxidizing factors are secreted into the surrounding environment leading to the precipitation of MnO_2 granules away from the sheath surface.

"*L. pseudo-ochracea*" Cells are more slender than those of the other *Leptothrix* species (Table 4), and are very motile by one thin polar flagellum. Even chains of 6–10 cells may show an undulatory locomotion after leaving their sheath. This characteristic may account for the relatively large number of empty sheaths in culture, compared with the number found in cultures of most other *Leptothrix* species; however, *L. ochracea* possesses even more empty sheaths. In slowly flowing ferrous iron-containing soil extract, the sheaths become impregnated with ferric oxide and appear yellow-brown. In this respect, the organism resembles *L. ochracea*. However, in media with added manganese compounds, the sheaths are covered with small granules of MnO_2, enabling an easy distinction from

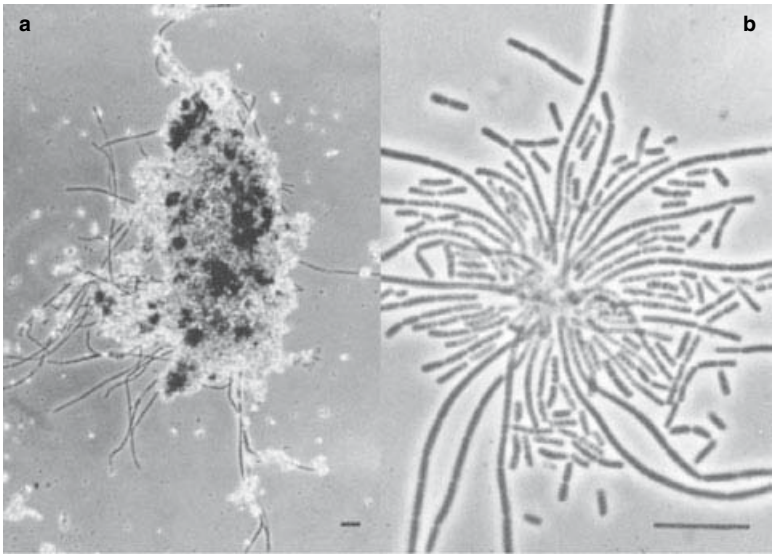

Fig. 6. *Leptothrix lopholea*. (a) Bacterial flocs with black-brown MnO_2 deposits. Reproduced with permission from Mulder and Deinema (1992). (b) Many trichomes radiating from common holdfasts. Bar = 10 µm. From Van Veen et al. (1978), with permission.

the sheaths of *L. ochracea* which are not impregnated with manganese oxides.

On Mn(II)-containing agar, the black-brown colonies are very filamentous and may exceed a width of 10 mm. On basal agar media containing 0.1% peptone and 0.1% glucose, the organism may grow in concentric rings.

The normal habitat of "*L. pseudo-ochracea*" is the slowly running, unpolluted, iron- and manganese-containing freshwater of ditches and brooklets. This species may also be found in slightly polluted water.

L. LOPHOLEA *Leptothrix lopholea* resembles *S. natans* to a greater extent than do the other *Leptothrix* species. It produces polytrichous subpolar flagellation and forms holdfasts and false branches. Strains may grow also in rich media. Cells usually develop short-sheathed filaments radiating from a cluster of holdfasts, giving rise to many tiny flocs when the cells are grown in liquid media (Fig. 6).

Deposition of iron and manganese oxides is more pronounced on holdfasts than on filaments. On Mn(II)-containing agar media, encrustation of sheaths with MnO_2 is retarded, so that colonies at first are white and later become black-brown. Cell growth responds poorly to an increased supply of organic nutrients. Strains that do show a good response oxidize manganese more slowly.

This species may be isolated from slowly flowing, unpolluted or polluted freshwater and from activated sludge.

L. CHOLODNII Cells of freshly isolated strains are usually found in long chains inside the sheaths. Single motile cells may be seen outside the sheaths. In the presence of Mn(II), the sheaths become covered with granular MnO_2 (Fig. 7a). At some sheath locations, the MnO_2 deposits may even exceed 10 µm. *Leptothrix cholodnii*, in contrast to other *Leptothrix* species, responds to an increased supply of organic nutrients (Table 2). This results in relatively large colonies (up to 5 mm in diameter) on nutrient-rich agar media. On Mn(II)-containing agar, black-brown hairy colonies are formed (Fig. 7b), particularly when the organism is seeded densely.

Most strains display a strong tendency to dissociate spontaneously and to produce smooth rather than the typical rough colonies (Fig. 7c). Such mutant strains are largely sheathless and oxidize manganese slightly or not at all (Mulder and Van Veen, 1963; Rouf and Stokes, 1964; Stokes and Powers, 1965).

In agreement with its nutritional requirements, *L. cholodnii* is found in slowly running iron- and manganese-containing unpolluted waters or in polluted waters, particularly in activated sludge.

L. DISCOPHORA Cells are relatively small compared to those of the other *Leptothrix* species described (Table 4). They may occur in narrow sheaths or be free-swimming; free cells are motile by a thin polar flagellum at one or both poles. The manganese-oxidizing and ferric oxide-storing capacities of this organism are very pronounced. In the presence of Mn(II), the sheaths are heavily but irregularly encrusted with MnO_2, giving rise to sheaths of sometimes 10 µm thickness. Under natural conditions, holdfasts may be formed and sheaths are covered with a slime capsule which tapers toward the growing tip and is impregnated with hydrated ferric oxides (Carlile and Dudeney, 2001). In enrichment media with both manganese (II) and iron (II), as in slowly

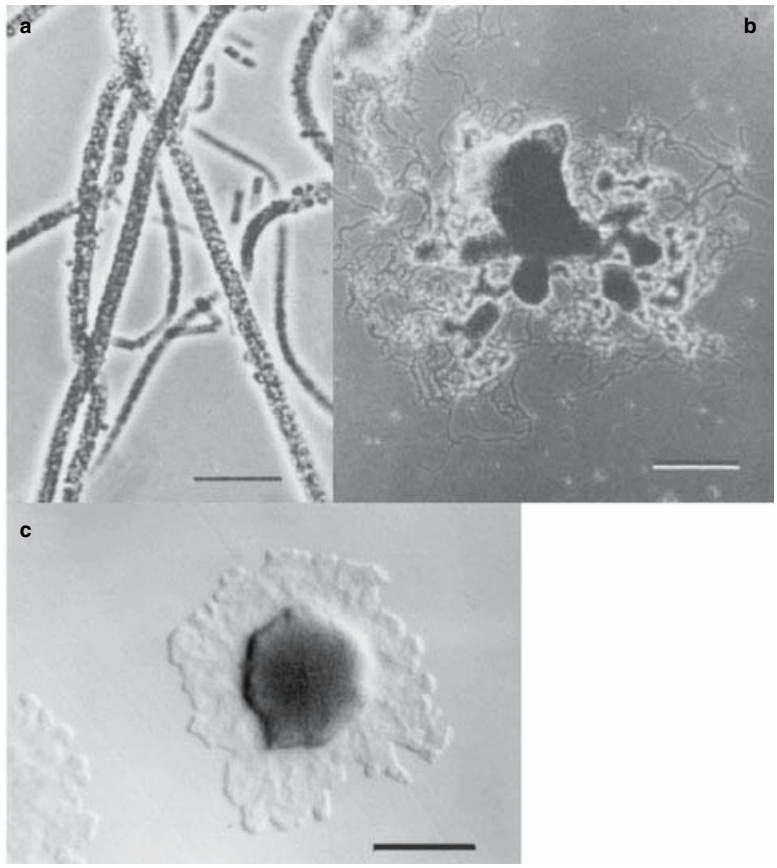

Fig. 7. *Leptothrix cholodnii*. (a) Sheaths encrusted with MnO_2. Bar = 10 μm. Reproduced with permission from Mulder and Deinema (1992). (b, c) Colony morphologies. (b) Filamentous colony on agar with $MnCO_3$ (medium 1, Mulder and Van Veen, 1963). Bar = 100 μm. Reproduced with permission from Mulder and Deinema (1992). (c) Smooth colony on the agar medium of Rouf and Stokes (1964). Bar = 500 μm.

flowing soil extract, the sheaths become covered with a thick, dark brown, fluffy layer of ferric oxide and MnO_2 which may increase the diameter of the trichomes up to about 20–25 μm (Fig. 8a).

Following isolation, the ability to form sheaths is easily lost in this species (Adams and Ghiorse, 1986). False branching is regularly observed, even with sheathless strains (Fig. 8b). In older cultures, coccoid bodies and cell evaginations are formed. Colonies on the solid medium of Rouf and Stokes (1964) are about 1 mm in diameter, more or less circular in shape, flat, and dark-brown (Fig. 8c). Under certain conditions, filamentous colonies may be formed. Increasing the supply of nutrients such as glucose, peptone, methionine, purine bases, vitamin B_{12}, biotin and thiamine only increases growth slightly. Visible aggregates are formed when grown in liquid media.

The normal habitat is slowly running, unpolluted, iron- and manganese-containing water of ditches, rivers or ponds.

L. MOBILIS Cells are similar in width to *L. discophora* cells (Fig. 9a), but are usually shorter in length. False branching, which is typical for *L. discophora*, is absent in cultures of *L. mobilis*. Cells are highly motile by a single polar flagellum. Sheaths are not formed under laboratory conditions. Colonies on the agar medium of Rouf and Stokes (1964) are about 1 mm in diameter, circular sometimes with frayed edges, flat, smooth and dark-brown (Fig. 9b). Visible aggregates are formed when grown in liquid media.

The species description is based on only one strain isolated from the sediment of a freshwater lake (Spring et al., 1996).

Preservation

Stock cultures can be stored on agar slants of the medium of Rouf and Stokes (1964) for about two months at 4°C. Rouf and Stokes (1964) reported a better survival of their strains if stored at room temperature instead of refrigerated. Most *Leptothrix* strains do not survive lyophilization. For the long-term preservation of these strains, freezing in liquid nitrogen is recommended using suspensions of cells in freshly prepared medium supplemented with 5% dimethyl sulfoxide (DMSO) as cryoprotectant.

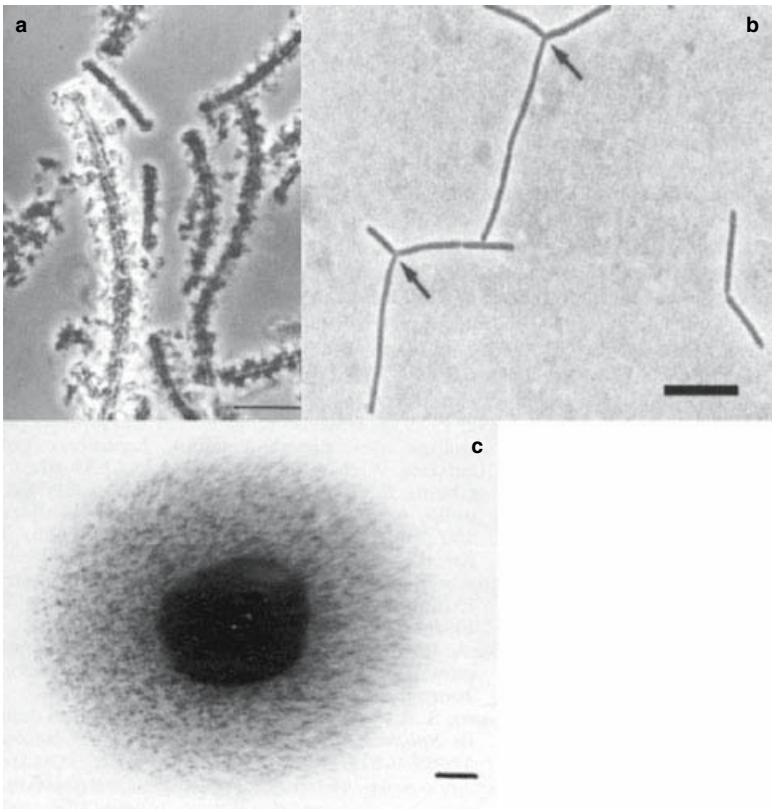

Fig. 8. *Leptothrix discophora*. (a) Sheaths covered with ferric hydroxide and manganese dioxide in running iron(II)- and manganese(II)-containing soil extract. Bar = 10 µm. From Mulder and Van Veen (1963), with permission. (b) Cells of a sheathless strain showing false branching. Bar = 10 µm. From Spring et al. (1996), with permission. (c) Smooth colony on $MnSO_4$-containing agar. MnO_2 is present within the colony and in a halo containing no bacteria. Bar = 100 µm. From Mulder and Van Veen (1963), with permission.

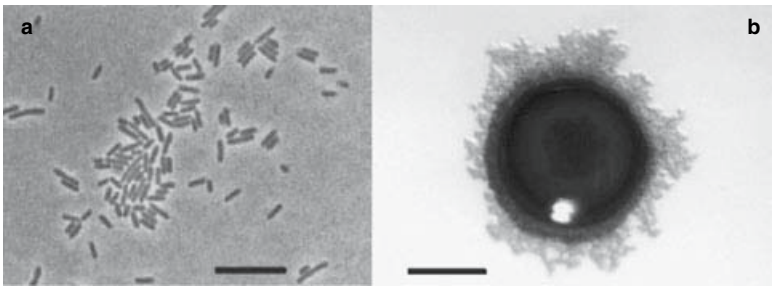

Fig. 9. *Leptothrix mobilis*. (a) Dimensions of single cells grown in the medium of Rouf and Stokes (1964). Bar = 10 µm. From Spring et al. (1996), with permission. (b) Colony morphology on agar medium of the same composition. Dark-brown, granular deposits of MnO_2 are visible in the frayed edge of the colony. Bar = 500 µm.

Physiology

The genus *Leptothrix* is characterized by two remarkable metabolic activities: Formation of sheaths and oxidation of iron and manganese.

OXIDATION OF Fe^{2+} AND Mn^{2+} It could be demonstrated in several studies that the deposition of metal-oxides in *Leptothrix* species is biologically controlled. Oxidation of manganese and iron is catalyzed by various metal-oxidizing factors actively secreted by the cell (De Vrind-de Jong et al., 1990).

At circumneutral pH, biological oxidation of iron is difficult to distinguish from the chemical oxidation by oxygen. Only with the identification of an iron-oxidizing protein with a molecular weight of 150 kDa in spent culture medium of the sheathless strain *Leptothrix discophora* SS-1, the capability of biological iron-oxidation in *Leptothrix* species could be clearly demonstrated (Corstjens et al., 1992). The function of iron-oxidation is still unknown, but it seems unlikely to play a role in the generation of energy. It has to be noted, however, that *Gallionella ferruginea*, which is also neutrophilic and microaerophilic, thrives in the same habitats as *L. ochracea* and is able to gain energy from chemolithoautotrophic iron-oxidation (Hallbeck et al., 1993).

Several manganese-oxidizing factors could be identified in culture supernatants of *Leptothrix* species. One component with an estimated molecular weight of 110 kDa could be purified and partly characterized. It is called "manga-

nese-oxidizing factor" (MOF) and consists of protein and probably polysaccharide (Emerson and Ghiorse, 1992). It is assumed that this component is part of a larger complex that originates from membranous blebs and is associated with the sheath structure (Brouwers et al., 2000). Antibodies raised against the MOF protein of *L. discophora* SS-1 allowed retrieval of the gene *mofA*, encoding a putative multi-copper oxidase. A strong indication for an active role of this copper-dependent enzyme in manganese-oxidation is the observation that addition of small amounts of copper to actively growing cultures enhances the rates of manganese oxidation considerably (Brouwers et al., 2000). In addition to multi-copper oxidases, *c*-type hemes could be involved in metal-oxidation by *Leptothrix* species. Genes encoding proteins with potential heme-binding sites were identified as part of the operon encoding *mofA*.

So far, the beneficial effects of manganese oxidation are largely unknown, but it is unlikely that the deposition of manganese oxides has been developed without conferring an important advantage to these bacteria. The benefits of manganese oxidation are probably so difficult to determine because they are only effective under natural conditions difficult to imitate in the laboratory. Traditionally, it was assumed that the positive effects of Mn(II)-oxidizing enzymes and manganese oxides are based on the detoxification of harmful oxygen species (superoxide and peroxide) or on the adsorption of toxic compounds (e.g., heavy metals). Recently, an interesting new perspective was introduced by Sunda and Kieber (1994). They found that manganese oxides are strong chemical oxidants able to attack complex organic compounds (e.g., humic substances), thereby leading to the release of small organic molecules which can be easily assimilated by bacteria.

Structure and Composition of the Sheath

The overall structure and chemical composition of sheaths formed by *Leptothrix* species resemble those of *Sphaerotilus natans*, although several distinguishing traits were found. One characteristic which can be used for the differentiation between both genera is the structure of the sheath surface. In electron micrographs of unstained preparations, the sheath surface appears rough in *Leptothrix* species and smooth in strains of *Sphaerotilus* (Figs. 10 and 12b).

Emerson and Ghiorse (Emerson and Ghiorse, 1993a; Emerson and Ghiorse, 1993b) performed a detailed investigation of the ultrastructure and chemical composition of the *Leptothrix* sheath with the sheath-forming strain *L. cholodnii* (formerly "*L. discophora*") SP-6. They reported that it consists of a condensed fabric of 6.5-nm-diameter fibrils underlying a more diffuse outer capsular layer. The inner sheath layer has a thickness of 30–100 nm and seems to be associated with the outer layer of the Gram-negative cell wall by membrane evaginations. The purified sheath substance contains approximately 34% polysaccharide, 24% protein, 8% lipid, and 4% inorganic material. As major components of the polysaccharide moiety, uronic acids and amino sugars, which are probably in the N-acetylated form, were identified, whereas neutral sugars could not be detected. The sheath proteins are rich in cysteine residues (6 mol%), which confer numerous disulfide- and sulfhydryl-groups to the sheath. The heteropolysaccharide and protein moieties appear to be tightly associated or connected. On the basis of these findings, it was concluded that the principal structural elements of the sheath are proteoglycan fibrils that are covalently linked to each other by interfibril disulfide bonds resulting in a stable fabric. It is possible that the difference between the condensed inner layer and diffuse capsular layer is caused by an increased amount of free sulfhydryl groups in the exterior sheath-layer. A further characteristic of the sheath is its negative charge due to free carboxyl groups, originating mainly from the uronic acids of the sheath-proteoglycans. Both chemical groups, the free sulfhydryl and the free carboxyl groups, provide the sheath with numerous sites for binding of metal cations, especially Mn^{2+} and Fe^{2+}. According to the working model

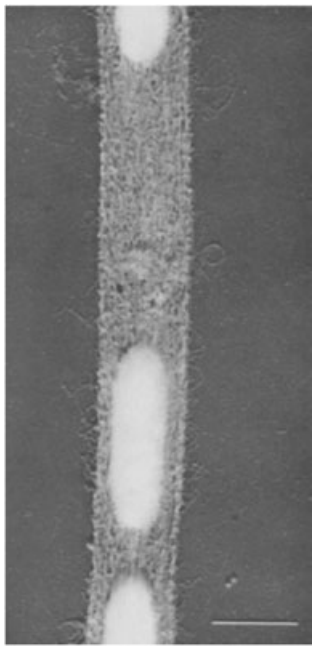

Fig. 10. Electron micrograph of a *Leptothrix* sheath showing a rough surface. Bar = 1 μm. Reproduced with permission from Mulder and Deinema (1992).

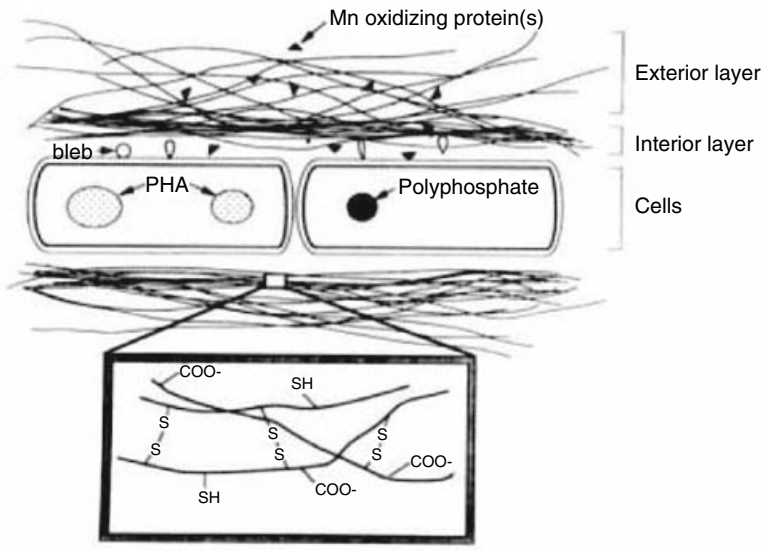

Fig. 11. Schematic model of the architecture and potential function of the sheath in *L. cholodnii* SP-6. PHA, polyhydroxyalkanoate. From Emerson and Ghiorse (1993a), with permission.

presented in Fig. 11, the anionic sheath-fibrils capture and concentrate soluble Mn(II) ions within the sheath, where they can be efficiently oxidized by excreted manganese-oxidizing factors associated with the sheath (Emerson and Ghiorse, 1993a). Manganese- and iron-oxidizing factors are probably excreted from the cell and transported to the sheath as membranous blebs containing protein complexes consisting of multi-copper oxidases and cytochromes (Emerson and Ghiorse, 1992).

The Genus *Sphaerotilus*

Habitat

Members of the genus *Sphaerotilus* are part of the natural microbial community in slowly running freshwater streams, ditches and ponds (Stokes, 1954). In contrast to *Leptothrix* sp., massive growth is typical in freshwater streams that receive high levels of organic pollution from sewage or industrial wastes, especially from paper, potato, dairy or other agricultural industries. When *Sphaerotilus* becomes established in a polluted river, it frequently proliferates so extensively that it lines the riverbed for long distances with a wooly, filamentous carpet (Dondero, 1975).

In poor settling activated sludge (so-called "bulking sludge"), *Sphaerotilus* is frequently found among other filamentous microorganisms (Eikelboom, 1975). Bulking or foaming of activated sludge is a common problem in water purification plants and prevents a ready separation of bacterial sludge flocs from the treated water. It appears that conditions which stimulate the growth of *Sphaerotilus natans* and/or other filamentous bacteria favor also the bulking of activated sludge. The correlation of conditions which favor the prolific growth of a distinct bacterial species with sludge bulking is however hardly possible owing to the high phenotypic diversity of filamentous bacteria which may be involved in this phenomenon (Kämpfer, 1997).

The uncontrolled growth of *Sphaerotilus* in artificial environments bears several risks of economic importance. It can contribute pyrogenic material to purified water for medical injection (Dondero, 1975) and cause damage to technical equipment or machines (Pellegrin et al., 1999). On the other hand, beneficial effects of the growth of *S. natans* are also discussed, e.g., the biosorption of heavy metals in wastewater at low pH values (Solisio et al., 2000; Esposito et al., 2001).

Isolation

Enrichment Procedures In many environments sheathed bacteria occur only in low numbers, for instance in activated sludge or nonpolluted water samples. The use of of enrichment cultures may facilitate the successful isolation of *Sphaerotilus* sp. from such habitats. For that purpose, variations of Winogradsky's hay infusion technique have been used (Mulder and Deinema, 1992). Extracted alfalfa straw (Stokes, 1954) or extracted pea straw (Mulder and Van Veen, 1963) serve as the nutrient material. Most of the soluble organic matter should be removed to prevent proliferation of undesirable organisms. This can be achieved by boiling and extracting the straw after it has been cut into small pieces. Suspensions of the extracted straw in tap water (1–8% [w/v]) are distributed in Erlenm-

eyer flasks and used as enrichment medium. After inoculation with 5–20% of a water sample or activated sludge and incubation for about one week at 22–25°C, filaments of *Sphaerotilus* may be seen in the medium upon microscopic observation. Often tufts of filaments can be found attached to the pieces of straw and also to the side of the flasks at or near the surface of the medium, thereby enabling easy removal using glass capillaries.

Pure cultures can be obtained from homogenized portions of the enriched material as described in the following paragraph.

DIRECT ISOLATION Often slimy masses of *Sphaerotilus* are found attached to submerged surfaces in polluted, slowly running water. Material from such sites can be successfully used for the direct isolation of *S. natans* or related strains by streaking on agar plates. To remove contaminating cells, the collected material should be washed with sterile tap water several times. Homogenization of the washed flocs by blending for a very short time may be useful. However, activated sludge flocs containing many filaments of *Sphaerotilus* should be streaked directly on the agar plates without homogenization step to avoid release of numerous, nonfilamentous cells by destroying the floc structure. The agar plates used should be dry and contain only low levels of nitrogen and carbon to limit the size of undesirable bacterial colonies, leaving large areas for the filamentous organisms. A further reason for the use of nutritionally poor media is the observation that *Sphaerotilus* often forms smooth colonies on nutrient-rich agar, instead of the typical rough colonies, which can be easily recognized. To inhibit the growth of contaminating fungi, the agar medium can be supplemented with cycloheximide (0.005 $g \cdot liter^{-1}$; Pellegrin et al., 1999).

A suitable isolation medium was proposed by Mulder (1989b), having the following basal composition per liter of distilled water: KH_2PO_4, 27 mg; K_2HPO_4, 40 mg; $Na_2HPO_4 \cdot 2\ H_2O$, 40 mg; $CaCl_2$, 50 mg; $MgSO_4 \cdot 7\ H_2O$, 75 mg; $FeCl_3 \cdot 6\ H_2O$, 10 mg; $MnSO_4 \cdot H_2O$, 5 mg; $ZnSO_4 \cdot 7\ H_2O$, 0.1 mg; $CuSO_4 \cdot 5\ H_2O$, 0.1 mg; $Na_2MoO_4 \cdot 2\ H_2O$, 0.05 mg; cyanocobalmin, 0.005 mg; peptone, 1 g; glucose, 1 g; and agar, 7.5 g.

Upon inoculation and incubation of these plates at 20–30°C, colonies of *Sphaerotilus* may be seen and tentatively identified within a few days by their characteristically flat, dull, cotton-like appearance. Confirmation of the identification may be achieved by microscopic observation.

Sphaerotilus may also be isolated by spread plate techniques with or without a previous centrifugation or homogenization step on a variety of agar media (Eikelboom, 1975; Williams and Unz, 1985; Ziegler et al., 1990), including the commercially available R2A agar (Seviour et al., 1994).

Identification

The genera *Sphaerotilus* and *Leptothrix* are closely related and share many phenotypic characteristics. Nevertheless, a clear differentiation of both genera is possible on the basis of phenotypic traits summarized in Table 2. The only recognized species of *Sphaerotilus* is currently *S. natans*. Typical morphological characteristics of this species are false branching of filaments, extensive slime production, attachment of filaments to solid surfaces by holdfast formation (Fig. 12a), deposition of hydrated ferric oxides on sheaths, and motility of cells by means of a bundle of subpolar flagella (Fig. 12b). In contrast to those of *Leptothrix*, the sheaths of *Sphaerotilus* occurring in natural habitats are usually thin and hyaline without encrustations by ferric or manganese oxides (Fig. 12c). However, the capability of Fe(II) oxidation can be easily demonstrated in this species by cultivation in media containing soluble iron compounds and low nutrient concentrations, e.g., soil extract enriched with ferrous iron. Under these growth conditions, the sheaths of *S. natans* turn yellow-brown and resemble in appearance those of *Leptothrix ochracea*. Most of the pronounced morphological characteristics of *S. natans* are largely dependent on the strain and cultivation conditions. Sheath formation can be inhibited by high levels of nutrients in the medium—peptones being more effective than carbohydrates—resulting in the formation of smooth instead of rough, filamentous colonies (Mulder and Van Veen, 1963). This loss of sheath-forming capability is reversible and can occur also spontaneously.

Growth of pure cultures in liquid media is usually flocculent, but sometimes pellicular or homogenous (Pellegrin et al., 1999). Upon prolonged incubation, large, circular bodies resembling protoplasts may appear in broth cultures. Their formation is probably due to the production of enzymes involved in the decomposition of cell walls during the death phase (Phaup, 1968).

The nutritional versatility of *S. natans* and related strains is remarkable. They can utilize a variety of carbon and nitrogen sources, tolerate a wide range of nutrient concentrations and can grow under low partial pressures of oxygen. Utilization of fructose, glucose, maltose, sucrose, lactate, pyruvate, and succinate as sole sources of carbon was reported by Stokes (1954), Mulder and Van Veen (1963), Kämpfer (1998), and Pellegrin et al. (1999). Numerous other carbon

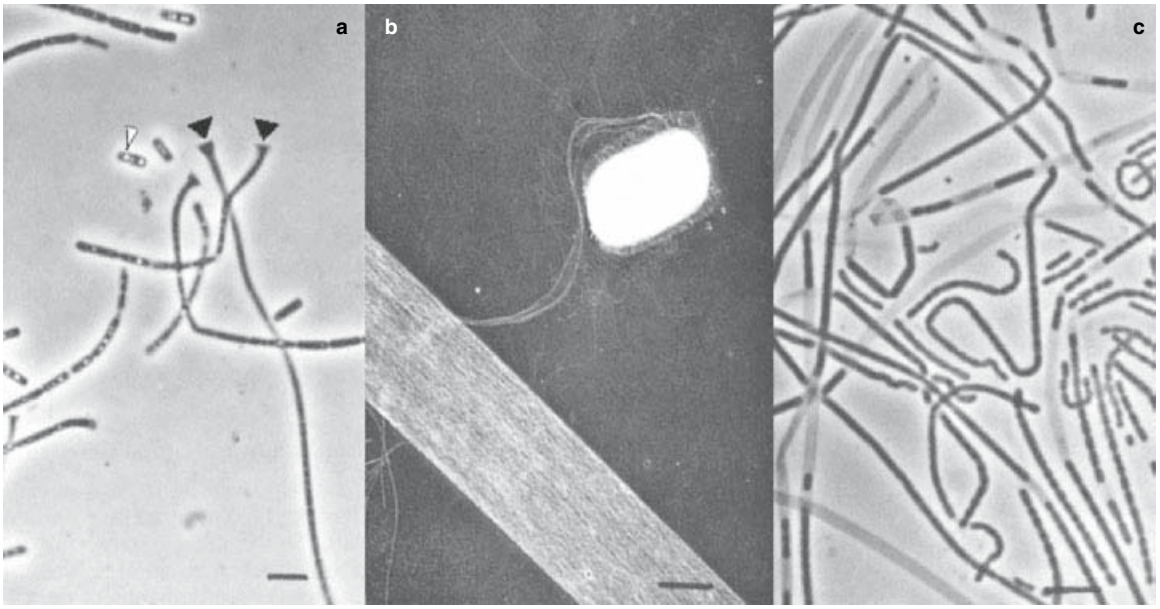

Fig. 12. *Sphaerotilus natans*. (a) Cells with internal granules of poly-β-hydroxybutyrate (white arrowhead) and filaments showing holdfasts (black arrowheads). Bar = 10 μm. (b) Electron micrograph of a single cell with a subpolar tuft of flagella and sheath with smooth surface. Bar = 1 μm. (c) Cells with and without a sheath and empty sheaths. Bar = 10 μm. All figures reproduced with permission from Mulder and Deinema (1992).

sources can be assimilated by strains of *S. natans*, but the substrate utilization patterns of strains within this species differ widely. In contrast to most *Leptothrix* strains, *S. natans* is able to assimilate relatively high concentrations of substrates from which it synthesizes considerable amounts of cellular material. Cells may contain large amounts of poly-β-hydroxybutyrate either as numerous small globules or as a few large globules (Fig. 12a). Polysaccharides may also accumulate. The synthesis of both reserve compounds is stimulated by a high carbon/nitrogen ratio in the medium or by oxygen deficiency (Mulder and Van Veen, 1963).

Preservation

Stock cultures of *S. natans* on agar slants of the previously described media can be stored for about 3 months at 4°C. Addition of 2–3 ml of sterile tap water to the agar slants may prolong the viability for another 3 months. Preservation for longer periods is accomplished by common lyophilization techniques; however, it must be stressed that some *Sphaerotilus* strains do not survive lyophilization. For the long-term preservation of these strains, freezing in liquid nitrogen can be applied.

Physiology

Metabolism *Sphaerotilus* is rarely found associated with deposits of metal oxides in natural environments and typically thrives in habitats with normal concentrations of ferrous iron. So far, the mechanism of iron oxidation in this microorganism is poorly understood and it could be that in *S. natans*, iron oxidation is only a side effect of a biological reaction with a different metabolic function.

Sphaerotilus natans is obligately aerobic and respiratory, but it can grow well with low concentrations of oxygen. It can readily adapt to various nutrient concentrations, growth temperatures (10–40°C) and pH values (pH 5.4–9.0), but seems to be sensitive to an increase in NaCl concentration (upper limit between 0.3 and 0.7%; Dondero, 1975). Glucose is dissimilated via the phosphogluconoate pathway and the tricarboxylic cycle. In the presence of glucose, the oxidation of other sugars, amino acids, and compounds of the tricarboxylic acid cycle seems to be repressed (Dondero, 1975).

Structure and Composition of the Sheath The sheath of *S. natans* is, like the sheath of *Leptothrix*, resistant to proteases and composed of a complex of polysaccharide, protein and lipid. Romano and Peloquin (1963) detected in purified sheath material 36% carbohydrate, 28% protein and 5.2% lipid, whereas Takeda et al. (1998) obtained values of 54.1, 12.2 and 1–3%, respectively. These discrepancies may be due to the investigation of different strains of *S. natans* or the application of various cultivation conditions or sheath purification methods. The surface

of the *S. natans* sheath is smooth and covered with an acidic exopolysaccharide composed of fucose, galactose, glucose and glucuronic acid (Gaudy and Wolfe, 1962). Slime production by exopolysaccharide secretion is much more pronounced in this species than in members of the genus *Leptothrix*. In contrast to the exopolysaccharide of the slime capsule, the sheath carbohydrate is free of acidic sugars and contains only glucose and galactosamine which is probably in its N-acetylated form. A heteropolysaccharide composed of glucose and galactosamine (in a molar ratio of 1 : 4) can be released from the sheath by hydrazine treatment which completely degrades the sheath structure (Takeda et al., 1998). The sheath of *S. natans* can be also degraded enzymatically by a kind of eliminase (produced by a *Paenibacillus* sp.) which attacks the polysaccharide moiety of the sheath (Takeda et al., 2000).

Although the sheath structure is resistant to protease treatment, part of the sheath protein appears to be sensitive to protease attack and can be removed from the sheath by treatment with agents that reduce disulfide bonds. However, in contrast to the *Leptothrix* sheath, disulfide bonds apparently do not play a role in maintaining the sheath structure of *S. natans*. In sheaths treated with protease, the most abundant amino acids were glycine and cysteine. Only three or four other major amino acids were detected, and it was concluded that the sheath protein may consist of repeating subunits of a small peptide which is crosslinked with a polysaccharide backbone (Takeda et al., 1998).

The revealed differences in the fine structure and composition of sheaths from *Sphaerotilus* and *Leptothrix* may be an indication for different functions of these structures for the respective microorganisms.

Acknowledgments. I am grateful to W. C. Ghiorse for providing unpublished photographs.

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Prokaryotes (2006) 5:778–811
DOI: 10.1007/0-387-30745-1_36

CHAPTER 3.2.12

The Lithoautotrophic Ammonia-Oxidizing Bacteria

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Introduction

The lithoautotrophic ammonia-oxidizing bacteria (AOB) are well defined by their fundamental metabolism. Ammonia serves as the sole energy source and carbon dioxide is used to fulfill the carbon need. Together with the lithotrophic nitrite-oxidizing bacteria (see the chapter Nitrite Oxidizing Bacteria in this Volume), the AOB catalyze the so-called "nitrification process" ($NH_3 \rightarrow NO_2^- \rightarrow NO_3^-$), which has a key position in natural nitrogen cycling. Details of the biochemistry of the AOB are reported in the chapter on Oxidation of Inorganic Nitrogen Compounds as an Energy Source in Volume 2. This chapter focuses on the phylogeny, taxonomy, and diversity of AOB as well as on their distribution in nature.

Winogradsky in his fundamental investigations on AOB already postulated the existence of a great diversity of species within this guild (Winogradsky, 1892; Winogradsky and Winogradsky, 1937; Winogradsky and Winogradsky, 1933). However, isolation of AOB turned out to be difficult and was neglected for several decades after Winogradsky's classical studies. Therefore, *Nitrosomonas europaea* was the only species in culture for a long period. Beginning in the 1960s, S. W. Watson from the Woods Hole Research Center (Woods Hole, MA, United States) and others started a new era of isolation and culturing of novel species of AOB. Since that time, 16 species of AOB have been described and named (Watson, 1965; Watson et al., 1971c; Harms et al., 1976; Jones et al., 1988; Koops et al., 1990; Koops et al., 1991), and the existence of many other cultured species has been reported in the literature (Koops and Harms, 1985; Stehr et al., 1995a). Meanwhile, the phylogeny of the described species and the majority of the yet unnamed cultured species has become well established (Woese et al., 1984; Woese et al., 1985; Head et al., 1993; Teske et al., 1994; Pommerening-Röser et al., 1996; Purkhold et al., 2000; Purkhold et al., 2003; see the section on Phylogeny of AOB in this Chapter), providing a sufficient basis for investigations of natural AOB populations employing classical as well as molecular techniques. In particular, the polymerase chain reaction (PCR)-assisted retrieval of 16S rRNA gene or *amoA* gene (encoding the active site subunit of the AOB key enzyme ammonia monooxygenase) sequences from the environment (e.g., Stephen et al., 1996; Purkhold et al., 2000) and the direct identification of AOB via fluorescence in situ hybridization FISH (e.g., Juretschko et al., 1998) allow the diversity of AOB to be monitored and their abundance in situ to be quantified. Results obtained from such investigations together with results from laboratory studies of cultured species have led to a better understanding of the ecology of this bacterial guild.

The following sections describe enrichment and isolation techniques of AOB (see the section on Enrichment, Isolation, and Maintenance in this Chapter), summarize the present knowledge on their taxonomy (see the section on General Characteristics of the Genera and Species of AOB in this Chapter) and phylogeny (see the section on Phylogeny of AOB in this Chapter), and provides an overview of available techniques for investigating AOB (see the "Methods Useful for In Situ Detection of AOB" in this Chapter). The last section aims to reveal possible correlation between ecophysiological characteristics and distribution patterns of the distinct species or groups of species of AOB (see the section on Dominant Populations of AOB in Different Environments in this Chapter).

Enrichment, Isolation, and Maintenance

Isolation procedures in general are targeted to the numerically most abundant AOB of the respective sample site. Therefore, the first step of enrichment should be a most probable number (MPN) dilution series in media, as far as possible reflecting the conditions of the environment under investigation. For example, in enrichments from acidic environments, low pH of the medium and addition of urea as the sole substrate for

energy generation is useful to isolate members of the dominant subpopulation of AOB. In general, the most important factors are the ammonia and the salt concentration in the medium. In some cases, temperature also may be of importance. Two alternative second steps on the way to isolation have been most successful. One option is additional series of dilutions, always using the highest positive dilution of the foregoing MPN series as inoculum. The other option is plating of the bacteria from the highest positive dilution of the original MPN dilution series. It is important to keep in mind that AOB sometimes grow extremely slowly. Therefore, a positive reaction in the highest dilution of an MPN series sometimes may become visible (via pH indicator) not before incubation for two or three weeks, and colony picking from agar plates should await a minimum growth period of 6–8 weeks.

Standard media, useful for enrichment cultures or pure cultures, are presented in Tables 1A and 1B. For stock cultures, calcium carbonate (5 g per liter) or *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES; 4.77 g per liter) can be used as a pH buffer. In working cultures, the pH should be adjusted by addition of a 10% solution of sodium hydrogen carbonate. This addition is either performed manually (with the aid of a pH indicator) or by using an automatic pH controller. Since ammonia and not ammonium is the substrate of AOB, the pH optimum in part depends on the concentration of the added ammonium salt. Ten mM ammonium salts at a pH of about 8.0 can be regarded as good standard for sufficient growth of AOB cultures. Large volume cultures should be stirred to guarantee a sufficient supply with oxygen. In general, 30°C is a suitable growth temperature. Stock cultures should be transferred to fresh medium every 3–4 months. Storage of AOB cells in liquid nitrogen is possible, whereas freeze-drying of AOB cells in general has not been successful.

General Characteristics of the Genera and Species of AOB

The taxonomic framework of the AOB stems from early investigations of Winogradsky (Winogradsky, 1892; Migula, 1900; Buchanan, 1917; Winogradsky and Winogradsky, 1933). The

Table 1A. Different growth media for lithotrophic ammonia oxidizers.

	Medium no.[b]				
Ingredient	1	2	3	4	5
Distilled water (ml)	1,000	1,000	1,000		600
Seawater (ml)				1,000	400
NH_4Cl (mg)			535.0	1,320.0	500.0
$(NH_4)_2SO_4$ (mg)	500.0	130.0			
$MgSO_4 \times 7H_2O$ (mg)	40.0	200.0	49.3	200.0	
$CaCl_2 \times 2H_2O$ (mg)	40.0	20.0	147.0	20.0	
KH_2PO_4 (mg)	200.0		54.4		50.0
K_2HPO_4 (mg)		87.0		114.0	
KCl (mg)			74.4		
NaCl (mg)			584.0		
Chelated iron (mg)		1.0		1.0	
$FeSO_4 \times 7H_2O$ (µg)			973.1		
Fe-EDTA (mg)	0.5				
$Na_2MoO_4 \times 2H_2O$ (µg)		100.0		1.0	
$(NH_4)_6Mo_7O_{24} \times 4H_2O$ (µg)			37.1		
$MnCl_2 \times 4H_2O$ (µg)		200.0		2.0	
$MnSO_4 \times 4H_2O$ (µg)			44.6		
$CoCl_2 \times 6H_2O$ (µg)		2.0		2.0	
$CuSO_4 \times 5H_2O$ (µg)		20.0	25.0	20.0	
$ZnSO_4 \times 7H_2O$ (µg)		100.0	43.1	100.0	
H_3BO_3 (µg)			49.4		
Phenol red, 0.5% (ml)	0.1	1.0		1.0	
Cresol red, 0.05% (ml)			1.0		1.0

[a]Suitable buffers are 5.0 g/l $CaCO_3$ and 4 g/l HEPES, respectively.
[b]Medium no. 1 is from Soriano and Walker (1968), for terrestrial strains. Medium no. 2 is from Watson (1971b), for terrestrial strains. Medium no. 3 is from Krümmel and Harms (1982), for terrestrial strains. Medium no. 3 is from Watson (1965), for marine strains. Medium no. 5 is from Koops et al. (1976), for brackish-water strains.

isolates originally were categorized into genera on the basis of the shape of cells. Later, the arrangement of their intracytoplasmic membranes was introduced as a second basic character. Using these criteria, the genera *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* have been established (Watson et al., 1981; Watson et al., 1989; Koops and Möller, 1992). In the following, a brief listing of the distinguishing morphological features of the five recognized genera of AOB is given and the distinct csultured species are described. Differential characteristics of the genera of AOB and of the species of AOB are presented in Tables 2 and 3, respectively.

The Genera

Genus *Nitrosomonas* (Winogradsky, 1892) Cells generally are rod shaped, but sometimes spherical. Extensive intracytoplasmic membranes are arranged as peripherally located flattened vesicles. Sometimes intrusions of membranes into the protoplasm are observed (Fig. 1).

Genus *Nitrosospira* (Winogradsky and Winogradsky, 1933) Cells are tightly closed spirals and occasionally vibrio shaped. Extensive intracytoplasmic membranes are missing. Intrusions of membranes into the protoplasm are sporadically observed (Fig. 2).

Genus *Nitrosovibrio* (Harms et al., 1976) Cells are vibrio shaped. Extensive intracytoplasmic membranes are missing. Intrusions of membranes into the protoplasm are documented (Fig. 3).

Genus *Nitrosolobus* (Watson et al., 1971c) Cells are pleomorphic lobes compartmentalized by the cytoplasmic membrane (Fig. 4).

Genus *Nitrosococcus* (Winogradsky, 1892) This genus solely represents the gamma-proteobacterial AOB. Both described species, *N. oceani* and *N. halophilus*, are characterized by large spherical to ellipsoidal cells revealing extensive intracytoplasmic membranes, arranged as a central stack of vesicles (Fig. 5).

Table 1B. Different media used to ascertain that cultures of ammonia oxidizing bacteria are free of heterotrophic contaminants.

	Medium no.[b]		
Ingredient	1	2	3
Distilled water	1,000ml	600ml	250ml
Seawater		400ml	750ml
Yeast extract (Difco)	0.5g	0.5g	0.5g
Peptone (Difco)	0.5g	0.5g	0.5g
Beef extract (Difco)	0.5g	0.5g	0.5g

[a]The pH of these media should be adjusted to 7.3.
[b]Media no. 1, 2 and 3 are from Koops et al. (1976). Medium no. 1 is for terrestrial and freshwater strains, respectively; no. 2 for brackish-water strains; and no. 4 for marine strains.

The Cultured Species

Since all AOB possess the same fundamental metabolism and morphological differences are per se limited within this group, identification on the species level often is difficult if exclusively phenotypic characters are used. The three genera *Nitrosolobus*, *Nitrosovibrio* and *Nitrosospira*, which phylogenetically form the *Nitrosospira* lineage (see the section on Phylogeny of AOB in this Chapter), are severely affected by this limitation. *Nitrosomonas* species within one of the distinct lineages of this genus often cannot be unambiguously identified using phenotypic characters, while species affiliated with different lineages can more easily be distinguished. In general, DNA-DNA hybridization is the method of choice to define closely related species. The above-mentioned phenotypic similarities among closely related AOB has hampered in many cases formal description of genotypically well-defined, cultured species. Below, important features of AOB species are listed.

Table 2. Characteristics of the genera of the ammonia-oxidizing bacteria.

Characteristics	*Nitrosococcus*	*Nitrosolobus*	*Nitrosomonas*	*Nitrosospira*	*Nitrosovibrio*
Cell shape	Spherical to ellipsoidal	Pleomorphic lobate	Straight rods	Tightly coiled spirals	Slender curved rods
Cell size (μm)	1.5–1.8 × 1.7–2.5	1.0–1.5 × 1.0–2.5	0.7–1.5 × 1.0–2.4	0.3–0.8 × 1.0–8.0	0.3–0.4 × 1.1–3.0
Flagellation of motile cells	Tuft of flagella	Peritrichous	Polar to subpolar	Peritrichous	Polar to subpolar
Arrangement of intracytoplasmic membranes	Central stacks of vesicles	Compartmentalizing membranes	Peripheral flattened vesicles	Invaginations	Invaginations

Table 3. Characteristics and preferred habitats of described species of the ammonia-oxidizing bacteria.

Species	G+C (mol%)	Carboxysomes	Urease activity	Substrate (NH_3) affinity (K, in µM)	Maximum ammonia tolerance NH_4Cl (in mN; pH 8.0)	Salt requirement	Maximum salt tolerance (in mM)	Preferred habitats
Nitrosomonas europaea	50.6–51.4	–	–	30–61	400	–	400	Sewage disposal plants, eutrophic freshwater and brackish water
Nitrosomonas eutropha	47.9–48.5	+	–		600	–	400	
Nitrosomonas halophila	53.8	+	–		400	+	900	
Nitrosococcus mobilis	49.3	–	–		250	+	500	
Nitrosomonas communis	45.6–46.0	–	–	14–43	250	–	250	Soils (not acid) eutrophic freshwater
Nitrosomonas nitrosa	47.9	+	+	19–46	100	–	300	
Nitrosomonas ureae	45.6–46.0	–	+	1.9–4.2	200	–	200	Oligotrophic freshwater and natural soils
Nitrosomonas oligotropha	49.4–50.0	–	+		50	–	150	
Nitrosomonas marina	47.4–48.0	–	+	50–52	200	+	800	Marine environments
Nitrosomonas aestuarii	45.7–46.3	–	+		400	+	600	
Nitrosomonas cryotolerans	45.5–46.1	–	+	42–59	400	+	550	Marine environments
Nitrosolobus multiformis	53.5	ND	+/–	ND	50	–	200	Soils (not acid)
Nitrosovibrio tenuis	53.9	ND	+/–	ND	100	–	100	Soils, rocks and freshwater
Nitrosospira briensis	54	ND	+/–	ND	200	–	250	Soils, rocks and freshwater
Nitrosococcus oceani	50–51	ND	+	ND	1000	+	1100	Marine environments
Nitrosococcus halophilus	50–51	ND	–	ND	500	+	1800	Marine environments and salt lakes

Symbols and Abbreviations: +, present; –, not present; +/–, present in some strains; and ND, no data.

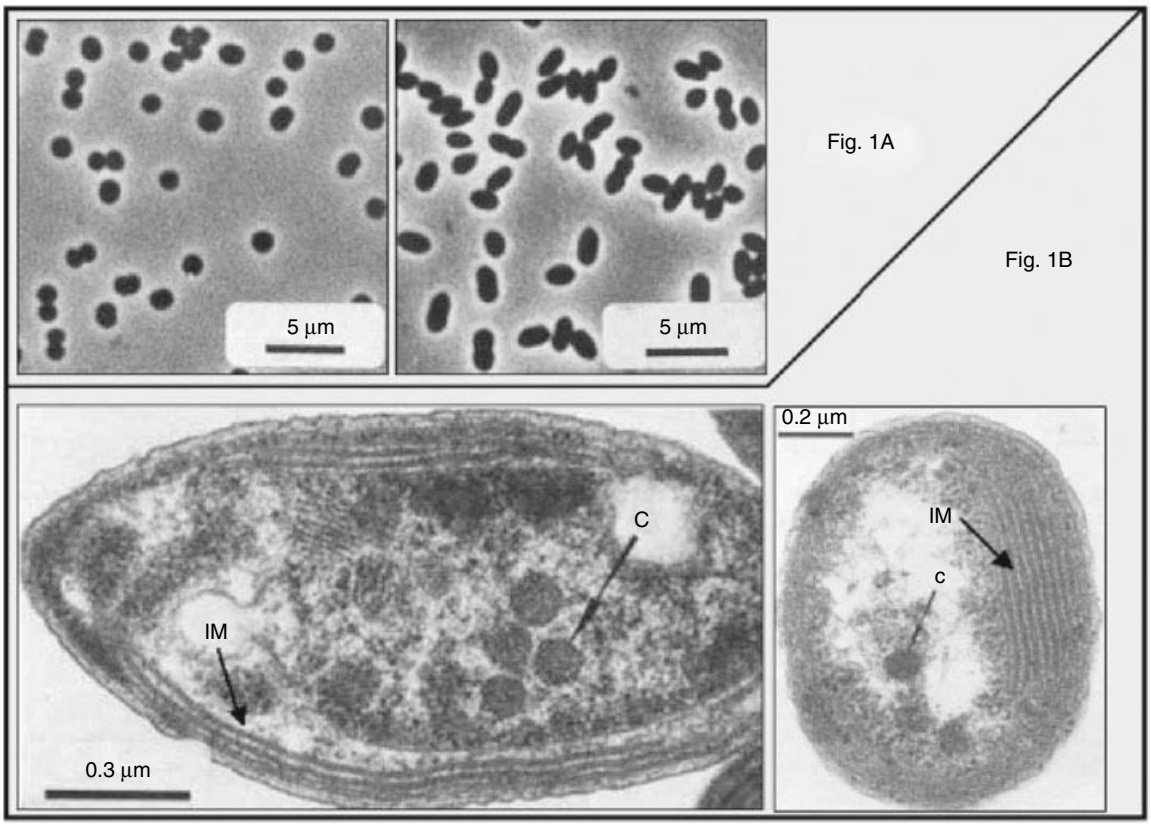


Fig. 1. The genus *Nitrosomonas*. Phase contrast photomicrographs (A) and electron micrographs of thin sections (B) of cells of different *Nitrosomonas* species showing the variability of shapes and sizes and the details of their ultrastructure (intracytoplasmic membranes [IM] and carboxysomes [C]).

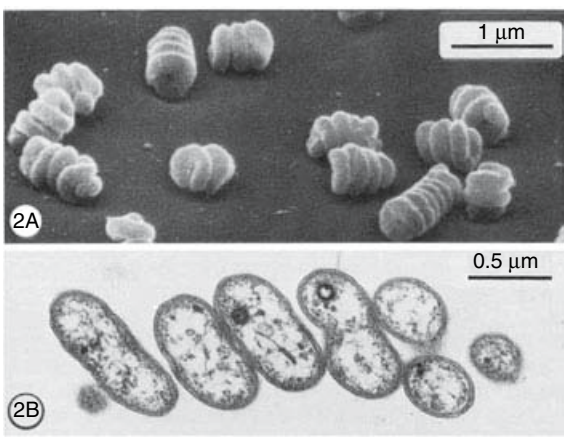


Fig. 2. The genus *Nitrosospira*. Scanning electron micrograph (A) and electron micrograph of thin sections (B) of cells of *Nitrosospira* species.

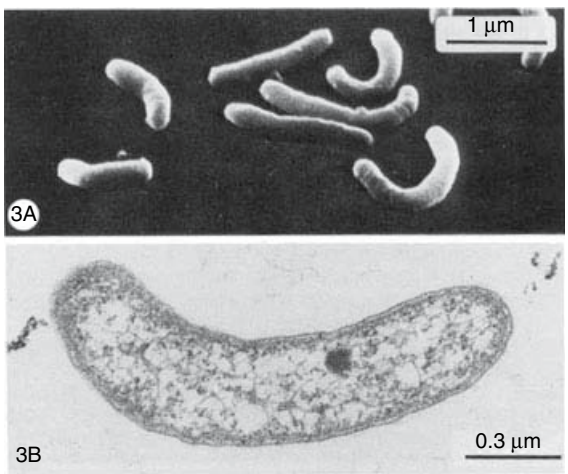


Fig. 3. The genus *Nitrosovibrio*. Scanning electron micrograph (A) and electron micrograph of thin sections (B) of cells of *Nitrosovibrio* species.

The Species of the Betaproteobacterial AOB

The Nitrosomonas *Group* The cultured species of this genus can be assigned to six distinct, phylogenetically definable lineages (see the section on Phylogeny of AOB in this Chapter), comprising 11 described species. Several further genospecies are in culture but cannot sufficiently be discriminated by phenotypic properties (e.g., Koops and Harms, 1985; Stehr et al., 1995a). In general, described species belonging to different lineages of *Nitrosomonas* are phenotypically well distinguishable (Table 3). However, within the distinct *Nitrosomonas* lineages, species discrimination in many cases is as impossible as observed for the *Nitrosospira* lineage.

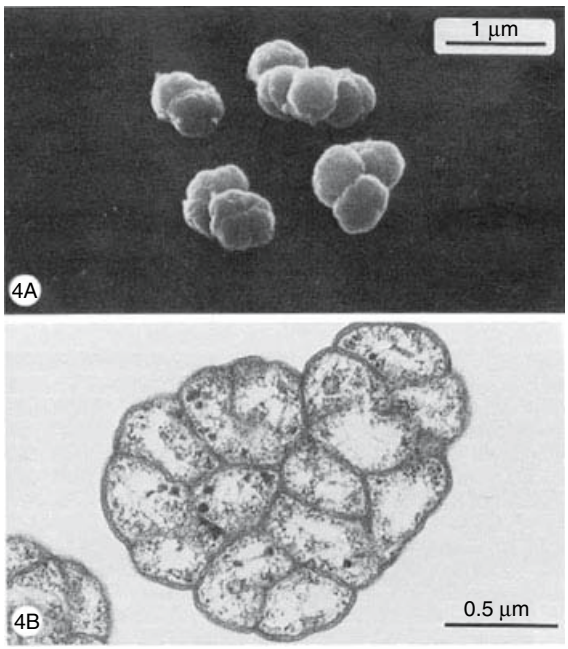


Fig. 4. The genus *Nitrosolobus*. Scanning electron micrograph (A) and electron micrograph of thin sections (B) of cells of *Nitrosolobus* species.

The Nitrosomonas europaea/Nc. mobilis *Lineage* This lineage comprises four described species, all being characterized by a relatively high salt tolerance. Two of these species, *N. halophila* and *Nc. mobilis*, have an obligatory but moderate salt requirement. All reveal relatively high affinity constants for ammonia (50–100 µM), and all cultured strains are urease negative. Consistent with these findings the members of this group seem to prefer eutrophicated aquatic environments with heightened ionic strength. The levels of phylogenetic interrelationships among the four species of this lineage are different. While *N. europaea* and *N. eutropha* reveal absolute close relationship with each other (15–16% DNA-DNA similarity), they show only minimal DNA-DNA similarity values with *N. halophila*, and DNA-DNA similarities are even missing with *Nc. mobilis*, as measured with the S1 nuclease technique (Pommerening-Röser et al., 1996).

Nitrosomonas europaea (Winogradsky, 1890; Watson, 1971a) Cells (0.8–1.1 × 1.0–1.7 µm) are short rods with pointed ends. Motility is not observed, and carboxysomes are missing. Often found in sewage disposal plants, cells are occasionally observed in eutrophicated freshwaters or fertilized soils. They have no obligate salt requirement, but do have a striking tolerance for

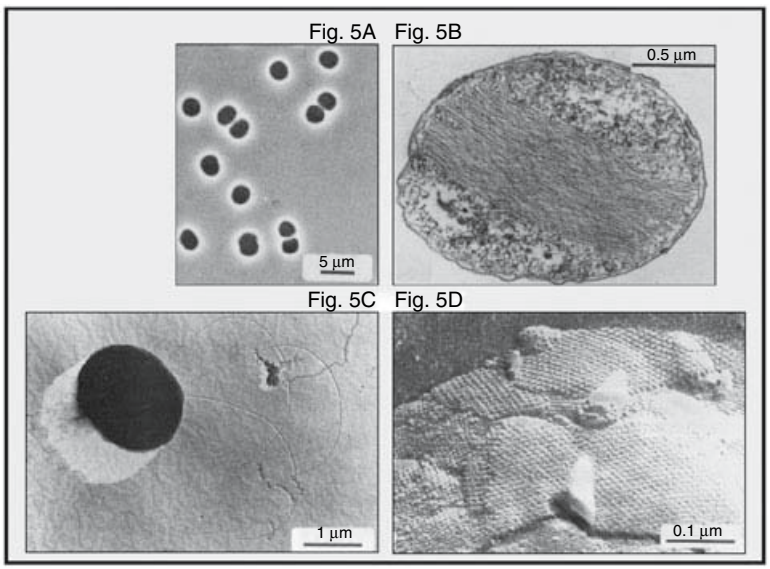


Fig. 5. The genus *Nitrosococcus*. Phase-contrast photomicrographs of whole cells (A) and electron micrographs of thin sections of cells (B) of *Nitrosococcus halophilus*. (C) Electron micrograph of a *Nitrosococcus halophilus* cell shadowed with chromium showing a tuft of flagella. (D) Freeze-etch electron micrograph of *Nitrosococcus oceani* showing the macromolecular arrangement of the two layers outside of the envelope.

increasing salt concentration up to 400 mM NaCl. The G+C content of DNA is 50.6–51.4 mol%.

Nitrosomonas eutropha (Koops et al., 1991) Cells occur singly or as short chains and are pleomorphic, rod- to pear-shaped, with one or both ends are pointed. Cells (1.0–1.3 × 1.6–2.3 μm) are motile, and carboxysomes are observed. The species is commonly distributed in sewage disposal plants, and occasionally it has been isolated from other eutrophicated environments. The species has no salt requirement but is tolerant to high salt concentration up to 400 mM NaCl. The G+C content of DNA is 47.9–48.5 mol%.

Nitrosomonas halophila (Koops et al., 1991) Cells (1.1–1.5 × 1.5–2.2 μm) occasionally appear coccoid. Motile cells possess a tuft of flagella, and carboxysomes are observed. Cells have an obligate salt requirement, and some isolates are alkali tolerant up to pH 10. Cells tolerate high salt concentration (up to 900 mM NaCl). Strains were isolated from the North Sea and from soda lakes located in Mongolia (Sorokin et al., 2001). The G+C content of DNA is 53.8 mol%.

Nitrosococcus (*Nitrosomonas*) *mobilis* (Koops et al., 1976) Cells (1.5–1.7 × 1.5–2.1 μm) are coccoid or rod shaped. Carboxysomes have not been observed. Motile cells possess a tuft of flagella. Cells have an obligate salt requirement. Isolates originate from the North Sea (Koops and Harms, 1985) and from an industrial wastewater treatment plant (Juretschko et al., 1998). The species seems to prefer eutrophic, aquatic environments. The G+C content of DNA is 49.3 mol%.

The Nitrosomonas communis *Lineage* According to phenotypic properties, this lineage is divided into two sublineages. This division is also supported by DNA-DNA similarity data (Pommerening-Röser et al., 1996). One subgroup contains *N. communis* together with two undescribed cultured genospecies (see the section on Phylogeny of AOB in this Chapter) that are phenotypically not well distinguishable. These three species are urease negative and prefer agricultural soils with neutral pH. The other subgroup is represented by a single species, *N. nitrosa*. In contrast to the first subgroup, all isolates of *N. nitrosa* are urease positive, and aquatic environments are the preferred habitats.

Nitrosomonas communis (Koops et al., 1991) Cells (1.0–1.4 × 1.7–2.2 μm) are relatively large rods with rounded ends. Motility is not observed, and carboxysomes are missing. Moderately eutrophicated, pH neutral soils seem to be the preferred habitat and freshwater only an occasional habitat. The G+C content of DNA is 45.6–46.0 mol%.

Nitrosomonas nitrosa (Koops et al., 1991) Cells (1.3–1.5 × 1.4–2.2 μm) are spheres or rods with rounded ends. Motility is not observed. Carboxysomes are present. Although eutrophicated freshwaters seem to be the preferred habitats, strains occasionally have been isolated from marine environments and a wastewater treatment plant. The G+C content of the DNA is 47.9 mol%.

The Nitrosomonas oligotropha *Lineage* Two described species and several undescribed genospecies (e.g., *Nitrosomonas* sp. Nm47, *Nitrosomonas* sp. Nm86, *Nitrosomonas* sp. Nm84, and *Nitrosomonas* sp. Nm59) belonging to this lineage exist in culture (see the section on Phylogeny of AOB in this Chapter). The absolute majority of strains originate from oligotrophic freshwaters, and only occasional isolates are from natural, often moderately acidic (pH about 6.0) soils. All strains investigated thus far reveal strikingly low affinity constants for ammonia (few μM) and almost all isolates are urease positive. All studied strains turned out to be salt sensitive.

Nitrosomonas oligotropha (Koops et al., 1991) Cells (0.8–1.2 × 1.1–2.4 μm) are rod shaped or spherical with rounded ends. Cell aggregates, obviously caused by extensive production of exopolymeric materials, are observed in the environment as well as in pure culture. Carboxysomes are missing, motility is not observed. The G+C content of DNA is 49.4–50.0 mol%.

Nitrosomonas ureae (Koops et al., 1991) Phenotypically very similar to *N. oligotropha* but distinguishable by a lower G+C content of the DNA (45.6–46.0 mol%).

The Nitrosomonas marina *Lineage* This lineage is represented by two described species and one undescribed genospecies (represented by the isolates Nm51 and Nm63; see the section on Phylogeny of AOB in this Chapter). All isolates originate from marine environments and reveal an obligate salt requirement. In general, isolates are urease positive.

Nitrosomonas marina (Koops et al., 1991) Cells (0.7–0.9 × 1.7–2.2 μm) are slender rods with rounded ends. Motility is not observed, and carboxysomes are missing. The G+C content of DNA is 47.4–48.0 mol%.

Nitrosomonas aestuarii (Koops et al., 1991) Phenotypically, this species is very similar to *N. marina*. Cells (1.0–1.3 × 1.4–2.0 μm) are rod shaped. The G+C content of DNA is 45.7–46.3 mol%.

The Nitrosomonas cryotolerans *Lineage* Only one species has yet been described, and only one strain is available in culture. The only cultured strain of *Nitrosomonas cryotolerans* (Jones et al., 1988) originates from surface water of the Kasitsna Bay (Alaska). The rod shaped cells (2.0–4.0 × 1.2–2.2 μm) requires salt and is urease positive. Carboxysomes are missing. Motility has not been observed. Intracytoplasmic membranes are not exclusively peripherally arranged, as is typical for the other members of the genus *Nitrosomonas*, but sometimes membranes intrude deep into the cytoplasm and often surround the nucleoplasm. Cultures can grow at temperatures as low as –5°C. The G+C content of DNA is 45.5–46.1 mol%.

The Nitrosomonas *sp. Nm143 Lineage* In total four isolates belonging to this lineage are available in culture (Ward, 1982; Ward and Carlucci, 1985; Purkhold et al., 2003; see the section on Phylogeny of AOB in this Chapter) but were not described by name. All isolates were obtained from marine systems.

The *Nitrosospira* Lineage Within this group of microorganisms, the three genera *Nitrosospira*, *Nitrosolobus*, *Nitrosovibrio* (each encompassing a single described species) and several genospecies (see the section on Phylogeny of AOB in this Chapter) have been described. While the genera show discriminative morphological characters, phenotypic species discrimination within the distinct genera is very difficult. However, the DNA G+C content differs significantly among genospecies or groups of genospecies (Koops and Harms, 1985). Different affinity constants for the binding of ammonia and different tolerances for increasing ammonia or salt concentrations could eventually serve as distinguishing features. Furthermore, temperature was observed as a factor that could be used to discriminate among species (Jiang and Bakken, 1999). However, no robust phenotypically based system for discriminating between species of the distinct genera is currently available. Unfortunately, this situation is consistent with the lack of sufficient resolution within 16S rRNA gene-based phylogenetic trees of the *Nitrosospira* lineage (see the section on Phylogeny of AOB in this Chapter).

THE GENUS *NITROSOSPIRA* This genus contains many cultured genospecies (see the section on Phylogeny of AOB in this Chapter) that cannot sufficiently be discriminated by phenotypic characteristics (Koops and Harms, 1985). However, two groups, containing two and three distinct species, respectively, can be distinguished on the basis of DNA G+C values around 53.5 and 55.2 mol%, respectively. The existence of these two groups later has been confirmed by DNA-DNA hybridizations applying the S1 nuclease technique (Pommerening-Röser, 1993). Unfortunately, only a few of the numerous cultured species have yet been included in such investigations. All cultured species are more or less pleomorphic, varying in shape between tightly closed spirals and vibrio-forms. All species, thus far studied, have urease-positive as well as -negative strains at similar frequencies. Species are commonly distributed in natural soils and, occasionally, occur in freshwater environments. Via molecular studies, representatives of *Nitrosospira* have also been detected in marine environments (McCaig et al., 1994; Stephen et al., 1996; Phillips et al., 1999; Bano and Hollibaugh, 2000; Horz et al., 2000; Hollibaugh et al., 2002; Nicolaisen and Ramsing, 2002). However, marine isolates do not exist and salt requirement has not yet been observed among cultured *Nitrosospira* strains.

Cells of *Nitrosospira briensis* (Winogradsky and Winogradsky, 1933) are motile cells and possess 1–6 peritrichous flagella. Strains of this species have been isolated from soils. The type strain is urease negative, but other isolates of this species are urease positive. The G+C content of DNA is about 54 mol%.

THE GENUS *NITROSOVIBRIO* Only one species is in culture. All strains of this species have exclusively vibrio-shaped cells. Pleomorphy, as observed with several genospecies of the genus *Nitrosospira*, has not yet been detected.

The three cultured strains of *Nitrosovibrio tenuis* (Harms et al., 1976) were isolated from natural soils and are urease positive. Cells are slender curved rods (0.3–0.4 × 1.1–3.0 μm). Motile cells possess 1–4 subpolar flagella. The G+C content of DNA is 53.9 mol%.

THE GENUS *NITROSOLOBUS* The genus is characterized by the pleomorphic, lobate cells which are compartmentalized by the cytoplasmic membrane. One described species and one genospecies (*Nitrosolobus* sp. NL5) are available. Both species are distinguishable by cell size and by a significantly different DNA G+C content of 53.5 and 56.5%, respectively (Koops and Harms, 1985). Most of the isolates of both species originate from soils, generally being pH-neutral and agriculturally used. One strain of *Nitrosolobus* sp. was obtained from a sewage disposal plant.

The cells of *Nitrosolobus multiformis* (1.0–1.5 × 1.0–2.5 µm; Watson et al., 1971c) are pleomorphic, motile, and possess 1–20 peritrichous flagella. Cell division is by constriction. The G+C content of DNA is about 53.5 mol%.

The Species of the Gammaproteobacterial AOB

The gammaproteobacterial AOB are represented by only two species of the genus *Nitrosococcus*: *N. oceani* and *N. halophilus*. Both species have a similar morphology. However, both are different in salt requirement, salt tolerance, and ammonia tolerance (Table 3). Moreover, the observation that all cultured strains of *N. oceani* possess urease whereas the two isolates of *N. halophilus* are urease negative indicates another discriminating character. However, more strains must be studied to verify these distinguishing features. A third species, *N. nitrosus*, is no longer available in culture.

Nitrosococcus nitrosus (Migula, 1900) This species is no longer available in culture, and nothing is known about its ecophysiology or its phylogenetic position. This species should be placed on the list of rejected names.

Nitrosococcus oceani (Watson, 1965; Watson, 1971a; Trüper and de Clari, 1997) This species originally was described as *Nitrosocystis oceanus* (Watson, 1965) and later renamed "*Nitrosococcus oceanus*" (Watson, 1971a), before the presently accepted name *Nitrosococcus oceani* was established (Trüper and de Clari, 1997). Spherical or ellipsoidal cells (1.8–2.2 µm in diameter) occur singly or as pairs. Motile cells possess 1–20 flagella (tuft). Two surface layers exist, being composed of subunits arranged in rectilinear and hexagonal arrays (Fig. 5). Intracytoplasmic membranes are arranged as a stack of parallel, flattened vesicles (Murray and Watson, 1965; Watson, 1965; Watson and Remsen, 1970). All cultured strains originate from marine environments, and all are urease positive. The G+C content of DNA is between 50 and 51 mol%.

Nitrosococcus halophilus (Koops et al., 1990) Both species are morphologically not distinguishable from *N. oceani*, but differ in salt requirement and salt tolerance as well as in ammonia tolerance (Table 3). The two cultured strains were isolated from an inland salt lake and from a salt lagoon in the Mediterranean Sea, respectively. In contrast to the cultured strains of *N. oceani*, the two isolates of *N. halophilus* are urease negative. The G+C content of DNA is 50–51 mol%.

Phylogeny of AOB

The phylogenetic framework of AOB has been established by comparative 16S rRNA gene sequence analysis of the cultured species. During the last years, this system has been significantly extended by including environmentally retrieved 16S rRNA gene sequences.

The Cultured AOB

The first phylogenetic analyses of AOB were carried out by Woese and co-workers in the 1980s (Woese et al., 1984; Woese et al., 1985) and demonstrated that two phylogenetically distinct groups of AOB exist. The major group, containing the genera *Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus*, belongs to the class Betaproteobacteria, while the second group of AOB, represented by two species of the genus *Nitrosococcus*, is affiliated with the class Gammaproteobacteria (see the chapter Oxidation of Inorganic Nitrogen Compounds as an Energy Source in Volume 2). These results, obtained from 16S rRNA oligonucleotide catalogues, were later confirmed and extended via comparative 16S rRNA gene sequence analysis (Head et al., 1993; Teske et al., 1994; Utaaker et al., 1995; Purkhold et al., 2000). Today, almost complete 16S rRNA gene sequences are available for the 14 described species of AOB affiliated with the Betaproteobacteria. Furthermore, more than 100 16S-rRNA gene sequences of other AOB isolates belonging to this class have been determined. Within the Betaproteobacteria, the AOB form a well-supported monophyletic evolutionary group (Head et al., 1993; Teske et al., 1994). The minimal 16S rRNA gene sequence similarity between recognized members of this group is 89.4% (*Nitrosococcus mobilis*/*Nitrosomonas nitrosa*). Figure 6 shows a phylogenetic 16S rRNA-based tree of those AOB (15 nitrosospiras and 19 nitrosomonads) demonstrated to represent different genospecies (DNA-DNA similarity less than 60% and/or 16S rRNA sequence similarity less than 97.5%). The genera *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* are closely related, and 16S rRNA phylogeny provides no convincing support for subdivision of this lineage into three genera, although morphological and ecophysiological differences exist between the different genera (see the section on General Characteristics of the Genera and Species of AOB in this Chapter). It has therefore been suggested these genera be lumped into the single genus *Nitrosospira* (Head et al., 1993). In contrast, the cultured nitrosomonads can be subdivided into six lineages which are consistently

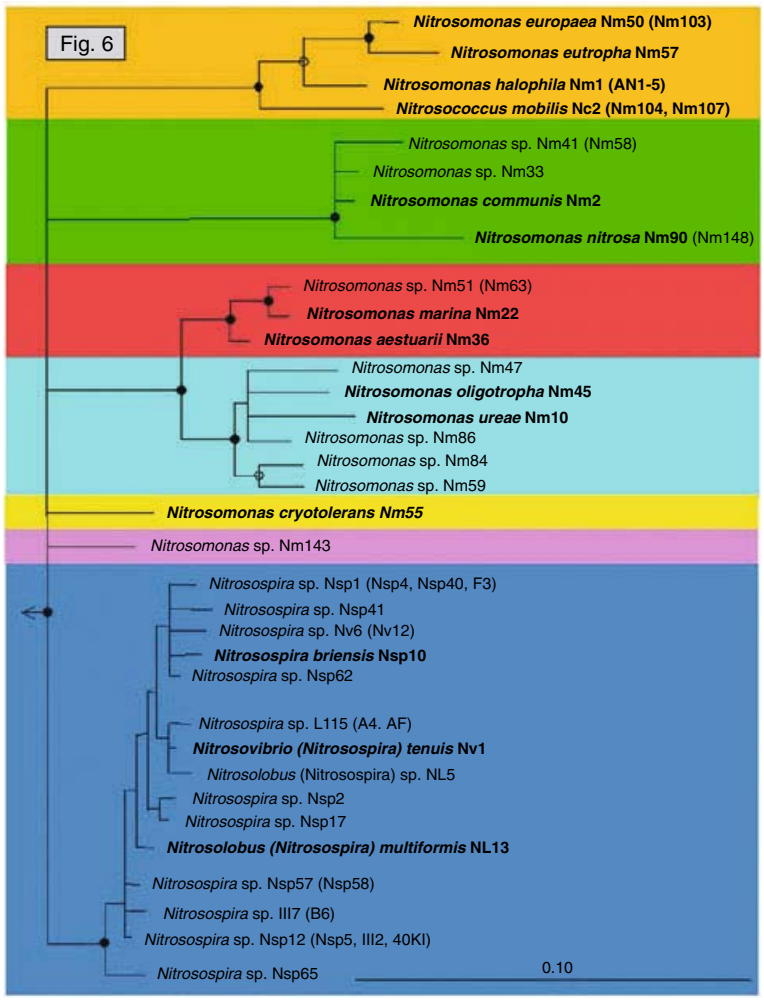


Fig. 6. 16S rRNA-based phylogenetic tree of the betaproteobacterial AOB. The tree includes only those AOB which have been demonstrated to represent different genospecies (DNA-DNA similarity < 60%) and for which 16S rRNA gene sequences longer than 1000 nucleotides are available. Strains with DNA-DNA similarity > 60% with each other are given in parenthesis after the respective species name. Described species are depicted in bold. Maximum likelihood, maximum parsimony, and neighbor-joining trees were calculated and merged. Multifurcations connect branches for which a relative order cannot be unambiguously determined by applying different treeing methods. Filled and empty dots indicate parsimony bootstrap values (100 resamplings) above 90% and 70%, respectively. Scale bar represents 10% estimated sequence divergence.

retrieved using different treeing methods and which (if consisting of more than one sequence) have parsimony bootstrap support of above 90%. This phylogenetic substructure is also retrieved if all betaproteobacterial AOB isolates (56 nitrosomonads and 48 nitrosospiras), for which a 16S rRNA sequence longer than 1000 bases has been determined, are included in the treeing analysis (Figs. 7 and 8). For the different groups within the nitrosomonads, different nomenclature systems are used in the literature (Pommerening-Röser et al., 1996; Stephen, 1996; Purkhold et al., 2000; Aakra et al., 2001b; see also Fig. 7). To avoid confusion, we herein propose a hierarchical system for subdivision of the betaproteobacterial AOB as displayed below for further use. New lineages should only be postulated if 1) almost full-length 16S rRNA sequences for the respective AOB exist and 2) the lineages can be retrieved with all treeing methods with high bootstrap support. The lineages are subdivided into recognized species and genospecies with each of these units possibly containing several strains. In the following, a brief list (consistent with the above rules) of those units within the betaproteobacterial AOB is presented:

Betaproteobacterial AOB
- Nitrosospira lineage
  - (Genus *Nitrosospira*)
  - (Genus *Nitrosolobus*)
  - (Genus *Nitrosovibrio*)
- *Nitrosomonas* group
  - *N. europaea*/*Nc. mobilis* lineage
  - *N. communis* lineage
  - *N. marina* lineage
  - *N. oligotropha* lineage
  - *N. cryotolerans* lineage
  - *Nitrosomonas* sp. Nm143 lineage

Although 16S rRNA phylogeny of the nitrosospiras does not reveal an obvious substructure, Stephen et al. (1996) suggested subdivision of this group into three "clusters" (clusters 2, 3 and 4, Fig. 8; cluster 1 contains no cultured species,

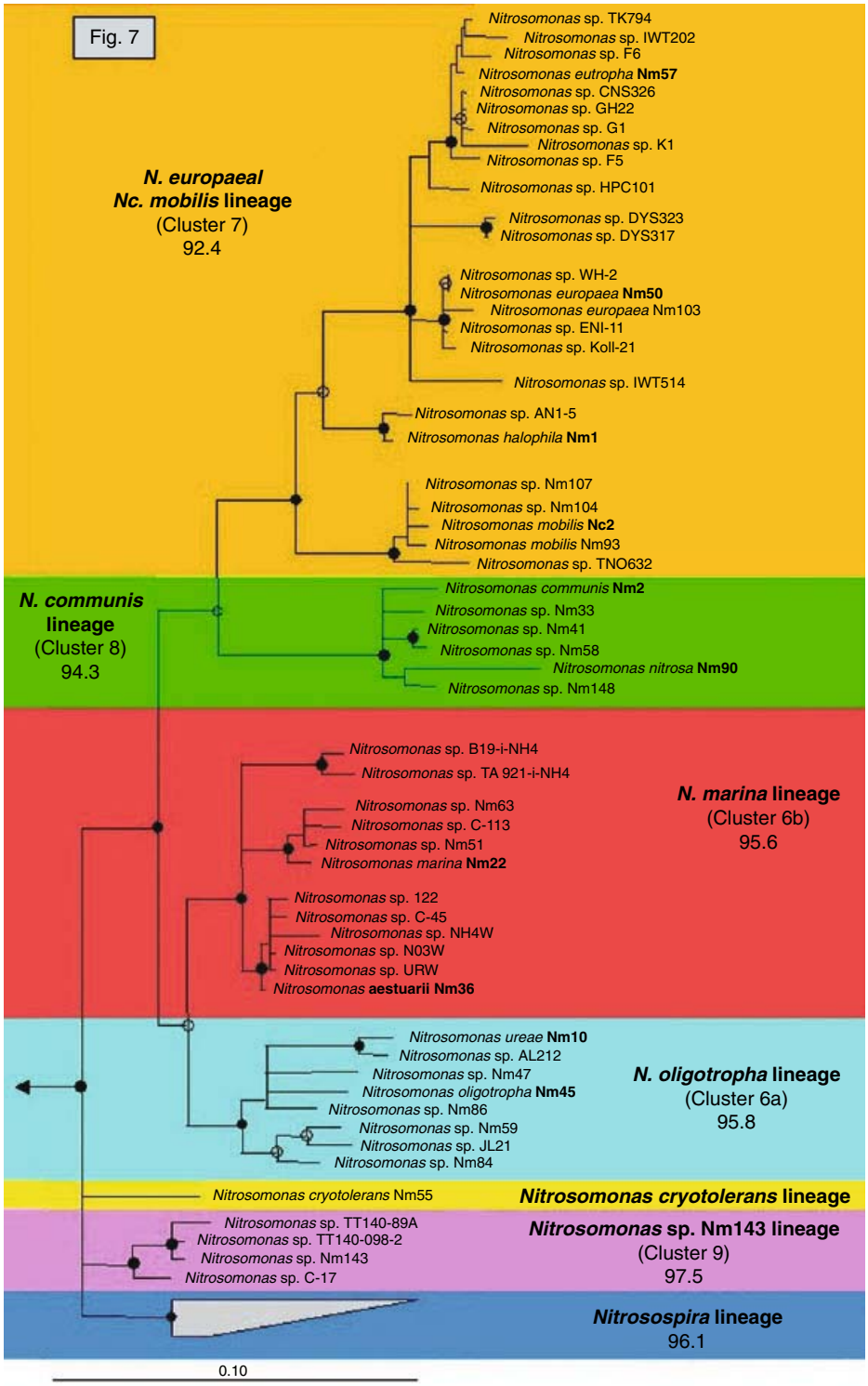


Fig. 7. 16S rRNA based phylogenetic tree of the nitrosomonads. The tree includes all isolates for which 16S rRNA gene sequences longer than 1000 nucleotides are available. Described species are depicted in bold. Maximum likelihood, maximum parsimony, and neighbor-joining trees were calculated and merged. Multifurcations connect branches for which a relative order cannot be unambiguously determined by applying different treeing methods. Filled and empty dots indicate parsimony bootstrap values (100 resamplings) above 90% and 70%, respectively. For each cluster, the minimum 16S rRNA sequence similarity between two of its members is depicted. Sequences included in the analysis were published by Head et al. (1993), Suwa et al. (1997), Juretschko et al. (1998), Sorokin et al. (1998), Aakra et al. (1999a), Aakra et al. (1999b), Yamagata et al. (1999), Purkhold et al. (2000), and Purkhold et al. (2002). Sequences of strains CNS326, G1, K1, IWT202, TK794, WH-2, Koll-21, DYS317, DYS323, IWT514, TNO632, marine bacteria C-45, NH4W, 122, URW, NO3W, C-113, TT140-098-2, TT140-89A, and estuarine bacteria TA 921-i-NH4, B19-i-NH4, C-17 are unpublished but available at GenBank. Scale bar represents 10% estimated sequence divergence.

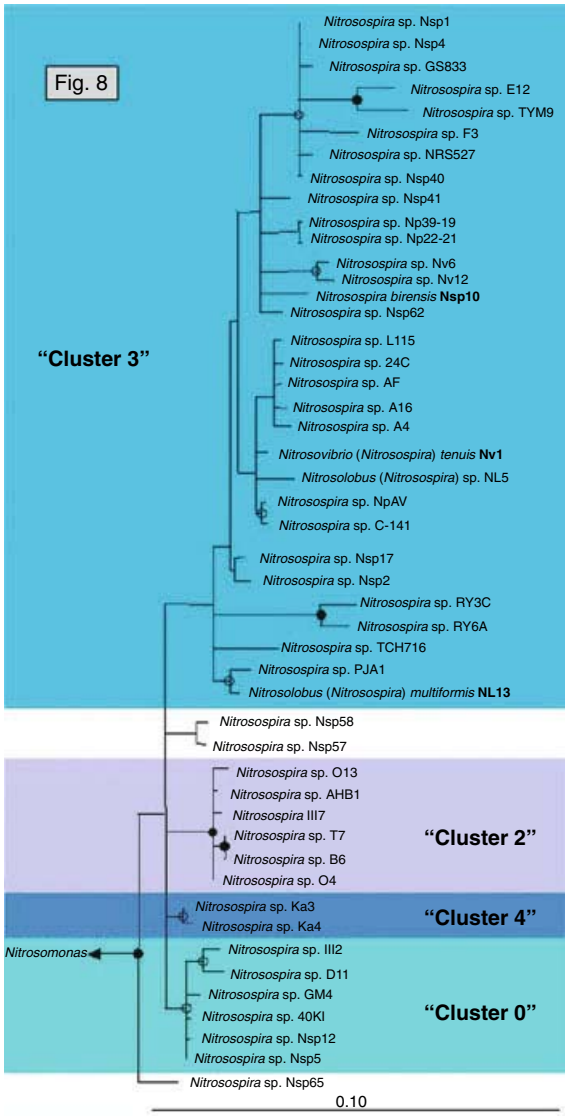


Fig. 8. 16S rRNA-based phylogenetic tree of the highly related genera *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio*. The tree includes all isolates for which 16S rRNA gene sequences longer than 1000 nucleotides are available. Described species are depicted in bold. Maximum likelihood, maximum parsimony, and neighbor-joining trees were calculated and merged. Multifurcations connect branches for which a relative order cannot be unambiguously determined by applying different treeing methods. Filled and empty dots indicate parsimony bootstrap values (100 resamplings) above 90% and 70%, respectively. Sequences included in the analysis were published by Head et al. (1993), Teske et al. (1994), Utaaker et al. (1995), Tokuyama et al. (1997), Aakra et al. (1999a), Aakra et al. (1999b), Aakra et al. (2001b), and Purkhold et al. (2002). Sequences of strains GS833, E12, NRS527, NpAV, RY6A, RY3C, TCH716, and PJA1 are unpublished but available at GenBank. Scale bar represents 10% estimated sequence divergence.

see the section on Environmental Sequences in this Chapter). Purkhold et al. (2000) subsequently extended this system by the proposing cluster 0. These nitrosospira clusters are found with all treeing methods but not all of them are well supported by bootstrap analysis. Therefore, clusters of the *Nitrosospira* lineage are not comparable with the distinct lineages within the *Nitrosomonas* group. This reflects the fact that the minimum 16S rRNA sequence similarity of 96.1% within the entire *Nitrosospira* group is higher than those within the individual, described *Nitrosomonas* lineages (Fig. 7). It should also be noted that DNA-DNA hybridization analyses suggest that the *Nitrosospira* clusters 0, 2 and 4 might each represent only one single species (H.-P. Koops, unpublished observation). Taken together, it is thus questionable whether subdivision of the *Nitrosospira* lineage into the above-mentioned clusters or other subdivisions (Aakra et al., 2001b) is justified.

Owing to the limited discriminatory power of comparative 16S rRNA analysis, in particular for the *Nitrosospira* lineage, other phylogenetic marker molecules were tested for phylogeny inference of AOB. Aakra and co-workers determined the 16S–23S rRNA gene intergenic spacer region (ISR) of several AOB and used these sequences for phylogenetic analysis (Aakra et al., 2001b). In that study, the authors postulated highly consistent ISR- and 16S rRNA-based tree topologies for AOB and thus suggested the usefulness of the ISR for future studies on AOB diversity and evolution. However, careful inspection of the phylogenetic trees presented in that publication reveal significant topological incongruencies between 16S rRNA and ISR trees. Owing to the high variability of the ISR, these sequences are very difficult to align and not suitable to reliably determine evolutionary relationships above the species level.

Recently, the *amoA* gene coding for the active site polypeptide of the ammonia monooxygenase has been used as an additional phylogenetic marker molecule for AOB (McTavish et al., 1993; Klotz and Norton, 1995; Rotthauwe et al., 1995; Suwa et al., 1997; Hommes et al., 1998; Alzerreca et al., 1999; Yamagata et al., 1999; Horz et al., 2000; Purkhold et al., 2000; Aakra et al., 2001a; Casciotti and Ward, 2001; Purkhold et al., 2003). PCR primers that allow amplification of a 453-bp fragment of this gene are generally used in these studies (Rotthauwe et al., 1997; modified by Stephen et al., 1999). Phylogeny inference based on the deduced amino acid sequence of the *amoA* gene fragment is overall consistent with the 16S rRNA phylogeny of AOB. Members of the genera *Nitrosospira*, *Nitrosolobus* and *Nitrosovibrio* form a tight monophyletic grouping with no obvious substructure. Within the

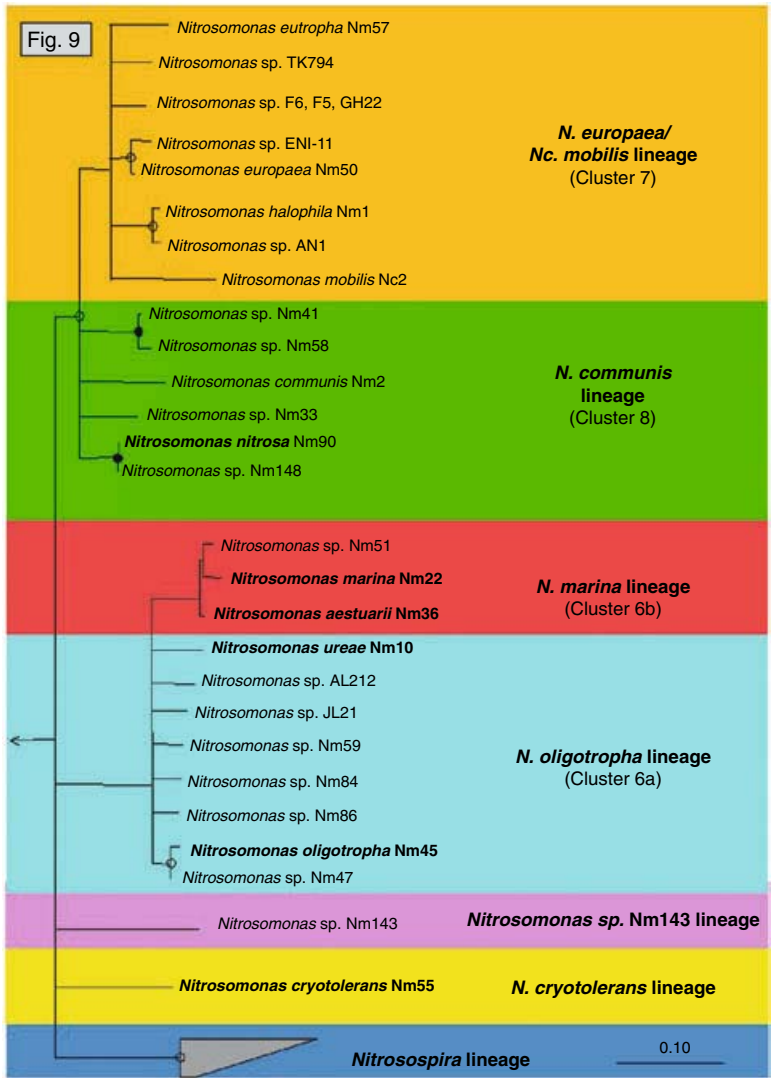


Fig. 9. AmoA-based phylogenetic tree of the betaproteobacterial AOB. Described species are depicted in bold. The 453-bp gene fragment obtainable with the most commonly used amoA PCR primers (Rotthauwe et al., 1995) was used for phylogeny inference. AmoA sequences shorter than 414 nucleotides were excluded from the analysis. Protein maximum likelihood, protein maximum parsimony, neighbor-joining, and Fitch trees were calculated and merged. Multifurcations connect branches for which a relative order cannot be unambiguously determined by applying different treeing methods. Filled and empty dots indicate parsimony bootstrap values (100 resamplings) above 90% and 70%, respectively. Sequences included in the analysis were published by McTavish et al. (1993), Holmes et al. (1995), Rotthauwe et al. (1995), Suwa et al. (1997), Yamagata et al. (1999), Aakra et al. (2001a), Casciotti et al. (2001), Sorokin et al. (2001), Norton et al. (2002), and Purkhold et al. (2003). Sequences of *Nitrosospira* sp. C-57 and *Nitrosomonas* sp. TK794 are unpublished but available at GenBank. Scale bar represents 10% estimated sequence divergence.

nitrosomonads the *N. europaea*/*Nc. mobilis* lineage and the *N. marina* lineage are also found with all treeing methods, while the *N. communis* and the *N. oligotropha* lineage are not always monophyletic (Fig. 9). If *amoA* nucleic acid sequences are used for phylogenetic analysis, basically the same picture emerges with the exception that the *N. europaea*/*eutropha* lineage is no longer monophyletic (data not shown). If compared to the 16S rRNA-based phylogeny of AOB, AmoA analysis does provide less resolution, reflecting that a relatively short (151 positions) and highly conserved (93 positions have an identical amino acid in at least 98% of the betaproteobacterial AOB) amino acid sequence stretch is used as marker. The information content of AmoA sequences could be significantly extended in future studies by the development of primers that allow the amplification of more complete *amoA* gene fragments. First attempts in this direction were recently published by Norton et al. (2002).

Correlation plots of *amoA* and AmoA similarity versus 16S rRNA similarity of all possible pairs of betaproteobacterial AOB isolates (Fig. 10) demonstrate that 1) 16S rRNA is more conserved than *amoA* or AmoA and 2) AOB showing below 80% *amoA* nucleic acid similarity (or 85% AmoA amino acid similarity) always possess less than 97.0% 16S rRNA sequence similarity (the currently accepted 16S rRNA threshold value for bacteria genospecies assignment; Stackebrandt and Goebel, 1994). AmoA sequences of a new AOB isolate with less than 80% nucleic acid similarity (or 85% amino acid similarity) to described AOB species are thus indicative of a previously undiscovered species. An *amoA* or AmoA sequence with a higher similarity to a described AOB species can represent multiple gene copies, different strains

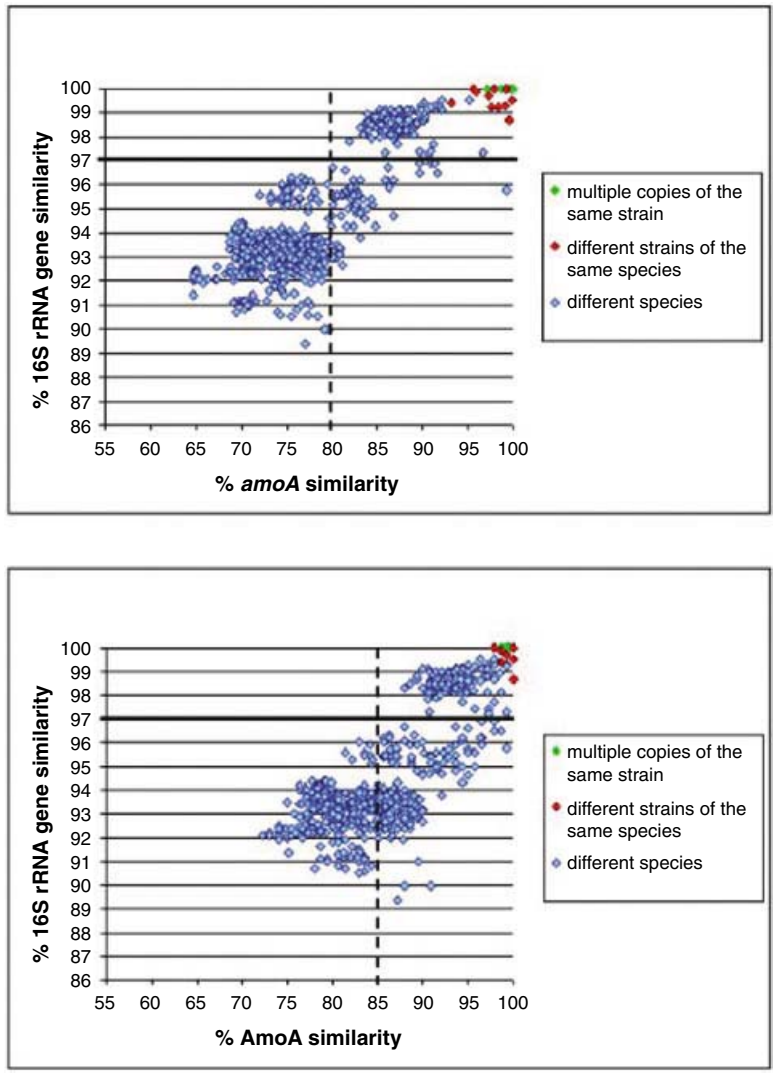


Fig. 10. Correlation plots of 16S rRNA similarity with amoA (panel A) and AmoA (panel B) similarity values. Solid lines indicate 16S rRNA threshold values for species delineation. Dotted lines indicate the amoA and AmoA threshold values below which amoA and AmoA sequences are indicative of novel AOB species.

of this species, or a novel AOB species. The latter possibility exists since 16S rRNA similarities between different species can be higher than 97%.

Environmental Sequences

Molecular diversity surveys of AOB almost exclusively focussed on betaproteobacterial AOB. Stephen et al. (1996) were the first to investigate the diversity of these bacteria in an environmental sample using directly retrieved 16S rRNA gene sequences (see the section on Molecular Techniques in this Chapter). Since then, AOB species richness of numerous natural and engineered systems has been analyzed by the 16S rRNA approach. However, the PCR primers applied in several of these studies do not cover all recognized AOB and thus probably provided a biased view of the natural AOB diversity and their environmental distribution (see the section on Molecular Techniques in this Chapter). Interestingly, of the 508 16S-rRNA clones affiliated with betaproteobacterial AOB (status August 2002), more than 83% cluster with cultured representatives of this guild. The remaining clones form two novel clusters, one within the nitrosomonads (environmental lineage 5) and one within the nitrosospiras (cluster 1; Fig. 11). On the basis of the results of the various AOB 16S rRNA diversity surveys, environmental distribution patterns of the different AOB clusters can be revealed and compared to the findings obtained with cultivation-based methods (Fig. 12). A more detailed discussion of environmental distribution patterns of the different AOB is provided in a separate section (see the section on Distribution of AOB in Nature in this Chapter).

The natural diversity of ammonia oxidizers in various environments also has extensively been studied by comparative sequence analysis

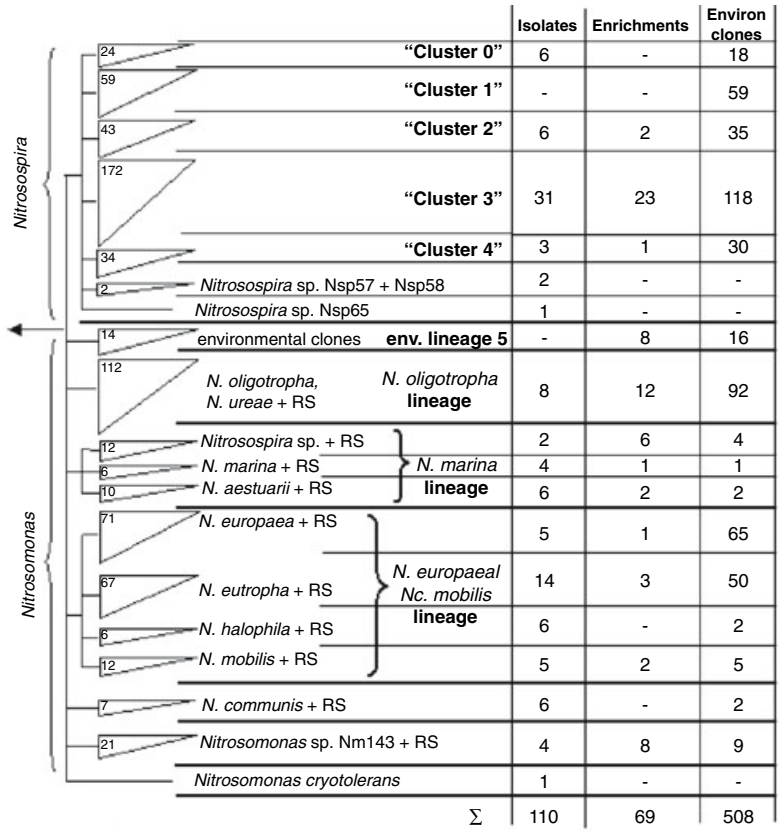


Fig. 11. Schematic 16S rRNA-based phylogenetic classification of the betaproteobacterial AOB. Multifurcations connect branches for which a relative order could not be unambiguously determined by applying different treeing methods. The height of each triangle represents the number of sequences in the cluster. It should be noted that 16S rRNA sequences shorter than 1000 nucleotides were only considered if they could be unambiguously assigned to one of the lineages. All other short 16S rRNA sequences had to be omitted because their phylogenetic analysis cannot reliably be performed (Ludwig et al., 1998). The 16S rRNA sequences used in this analysis were published by Takahashi et al. (1992), Head et al. (1993), McCaig et al. (1994), McCaig et al. (1999), Teske et al. (1994), Rotthauwe et al. (1995), Utaaker et al. (1995), Pedersen et al. (1996), Stephen et al. (1996), Kowalchuk et al. (1997), Kowalchuk et al. (1998), Kowalchuk et al. (2000a), Kowalchuk et al. (2000b), Suwa et al. (1997), Tokuyama et al. (1997), Juretschko et al. (1998), Logemann et al. (1998), Princic et al. (1998), Speksnijder et al. (1998), Aakra et al. (1999a), Aakra et al. (1999b), Aakra et al. (2000), Aakra et al. (2001b), Bruns et al. (1999), Mendum et al. (1999), Philips et al. (1999), Philips et al. (2000), Radeva et al. (1999), Whitby et al. (1999), Whitby et al. (2001a), Whitby et al. (2001b), Yamagata et al. (1999), Abd El Haleem et al. (2000), Bano et al. (2000), Ward et al. (2000), Bollmann et al. (2001), Burrell et al. (2001), Chang et al. (2001), de Bie et al. (2001), Daims et al. (2001a), Gieseke et al. (2001), Smith et al. (2001), Sorokin et al. (2001), Hollibaugh et al. (2002), Nicolaisen et al. (2002), Regan et al. (2002), and Purkhold et al. (2003). In addition, unpublished 16S rRNA sequences (Accession numbers: AF107527, AJ441258, AJ441259, AJ441260, AJ441261. AJ441262, AJ441263, AJ441264, AJ441265, AJ441266] AJ441267, AJ441268, AJ441269, AJ441270, AJ441271, AJ441272], AJ441273, AJ441274, AJ441275, AJ441276, AJ441277, AJ441278, AJ441279, AJ441280, AJ441281, AJ441282, AJ441283, AJ441284, AJ441285, AF034139, AF034140, AF034141], AF034142, AF034143, AF034144, AF034147, AJ318197, U57617, AF510862, AF510863, AF510864, AF510865, AJ245751, AJ245752, AJ245753, AJ245754, AJ245755, AJ245756, AJ245757, AJ245758, AJ245759, AJ245760, AJ431350, AJ431351], AF414581, Y10128, Y10127, AJ224941, AF353155, AF353156, AF353157, AF353158, AF353159, AF353161, AF353162, AF353163, AF353164, AF359341, AF363287, AF363289, AF363290, AF363291, AF363292, AF363293, and A036898) available in GenBank were analyzed. RS: related sequences.

of environmentally retrieved *amoA* clones (Rotthauwe et al., 1997; Helmer et al., 1999; Stephen et al., 1999; Baribeau et al., 2000; Horz et al., 2000; Ivanova et al., 2000; Nold et al., 2000; Purkhold et al., 2000; Schmid et al., 2000; Chang et al., 2001; Gieseke et al., 2001; Oved et al., 2001; Dionisi et al., 2002; Sakano et al., 2002). In total, 383 *amoA* clones are currently available from these studies (status August 2002). Consistent with the 16S rRNA-based AOB diversity surveys, most *amoA* clones obtained are affiliated with sequence clusters

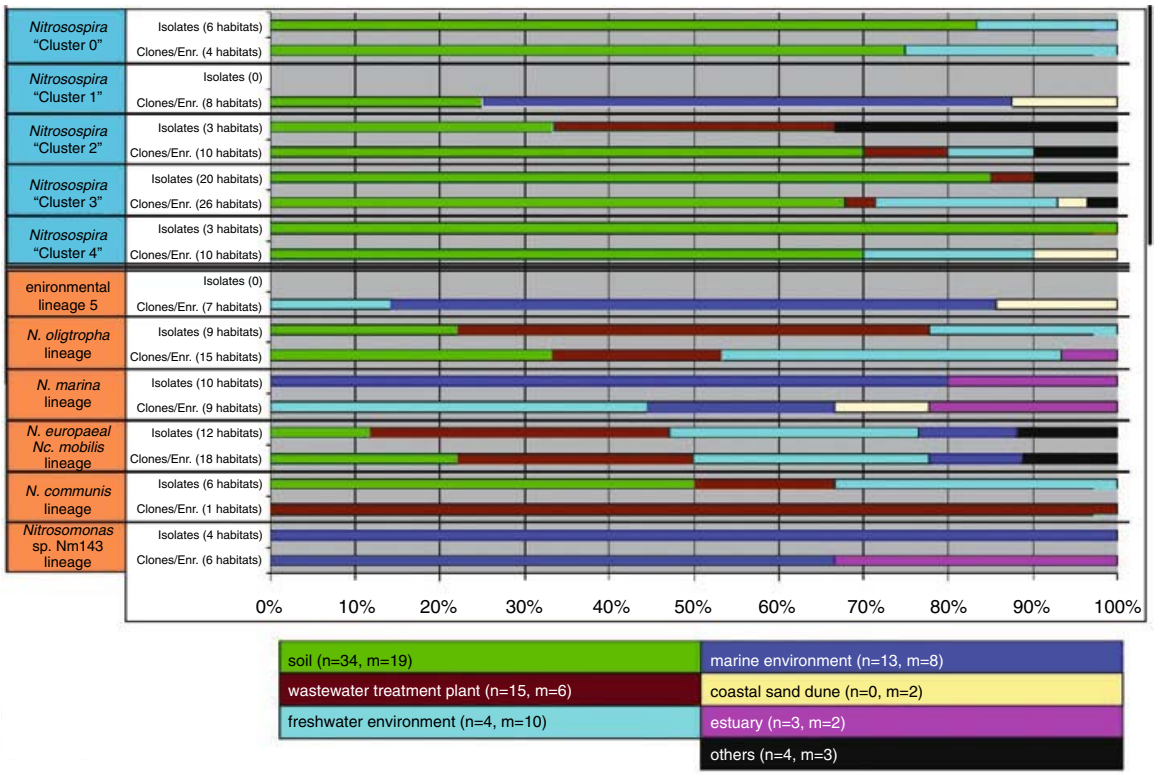


Fig. 12. Environmental distribution patterns of AOB clusters. For each cluster, the origin (sampling site) of each 16S rRNA sequence belonging to this cluster was determined. Subsequently, we counted the number of different sampling sites in which members of a particular cluster were detected. This number of habitats, which is given in parentheses, was set to 100%. In the next step, the sampling sites were grouped into the categories wastewater treatment plants, soil (including a rhizoremediation plant), freshwater (including sediment), marine (including sediment), estuary, and coastal sand dune. All other habitats were lumped together. For each cluster the proportion of each category is shown. For comparison, an identical analysis was performed of the available isolates for each cluster. The displayed legend explains the color-coding of the habitats. In addition, the number of different samples of a particular habitat category analyzed by the 16S rRNA approach (n) or cultivation (m) is given. For example, 16S rRNA AOB clones are available from 34 different soil samples and 19 soils were examined by AOB cultivation.

defined by cultured ammonia oxidizers (Table 4). The environmental distribution patterns of AOB inferred from AmoA analysis (Fig. 13) confirm the results from the 16S rRNA studies (Fig. 12), although the limited resolution provided by AmoA does not allow specific data for the individual *Nitrosospira* clusters to be extracted or the *N. oligotropha* to be distinguished from the *N. marina* cluster. According to the threshold values inferred from the *amoA*/16S rRNA correlation plots (Fig. 10), only two (clone GLII-9 from a wastewater treatment plant [wwtp] and clone Plus8ee from a lake) of the environmental *amoA* clones probably represent novel AOB species. These observations confirm that ammonia oxidizers already deposited in culture collections might be surprisingly representative of the natural diversity within this guild. However, it is important to stress that the sequences from 16S rRNA or *amoA* gene libraries from environmental samples rarely contain sequences completely identical to those of cultured organisms. Since no DNA-DNA hybridization data can be obtained for noncultured organisms, it can, according to the current species definition in bacteriology (Stackebrandt et al., 1988), not be decided whether these sequences represent novel or known ammonia oxidizer species. In this context, one should however bear in mind that PCR and cloning procedures introduce microvariation artifacts in cloned sequences (Speksnijder et al., 2001) and thus might cause (at least a part of) the high degree of microheterogeneity observed for AOB sequence clusters detected in environmental 16S rRNA and *amoA* clone libraries.

Although the 16S rRNA and *amoA* library approaches provided valuable insights into the natural diversity of AOB, the dependency of these techniques on nucleic acid extraction, PCR and cloning heavily biases the results (Wintzigerode and Goebel, 1997). Understand-

Table 4. Phylogenetic affiliation of amoA sequences obtained from environmental samples. AmoA-sequences analyzed for this Table were obtained from Baribeau et al. (2000), Chang et al. (2001), Dionisi et al. (2002), Gieseke et al. (2001), Ivanova et al. (2000), Helmer et al. (1999), Henckel et al. (1999), Holmes et al. (1999), Horz et al. (2000), Nold et al. (2000), Oved et al. (2001), Purkhold et al. (2000), Purkhold et al. (2003), Rotthauwe et al. (1997), Sakano et al. (2002), Schmid et al. (2000), and Stephen et al. (1999). In addition, unpublished environmental amoA sequences determined by the authors or available in GenBank (Accession numbers: AJ277459], AF338319, AF338320], AF239878, AF239879, AF239880, AF239881, AF239882, AF239883, AF239884], AJ388568, AJ388569, AJ388570, AJ388571, AJ388572, AJ388573, AJ388574, AJ388575, AJ388576, AJ388577, AJ388578, AJ388579, AJ388580, AJ388581, AJ388582, AJ388583, AJ388588, and AJ388589) were used.

	Total	Isolates	Enrichments	Environmental clones
Nitrosospira cluster	152	37	3	112
N. marina and *N. oligotropha* cluster	116	16	0	100
N. europaea/Nc. mobilis cluster	156	16	0	140
N. communis cluster	37	6	0	31
Nitrosomonas sp. Nm143 cluster	1	1	0	0
N. cryotolerans cluster	1	1	0	0
Σ	463	77	3	383

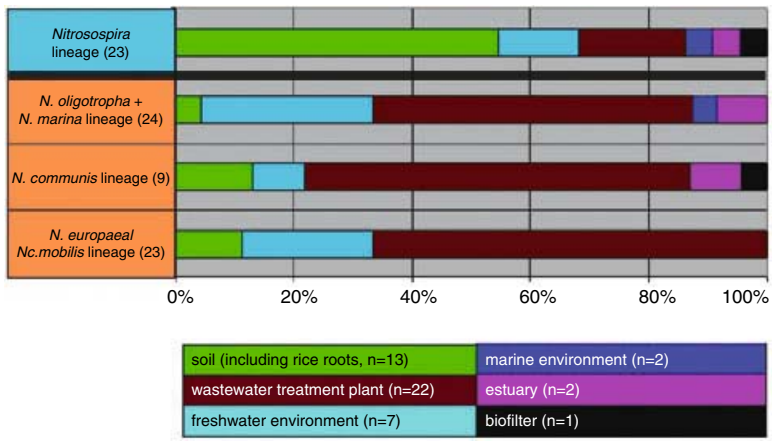


Fig. 13. Environmental distribution patterns of AOB clusters based on AmoA analysis. For each cluster, the origin (sampling site) of each AmoA sequence belonging to this cluster was determined. This analysis only included sequences from enrichments and sequences directly retrieved from the environment. Subsequently, we counted the number of different sampling sites in which members of a particular cluster were detected. This number, which is given in parentheses after the cluster designation, was set to 100%. In the next step, the sampling sites were grouped into the categories wastewater treatment plants, soil, freshwater (including sediment), estuary, biofilter, and marine (including sediment). For each cluster, the proportion of each category is shown. The displayed legend explains the color-coding of the habitats. In addition, the number of different sampling sites of a particular habitat category analyzed by the 16S rRNA approach (n) is given. Please note that the depicted grouping does not reflect the phylogenetic relation but results from the poor resolution of the amplified amoA gene fragment.

ing the ecology of AOB requires quantitative data on their in situ abundance and activity in ecosystems. The AOB community composition in a system can be quantitatively analyzed by using rRNA-targeted probes in combination with dot blot or in situ hybridization techniques (see the section on Molecular Techniques in this Chapter). However up to now, the AOB composition of only wastewater treatment reactors and full-scale plants has been characterized with these methods (Wagner et al., 1995; Mobarry et al., 1996; Juretschko et al., 1998; Schramm et al., 1999; Schramm et al., 2000; Daims et al., 2001b).

Methods Useful for In Situ Detection of AOB

Classical Techniques

Traditionally, the abundance, diversity and ecology of AOB were investigated by cultivation-dependent methods. For the enumeration of

viable AOB, the most probable number (MPN) technique often has been used, sometimes in parallel with other techniques. The efficiency of MPN has been calculated from comparisons with results obtained with fluorescent antibody (FA) counting (Belser, 1979; Ward, 1982) or calculations of activity per cell (Belser and Mays, 1982). Results obtained from these comparisons have led to the suggestion that the MPN efficiency may range between 0.001 and 0.05% (Hall, 1986). One important reason of underestimating AOB with MPN may be that AOB often form cell aggregates in the environment (Wagner et al., 1995; Stehr et al., 1995b; Juretschko et al., 1998; Koops and Pommerening-Röser, 2001). Another reason may be that the media used in MPN counting of AOB often have not been well adapted (ammonia concentration, salinity, and pH value) to the conditions in the environment under investigation (Koops and Pommerening-Röser, 2001).

Qualitative characterization of natural AOB populations is a very time consuming process. This especially holds true if classical methods are applied. The isolation of numerically dominant species will take at least some months (see the section on Enrichment, Isolation, and Maintenance in this Chapter). Moreover, considerable experience in working with AOB in the laboratory is required. However, this approach is currently the only option that allows the physiological properties of those species representing the numerically dominant AOB population in the respective environment to be completely investigated. These findings can help determine why distinct species of AOB are dominant in certain environments.

Phenotypic identification of new isolates of AOB is possible if the strain belongs to one of those species already being described in the literature. Useful parameters are the physiological properties (Ks values of ammonia-oxidation, requirement or tolerance of increased salt concentrations, and possession of urease), morphological characteristics (shape and size of cells, arrangement of intracytoplasmic membranes, and possession of carboxysomes), and G+C content of the DNA. For unambiguous identification, DNA-DNA hybridization with the type strain of the respective species should be carried out.

Immunological Techniques

In the 1970s, the cultivation-dependent approach was complemented by the fluorescent antibody technique, which allowed (for the first time) members of this guild to be detected in situ. Generally, polyclonal antibodies against several AOB were produced by injecting rabbits with the respective cells. Several studies demonstrated in situ visualization of AOB in environmental samples using these antisera for immunofluorescence and claimed that this approach allows a more rapid and accurate enumeration of AOB compared to enrichment culture methods (Belser, 1979; Ward and Perry, 1980; Ward and Carlucci, 1985; Abeliovich, 1987; Völsch et al., 1990). Although the fluorescent antibody (FA) approach represented a significant step forward in the history of research on nitrifier ecology, this technique has several disadvantages. Most importantly, pure cultures of AOB from the environment under investigation are required to produce the antibodies, since different serological groups exist within at least some of the AOB species. Therefore, the FA-technique, although allowing direct in situ detection of AOB, is indirectly dependent on successful cultivation of the target organisms. Consequently, this technique will not allow identification of novel, previously uncultured AOB species. Furthermore, the various polyclonal antibodies raised in different laboratories against different AOB are not easily available and thus their use is often restricted to a few laboratories. Another major disadvantage of the FA-technique is that the specificity of the obtained antibodies cannot be tailored in silico (i.e., using a computer) and must be determined in time-consuming experimental screening assays using different pure cultures. Finally, the FA-technique potentially suffers from false negative and false positive signals. The false negative signals might result from penetration problems of the relatively large antibodies in dense flocs or biofilms. False-positive signals were described as a result from nonspecific antibody binding to extracellular material or detritus (Szwerinski et al., 1985).

Recently, polyclonal antibodies against the purified β subunit of the ammonia monooxygenase (AmoB) of *Nitrosomonas eutropha* were shown to target all betaproteobacterial AOB by Western blot analysis (Pinck et al., 2001). After appropriate AOB cell permeabilization (Bartosch et al., 1999), the α AmoB antibodies are also suitable for immunofluorescence-based group-specific detection of betaproteobacterial AOB (E. Spieck, personal communication). Since these polyclonal antibodies target a key enzyme of the betaproteobacterial AOB, this assay is expected to be highly specific and will probably also allow yet unknown members of this group to be targeted. In the future, this technique most probably can be standardized to become the first choice method for general counting of betaproteobacterial AOB in those environmental samples that are well suited for FA application.

Molecular Techniques

After the establishment of 16S rRNA-based phylogeny of cultured AOB (Head et al., 1993; Teske et al., 1994; Pommerening-Röser et al., 1996; see the section on Phylogeny of AOB in this Chapter), many different 16S rRNA-targeted PCR primers and probes for dot blot or fluorescence in situ hybridization (FISH) have been developed (Table 5). Most frequently the natural diversity of AOB has been investigated by comparative analyses of PCR amplified and cloned AOB 16S rRNA gene fragments (see the section on Distribution of AOB in Nature in this Chapter). Apparently, the specificity of the applied PCR primers dramatically determines the outcome of such molecular AOB diversity surveys. In this context, it is important to note that the actual specificity of many primers used in previous investigations significantly differs from the indicated (intended) specificity listed in the original publications (Tables 6 and 7). These inconsistencies were detected after re-evaluating the primer/probe specificities based on the current, encompassing AOB 16S rRNA gene database. For example the PCR primer NitA, which is frequently used for AOB diversity surveys (Abd El Haleem et al., 2000; Ward et al., 2000; Hollibaugh et al., 2002), shows mismatches to all cultured nitrosospiras, all members of the *N. communis* and *N. oligotropha* lineage, and most members of the *N. marina* lineage. To avoid such pronounced biases, the use of PCR primers with high sensitivity (targeting all known or at least most betaproteobacterial AOB) but relatively low specificity (not perfectly excluding other bacteria) is recommended. Furthermore, the amplified 16S rRNA gene fragment must have a size larger than 1000 nucleotides to allow reliable phylogeny inference. The above-mentioned criteria are currently best fulfilled by the β AMOf and β AMOr primer pair. This approach accepts the amplification of non-AOB 16S rRNA gene fragments, which subsequently have to be identified by phylogenetic analysis or hybridization with probes with excellent specificity (e.g., probe Nso1225).

Recently, the toolbox for AOB detection has been extended by analyzing PCR amplified 16S rRNA gene fragments with denaturing gradient gel electrophoresis (DGGE; Kowalchuk et al., 1997; Nicolaisen and Ramsing, 2002). This fingerprinting approach allows a rapid comparative assessment of AOB diversity in many samples but requires subsequent controls like cloning and sequencing of bands or hybridization of membrane-blotted DGGE gels with suitable probes. Furthermore, the length of PCR amplificates that can be analyzed is limited to approximately 500 bp, which limits primer selection and hampers phylogeny inference.

Quantitative analysis of AOB community composition and abundance in the environment requires the use of methods not affected by PCR biases (Wintzigerode and Goebel, 1997). For this purpose, both dot blot and FISH methods have been developed and applied for AOB diversity research (Wagner et al., 1995; Mobarry et al., 1996; Schramm et al., 1996; Juretschko et al., 1998; Okabe et al., 1999; Liebig et al., 2001; Nogueira et al., 2002). The major advantage of dot blot analysis is that it can be applied to almost all environmental samples, while FISH does not work quantitatively in samples having a high autofluorescence or containing nontransparent particles (like soils). On the other hand, FISH allows visualization of the target bacteria and quantification of the AOB community composition and absolute AOB cell numbers (Daims et al., 2001c), while dot blot analysis only provides information on the relative or absolute abundance of probe target rRNA molecules. Whether, for example, a high rRNA content of a certain AOB species in an environmental sample originates from many target cells with a low rRNA concentration or a few cells with a high rRNA concentration cannot be figured out by dot blot hybridization.

It has been postulated that the rRNA content of a bacterial cell is directly related to its physiological activity. It must however be noted that this link does not always hold true for AOB. Quantitative FISH experiments showed that AOB have high cellular ribosome contents even after prolonged complete physiological inhibition (Wagner et al., 1995) or starvation (Morgenroth et al., 2000). Therefore, physiological activity of AOB cannot reliably be inferred from the above-mentioned methods and must be determined for example by combining FISH and microautoradiography (Lee et al., 1999), FISH and microelectrodes (Schramm et al., 1999), or stable isotope probing (Whitby et al., 2001a).

In addition to rRNA-based methods, the gene encoding the active site polypeptide of the ammonia monooxygenase (*amoA*) is frequently used as phylogenetic marker for AOB in environmental diversity surveys. Like the 16S rRNA database, the database of *amoA* sequences of cultured AOB is encompassing (see the section on Phylogeny of AOB in this Chapter). Several primer pairs for specific amplification of *amoA* have been published (Holmes et al., 1995; Rotthauwe et al., 1995; Sinigalliano et al., 1995; Mendum et al., 1999; Webster et al., 2002) but only the primer pair described by Rotthauwe et al. (1995), amplifying 55% of the *amoA* gene and slightly modified later by Stephen et al. (1999), has been shown to be suitable for detecting all tested betaproteobacterial AOB (Purkhold et al., 2000). The *amoA* approach for

Table 5. List of published 16S rRNA-targeted primers and probes. Probes that have been successfully used for FISH are shaded. Listed are specificities of primers and probes as given in the original publications. For an overview of the actual number of mismatches of each primer or probe with the 16S rRNA of all ammonia-oxidizer isolates please refer to Tables 6 and 7.

Probe/primer	Target site	Specificity[a]	References
NM-75	67–86	Terrestrial *Nitrosomonas* spp./*Nitrosococcus mobilis*	Hiorns et al., 1995
NS-85	76–95	*Nitrosospira* spp.	Hiorns et al., 1995
NmII[b]	120–139	*Nitrosomonas communis* lineage	Pommerening-Röser et al., 1996
NSMR32f	125–141	*Nitrosospira tenuis*-like AOB	Burrell et al., 2001
NSMR71f	126–143	*Nitrosomonas marina*-like AOB	Burrell et al., 2001
NSMR34	131–149	*Nitrosospira tenuis*-like AOB	Burrell et al., 2001
NSMR76[b]	132–149	*Nitrosomonas marina*-like AOB	Burrell et al., 2001
NitA	136–158	βAOB	Voytek and Ward, 1995
βAMOf	142–162	βAOB	McCaig et al., 1994
NSPM	145–162	βAOB	Silyn-Roberts and Lewis, 2001
Nm0	148–165	*Nitrosomonas* spp.	Pommerening-Röser et al., 1996
Nsm 156[b]	155–173	*Nitrosomonas* spp./*Nitrosococcus mobilis*	Mobarry et al., 1996
NmV[b]	174–191	*Nitrosococcus mobilis*	Pommerening-Röser et al., 1996
CTO189f A/B-GC	189–207	βAOB	Kowalchuk et al., 1997
CTO189f C-GC	189–207	βAOB	Kowalchuk et al., 1997
Nso 190[b]	189–207	βAOB	Mobarry et al., 1996
Noli191[b]	191–208	*Nitrosomonas* cluster 6a	Gieseke et al., 2001; Rath, 1996
TAOfwd	192–208	Terrestrial AOB	Chandler et al., 1997
NM198	198–218	*Nitrosomonas ureae* + *Nitrosomonas* sp. AL212	Suwa et al., 1997
NmoCL6a_205	205–221	*Nitrosomonas* cluster 6a	Stephen et al., 1998
NmI	210–225	*Nitrosomonas europaea*-lineage	Pommerening-Röser et al., 1996
Nmo218[b]	218–236	*Nitrosomonas oligotropha*-lineage	Gieseke et al., 2001
TMP1	226–253	AOB	Hermansson and Lindgren, 2001
β-AO233	233–249	βAOB	Stephen et al., 1998
NspCL1_249	249–266	*Nitrosospira* cluster 1	Stephen et al., 1998
Nmo254a	254–271	All *Nitrosomonas*	Stephen et al., 1998
Nmo254	254–271	All *Nitrosomonas*	Stephen et al., 1998
RT1r	283–304	AOB	Hermansson and Lindgren, 2001
AAO258	258–277	Terrestrial βAOB	Hiorns et al., 1995
Primer 356f	356–372	Nested PCR in NitAB amplicons	Hollibaugh et al., 2002
NmoCL6b_376	376–392	*Nitrosomonas* cluster 6b	Stephen et al., 1998
Nsp436	436–453	All *Nitrosospira*	Stephen et al., 1998
NmoCL7_439	439–456	*Nitrosomonas* cluster 7	Stephen et al., 1998
Nm439	439–459	*Nitrosomonas ureae* + *Nitrosomonas* sp. AL212	Suwa et al., 1997
NitD	439–461	*Nitrosomonas europaea*	Ward et al., 1997
NMOB1f	442–461	*Nitrosococcus mobilis*-like AOB	Burrell et al., 2001
NSMR52f	443–461	*Nitrosomonas europaea*-like AOB	Burrell et al., 2001
Nsv 443[b]	443–461	*Nitrosospira* spp.	Mobarry et al., 1996
NspCL4_446	446–463	*Nitrosospira* cluster 4	Stephen et al., 1998
Nsp0	452–469	*Nitrosospira* spp.	Pommerening-Röser et al., 1996
NspCL3_454	454–471	*Nitrosospira* cluster 3	Stephen et al., 1998
NspCL2_458	458–475	*Nitrosospira* cluster 2	Stephen et al., 1998
Nlm 459r	458–477	*Nitrosospira multiformis*/*Nitrosospira* sp. C-141	Hastings et al., 1997
NSM1B	478–494	*Nitrosomonas europaea*-lineage/*Nitrosococcus mobilis*	Hovanec and DeLong, 1996
Primer 517r	517–533	Nested PCR in NitAB amplicons	Hollibaugh et al., 2002
TAOrev	632–649	Terrestrial ammonia oxidizers	Chandler et al., 1997
CTO654r	632–653	βAOB	Kowalchuk et al., 1997
NITROSO4E[b]	638–657	βAOB	Hovanec and DeLong, 1996
NEU[b]	651–668	Most halophilic and halotolerant *Nitrosomonas*	Wagner et al., 1995
Amβ	738–758	βAOB	Utaaker and Nes, 1998
NitF	844–862	βAOB	Ward et al., 1997
NitC	846–862	βAOB	Voytek and Ward, 1995
NmIII	998–1018	*Nitrosomonas marina*-lineage	Pommerening-Röser et al., 1996
NSMR53r	999–1017	*Nitrosomonas europaea*-like AOB	Burrell et al., 2001
NSMR74r	1000–1017	*Nitrosomonas marina*-like AOB	Burrell et al., 2001
NMOB1r	1006–1026	*Nitrosococcus mobilis*-like AOB	Burrell et al., 2001
NSMR33r	1006–1021	*Nitrosospira tenuis*-like AOB	Burrell et al., 2001
RNM-1007	1005–1028	Terrestrial *Nitrosomonas* spp.	Hiorns et al., 1995
NS-1009	1007–1026	*Nitrosospira* spp.	Hiorns et al., 1995
NmIV[b]	1004–1022	*Nitrosomonas cryotolerans*-lineage	Pommerening-Röser et al., 1996
NitB	1213–1233	βAOB	Voytek and Ward, 1995
Nso 1225[b]	1224–1243	βAOB	Mobarry et al., 1996
βAMOr	1295–1314	βAOB	McCaig et al., 1994
Nse 1472[b]	1472–1489	*Nitrosomonas europaea*-lineage	Juretschko et al., 1998

[a]Listed are specificities of primers and probes as given in the original publications. For an overview of the actual number of mismatches of each primer or probe with the 16S rRNA of all ammonia-oxidizer isolates, please refer to Tables 6 and 7.
[b]Probe has been successfully used for FISH.

Table 6. Specificity and sensitivity of published 16S rRNA (gene) targeting PCR primers and hybridization probes for nitrosospiras. Abbreviations: NTO, number of non-target organisms outside the AOB with no mismatch (0 MM) or one mismatch (1 MM) in its 16S rRNA (gene) to the respective primer/probe.[a]

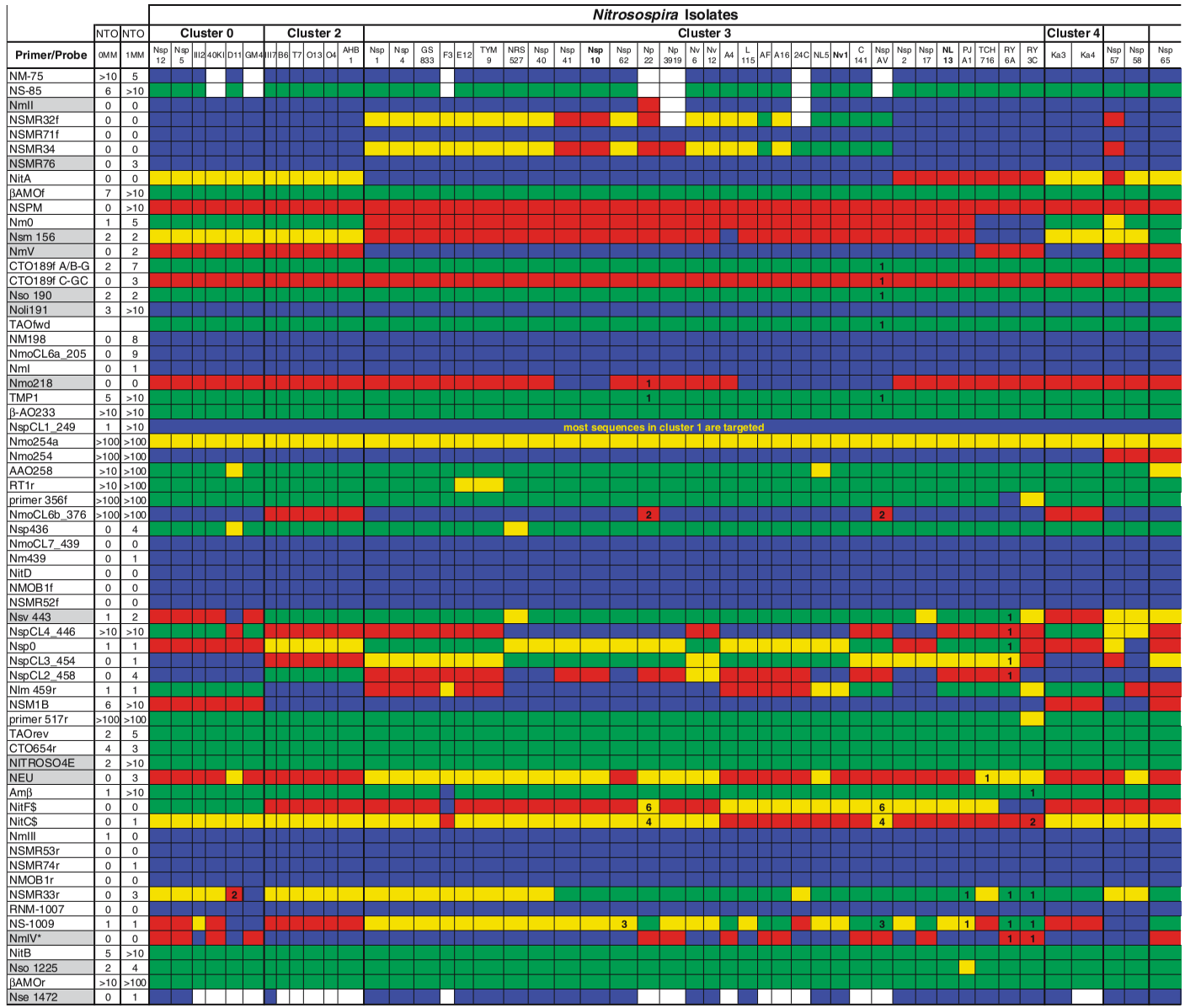
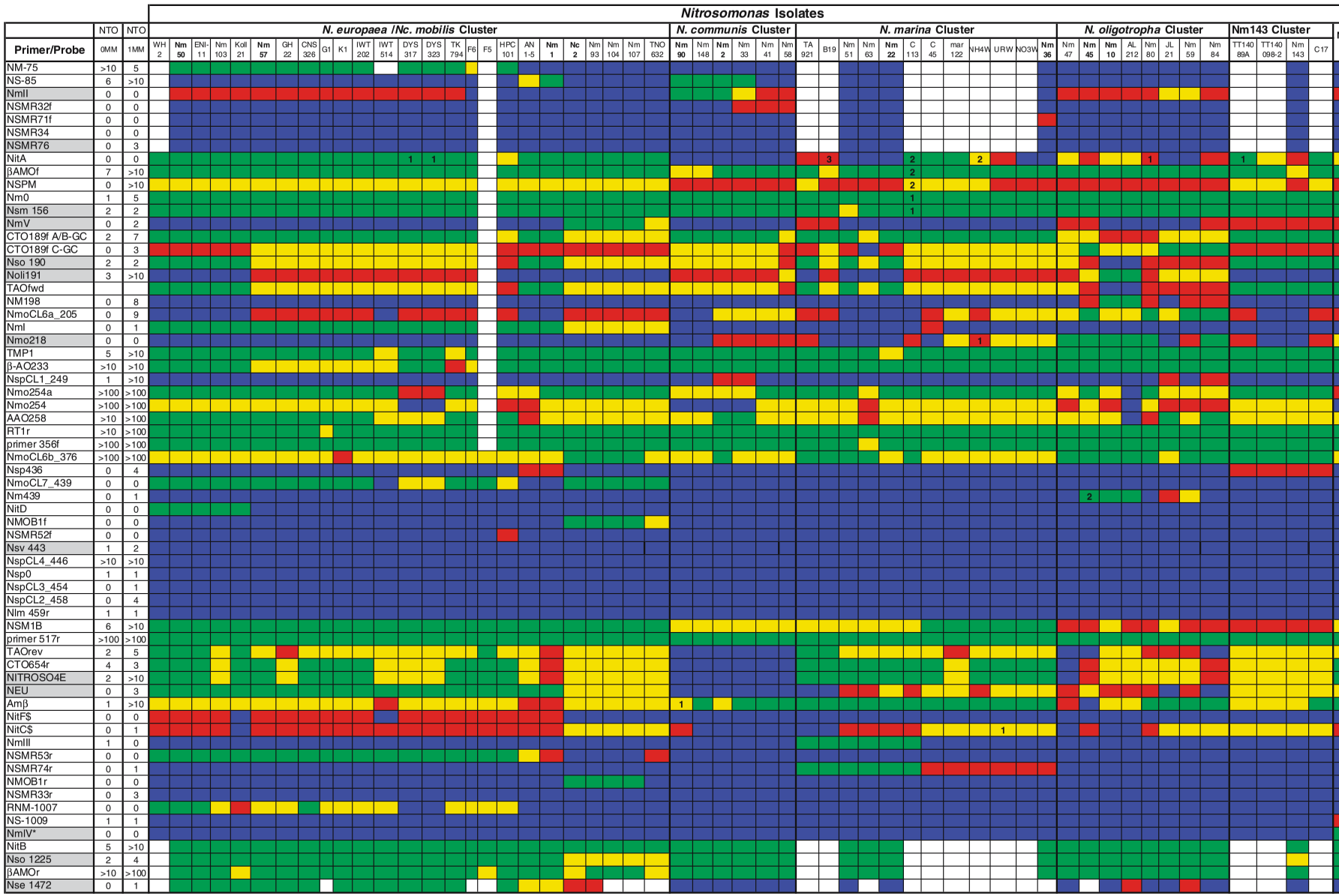


Table 7. Specificity and sensitivity of published 16S rRNA (gene) targeting PCR primers and hybridization probes for nitrosomonads. Abbreviations: NTO, number of non-target organisms outside the AOB with no mismatch (0 MM) or one mismatch (1 MM) in its 16S rRNA (gene) to the respective primer/probe.[a]



AOB diversity research impresses by its specificity and sensitivity but suffers from PCR-biases and relatively low phylogenetic information content (see the section on Phylogeny of AOB in this Chapter). Several fingerprinting techniques like gel retardation, DGGE, and terminal restriction fragment length polymorphism (T-RFLP) have been combined with the *amoA* approach and allow first insights into the diversity of an *amoA* PCR amplificate without time-consuming cloning and sequencing analysis (Kowalchuk et al., 1998; Horz et al., 2000; Schmid et al., 2000; Nicolaisen and Ramsing, 2002). Furthermore, an in situ PCR technique is available which allows direct detection of the *amoA* gene in AOB (Hoshino et al., 2001).

Significant method development for AOB diversity research is underway. Several quantitative PCR methods for AOB detection in the environment have been developed (Mendum et al., 1999; Dionisi et al., 2002). Furthermore, absolute AOB numbers in complex samples can also be determined by applying a novel FISH method in combination with confocal laser scanning microscopy and digital image analysis (Daims et al., 2001c). In the future, DNA microarrays composed of several hundred rRNA- or *amoA* -targeted oligonucleotide probes will almost certainly become available for parallel analyses of AOB community structures. Studies on first generation microarrays carrying a relatively limited number of AOB-targeted probes have already been published (Guschin et al., 1997; Wu et al., 2001).

Distribution of AOB in Nature

The distribution of AOB in the environment has traditionally been analyzed by cultivation-dependent methods (see the section on Classical Techniques in this Chapter). Recently, these studies were complemented by molecular diversity surveys of various man-made and natural systems (see the section on Molecular Techniques in this Chapter). The combination of both approaches is strongly recommended because each approach is characterized by its specific limitations. MPN-based isolation of AOB target the numerically most important AOB but might overlook not yet cultured AOB species that cannot thrive in the cultivation medium offered (although any clear indication for that is missing in the literature). On the other hand, it is important to interpret PCR-based molecular AOB distribution data with caution because the mere retrieval of a 16S rRNA sequence of an AOB proves neither that this organism is abundant nor that it is physiologically active. This problem is particularly severe for AOB (compared to many other bacteria) because they can survive very long time periods under unfavorable conditions (Johnstone and Jones, 1988; Wilhelm et al., 1998; Pinck et al., 2001). Therefore, molecular studies aiming at identification of dominant AOB species in an environment should focus on PCR amplificates of the highest positive DNA dilution, an approach analogous to the MPN technique (Feray et al., 1999), or even better include quantitative techniques (see the section on Molecular Techniques in this Chapter). Independent of the method used, the distinction between autochthonous and allochthonous AOB of a particular ecosystem is not trivial, making the natural distribution patterns of the distinct AOB species difficult to define. This problem is particularly severe because many of the environments are strongly influenced by human activity that eventually causes transmission of significant amounts of AOB to foreign systems. For example, AOB typically found in activated sludge also might be detectable in freshwater systems, if effluents of a wastewater treatment plant are disposed close to the sampling site. In this context, the importance of a careful selection of representative sampling sites is obvious. It is also interesting to note that isolations of strains via MPN series in general define more restricted distribution patterns of the distinct species of AOB than obtained from comparable molecular investigations.

AOB are present in most aerobic environments including rocks and building stones but can also survive in anaerobic systems (see the section on Dominant Populations of AOB in Different Environments in this Chapter). Since ammonia is the essential energy source for these organisms, their distribution in nature is coupled to geological, biological and anthropogenic sources of reduced nitrogen. Consequently, they have adapted to a broad range of different ammonia concentrations in the diverse environments, reflected by different affinity constants for ammonia. This is one of the most important aspects influencing the distribution patterns of AOB in nature (Suwa et al., 1994; Suwa et al., 1997; Stehr et al., 1995a; Koops and Pommerening-Röser, 2001). On the other side, ammonia is a toxic compound. Therefore, the tolerance of increasing ammonia concentrations (Table 3) is another aspect shaping the distribution patterns of AOB (Bollmann and Laanbroek, 2001).

Furthermore, the presence or absence of urease activity was observed to be of ecophysiological relevance for AOB. This property is of special importance in acidic environments, where free ammonia is missing as substrate because it is nearly quantitatively ionized to ammonium. Under such conditions, only those AOB species that can use urea as an alternative

ammonia source can build up stable populations (De Boer and Laanbroek, 1989; De Boer et al., 1991; Jiang and Bakken, 1999; Burton and Prosser, 2001). Possession of urease seems also to be an essential property for AOB that successfully colonize oligotrophic soils or aquatic environments.

Distribution of AOB in nature also is affected by different salt requirements, salt tolerances and salt sensitivities of the distinct species (Table 3). This is of special importance for their distribution patterns in aquatic systems (such as rivers, lakes, estuaries, marine environments and salt lakes) that significantly differ in salinity (Koops et al., 1990; Koops et al., 1991).

The AOB are aerobes. Although their oxygen affinity constants are relatively high (Painter, 1986), AOB possess nitrifying activity not only at oxygen saturation but also at extremely low oxygen concentrations (Goreau et al., 1980). Even ammonia oxidation under anaerobic conditions is being discussed (Schmidt and Bock, 1997; Schmidt and Bock, 1998; Zart et al., 2000). However, specific selection of distinct AOB species by different oxygen concentrations has not yet been reported in the literature.

Temperature also may be of importance for distribution patterns of AOB in nature. This has been indicated in some publications (Golovacheva, 1976; Jones et al., 1988; Jiang and Bakken, 1999). Beside environments characterized by constant high or low temperatures, such as hot springs or permafrost soils, environments showing pronounced temperature changes, such as rock surfaces, might harbor interesting AOB. However, these systems have not been studied intensively enough to allow general conclusions on their AOB diversity.

The Betaproteobacterial AOB

The betaproteobacterial AOB are divided into two main subgroups, the *Nitrosomonas* group (containing six distinct lineages) and the *Nitrosospira* lineage (containing the genera *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus*). Within the *Nitrosomonas* group, a relatively strong correlation between the phylogenetically defined lineages (see the section on Phylogeny of AOB in this Chapter) and the distribution patterns of the respective species exists. Within the *Nitrosospira* lineage, this correlation is not as obvious.

The *Nitrosomonas* Group

The N. europaea/Nc. mobilis *Lineage* The four cultured species of the *N. europaea/Nc. mobilis* lineage are moderately halophilic and all show striking halotolerance (Table 3). Most of the cultured strains of the three species *N. europaea*, *N. eutropha* and *Nitrosococcus mobilis* (which is phylogenetically a member of the genus *Nitrosomonas*) have been isolated from wastewater treatment plants and their molecular signatures are also frequently retrieved from these systems (Figs. 12 and 13). In addition, quantitative FISH results demonstrated that *Nc. mobilis* and *N. europaea* are dominant in several wastewater treatment plants (Juretschko et al., 1998; Daims et al., 2001b). Some isolates of this lineage were obtained from strongly fertilized agricultural soils. All these environments are more or less eutrophicated and influenced by anthropogenic activity, frequently rendering the conditions more favorable for these AOB. The successful colonization of these systems requires tolerance of increasing ionic strength and, in particular, of high concentrations of toxic compounds such as ammonia. Consequently, these properties are found in all members of these AOB lineages (Table 3). The natural habitat for members of these lineages has not yet been identified. However, in a recent molecular investigation the presence of *N. europaea* -like and *N. eutropha* -like organisms in a salt lake was shown (Ward et al., 2000). This is of interest, since strains belonging to the fourth species of the *N. europaea/Nc. mobilis* lineage, *N. halophila*, recently could be isolated from salt lakes in Mongolia (Sorokin et al., 2001). Sporadically, isolates of the *N. europaea/Nc. mobilis* lineage were obtained from the North Sea (*N. halophila* and *N. mobilis*), from the river Elbe estuary (*N. europaea*) and from freshwater sediments (*N. europaea* and *N. eutropha* ; Stehr et al., 1995a; Koops and Pommerening-Röser, 2001). Consistent with these findings, molecular investigations detected members of the *N. europaea/Nc. mobilis* lineage in fertilized soils or natural fresh water environments (Voytek, 1996; Holmes et al., 1999; Campbell, 2000; Horz et al., 2000; Ivanova et al., 2000; Phillips et al., 2000; Burrell et al., 2001; Oved et al., 2001; Smith et al., 2001; Whitby et al., 2001a; Morris et al., 2002; Radajewski et al., 2002; Webster et al., 2002).

The N. communis *Lineage* The *N. communis* lineage comprises two entities, the *N. communis* and the *N. nitrosa* sublineages, which are currently phylogenetically not clearly distinguishable but have significantly different distribution patterns in nature. The *N. communis* sublineage contains, in addition to *N. communis*, three other cultivated species, not yet described by name (Fig. 9). The majority of strains of this sublineage were isolated from approximately pH neutral soils, often agriculturally utilized. Since all three species of this sublineage possess relatively high

affinity constants for ammonia and lack urease activity, they probably need relatively high ammonia concentrations in the environment. Most likely this is an important reason that these species seem to be missing in oligotrophic natural soils and in substrate-low natural freshwaters. In contrast to the above subgroup, strains of the *N. nitrosa* subgroup are commonly distributed in more or less eutrophicated freshwaters. Since the affinity constants for ammonia are similar to those of the species of the *N. communis* sublineage, the possession of urease might be responsible for this different distribution. Strains of *N. nitrosa* also have been isolated from marine environments, a fact which is in apparent contrast to the relatively low salt tolerance of these strains observed in laboratory studies (Table 3). Surprisingly, only two 16S rRNA gene clones of the *N. communis* lineage have been detected in environmental diversity surveys (Gieseke et al., 2001; Fig. 11). This low retrieval rate may have been caused by the fact that many of the commonly applied primers possess mismatches with the 16S rRNA gene of members of this lineage (Table 7). In contrast, *amoA* sequences affiliated with this lineage were detected in soil, freshwater and wastewater treatment plants (Ivanova et al., 2000; Purkhold et al., 2000; Gieseke et al., 2001; Oved et al., 2001; Dionisi et al., 2002; Nogueira et al., 2002; Fig. 11).

The N. oligotropha *Lineage* The *N. oligotropha* lineage comprises several species, although only *N. oligotropha* and *N. ureae* are described by name. A total of six species of this lineage is available in culture (Fig. 7), and the existence of more species has been indicated by molecular environmental studies (Speksnijder et al., 1998) and several unpublished sequences publicly accessible via GenBank. The majority of cultured strains have been isolated from rivers and lakes (Koops and Pommerening-Röser, 2001). Some isolates originate from sewage disposal plants (Koops and Harms, 1985; Suwa et al., 1997) and from soil samples. The 16S rRNA sequences of this cluster were frequently detected in soil and freshwater (Stephen et al., 1996; Speksnijder et al., 1998; Bruns et al., 1999; Radeva et al., 1999; Kowalchuk et al., 2000b; Phillips et al., 2000; Bollmann and Laanbroek, 2001; Whitby et al., 2001b; Regan et al., 2002). Among the cultivated species of AOB, the representatives of the *N. oligotropha* lineage reveal the lowest affinity constants for ammonia, being in the range of a few μM. All investigated strains are urease positive. These observations are in accordance with the generally low ammonia concentration in their natural habitats. It is also notable, that representatives of this lineage often occur floc- or biofilm-attached in the environment (Stehr et al., 1995a), as well as in laboratory studies (Bollmann and Laanbroek, 2001). Consistent with this observation, at least some members of this lineage can produce remarkable amounts of exopolymeric substances (Stehr et al., 1995b).

The N. marina *Lineage* The *N. marina* lineage comprises three cultivated species, *N. marina*, *N. aestuarii* and *Nitrosomonas* sp. (represented by the isolates Nm 51 and Nm 63). All isolates originate from marine environments. Molecular signatures of this lineage have also been detected in marine samples (Stephen et al., 1996; Casciotti and Ward, 2001) as well as in enrichment cultures from such environments (Stephen et al., 1996). The presence of members of this lineage has been observed with molecular techniques in estuarine environments, coastal sand dunes and even in freshwater (aquaria) and a wastewater treatment plant (Kowalchuk et al., 1997; Speksnijder et al., 1998; Purkhold et al., 2000; Burrell et al., 2001; de Bie et al., 2001; Nicolaisen and Ramsing, 2002). Some authors, unfortunately, have lumped together the *N. oligotropha* lineage and the *N. marina* lineage (Kowalchuk and Stephen, 2001), although both lineages are phylogenetically distinct (Figs. 6 and 7) and the species of the two lineages show pronounced differences in ecophysiological characteristics and distribution patterns.

The N. cryotolerans *Lineage* The *N. cryotolerans* lineage is represented by only one species, *N. cryotolerans*, and only one isolate exists in culture. This strain has been isolated from a marine environment and is obligatorily halophilic and urease positive. In molecular in situ analyses of AOB populations, *N. cryotolerans* has not yet been detected, although most of the commonly applied 16S rRNA gene primers do match this species.

The Nm 143 Lineage Recently, a new lineage was identified within the genus *Nitrosomonas* (Purkhold et al., 2003; Fig. 7). This lineage has been provisionally named after *Nitrosomonas* sp. Nm143 and contains three additional isolates. It is not known whether these four isolates represent one or more than one species. All isolates were obtained from marine systems. Molecular signatures of this lineage were found in several marine and estuary systems (McCaig et al., 1994; Stephen et al., 1996; de Bie et al., 2001; Nicolaisen and Ramsing, 2002).

Uncultured Lineage The so-called "environmental lineage 5" is the only major evolutionary lineage within the *Nitrosomonas* group which does not yet contain a cultured representative

(Stephen et al., 1996; Purkhold et al., 2000). The 16S rRNA sequences of this lineage were obtained from freshwater environments, marine systems and a coastal sand dune (McCaig et al., 1994; McCaig et al., 1999; Stephen et al., 1996; Kowalchuk et al., 1997; Speksnijder et al., 1998; Bano and Hollibaugh, 2000).

The Nitrosospira *Lineage* Members of the *Nitrosospira* lineage were postulated by some authors to be the dominant AOB in nature (Hiorns et al., 1995; Hastings et al., 1997; Ceccherini et al., 1998). However, this postulate is rather unsecured because it is based on findings obtained with nonquantitative PCR-dependent methods often involving primers and probes not sufficiently covering some of the *Nitrosomonas* lineages (Tables 6 and 7). The *Nitrosospira* lineage represents a phylogenetically young group of AOB. Therefore, no highly reproducible phylogenetic substructure can be revealed within this group based on comparative 16S rRNA or *amoA* sequence analysis (see the section on Phylogeny of AOB in this Chapter). Nevertheless, a provisional subdivision system of this group has been suggested (Stephen et al., 1996; extended by Purkhold et al., 2000; Fig 8) and widely applied for interpretation of data from molecular AOB diversity surveys (for a review, see Kowalchuk and Stephen, 2001). However, it is questionable whether the suggested clustering defines units characterized by particular ecophysiological traits.

Most isolates of the *Nitrosospira* lineage were obtained from terrestrial systems. Consistent with this finding, the current molecular dataset suggests that members of the *Nitrosospira* clusters 0, 2, 3 and 4 are predominantly found in terrestrial habitats (Fig. 12). Whether the reported occurrence of these clusters in freshwater habitats reflects that these bacteria are autochthonous freshwater inhabitants or soil-borne organisms transferred to the water bodies cannot be decided. Nitrosospiras of cluster 1, for which no isolate exists, also occur in soil but are more frequently found in marine systems. The analyzed wastewater treatment plants rarely harbored nitrosospiras, and only members of clusters 2 and 3 were detected. Constructed wetlands might represent an exception to this rule, since mainly *Nitrosospira* -related 16S rRNA sequences were retrieved from a plant using rhizoremediation technology (Abd El Haleem et al., 2000). *Nitrosospira* strains Nsp57, 58 and 65, which were isolated from masonry and form two novel branches within this group (Fig. 8), are the only *Nitrosospira* isolates for which yet no closely related sequences were directly obtained from an environment.

At least within the so-called "*Nitrosospira* cluster 3," profound differences regarding physiology and distribution patterns exist because this cluster, in contrast to the other four clusters, comprises many morphologically as well ecophysiologically different species.

The Gammaproteobacterial AOB

The cultured strains of the two species of the genus *Nitrosococcus*, representing the gammaproteobacterial AOB, have been isolated from marine environments (all strains of *N. oceani*) and from a salt lagoon or from a salt lake (two isolates of *N. halophilus*), respectively. This habitat range is in accordance with the salt requirements and with the salt tolerances of the two species in laboratory experiments. Using immunological methods and molecular techniques, *N. oceani* was detected exclusively in marine environments but shown to be widely distributed (Ward and Perry, 1980; Ward, 1982; Ward and O'Mullan, 2002). *Nitrosococcus halophilus* has not yet been observed in nature via molecular techniques.

Dominant Populations of AOB in Different Environments

Attempts to describe the dominant AOB populations of an environment should always take into careful consideration whether the retrieved species are autochthonous or allochthonous members of the analyzed ecosystem. Often species are introduced into environments from adjacent habitats. Since ecophysiological characteristics of distinct species of AOB often differ only minimally, introduced populations have a good chance to survive in the novel environment. Moreover, anthropogenic influence has created many new environments colonized by AOB that had no or only little time to adapt evolutionarily to these systems. In such cases, the sum of ecophysiological parameters will determine finally which of the species competing with each other will become the dominant representative.

Marine Environments

A relatively clear situation is found in marine environments. In these ecosystems, generally those AOB are dominant which have a compatible salt requirement and a suitable salt tolerance. Consequently, the gammaproteobacterial AOB, *N. oceani* together with representatives of the *Nitrosomonas marina* lineage are the only AOB isolated with high frequency from marine environments. The second species of *Nitrosococ-*

cus, *N. halophilus*, seems to prefer environments with higher salt concentrations (salt lagoons or even salt lakes). Most of the above-mentioned organisms (except *N. halophilus*) can also be detected in marine systems using molecular methods (Stephen et al., 1996; Ward and O'Mullan, 2002). It is not clear why only one strain of *N. cryotolerans*, which also is ecophysiologically well adapted to these systems, has been isolated from marine samples. Members of the Nm143 lineage also seem to be distributed exclusively in marine environments. However, the ecophysiological properties of the latter group have not yet been investigated.

Occasionally, species of the *Nitrosomons europaea*/*Nc. mobilis* lineage, owing to their relatively pronounced salt tolerance, were cultured from marine samples (Koops et al., 1976; Koops and Pommerening-Röser, 2001). Using quantitative dot blot, Hovanec and DeLong (1996) found that *N. europaea* -like bacteria were a major component of the microbial community in filters from seawater aquaria, but the primer (NSM1B) used for this analysis also has a full match to several members of the *N. marina* lineage (Tables 6 and 7). The 16S rRNA sequences of the *Nitrosomonas* environmental lineage 5 and *Nitrosospira* cluster 1, which both contain no cultured representatives, were frequently detected in marine environments (McCaig et al., 1994; McCaig et al., 1999; Stephen et al., 1996; Phillips et al., 1999; Bano and Hollibaugh, 2000; Hollibaugh et al., 2002; Fig. 12), but quantitative molecular data are missing. The retrieval of nitrosospiras in systems with elevated salt concentrations is surprising, since all cultured species of this lineage have a low salt tolerance in laboratory studies. The mere detection of 16S rRNA sequences of *Nitrosospira* cluster 1 in a marine environment does not prove that these organisms indeed possess a high salt tolerance and thrive in these systems. Alternatively, these nitrosospiras might be allochthonous inhabitants able to survive for extended periods in the ocean without significant physiological activity. It is thus important 1) to isolate and study a strain of this cluster and/or 2) to prove physiological activity of members of this cluster in situ for example by combination of FISH and microautoradiography (Lee et al., 1999).

Salt Lakes

In salt lakes, the pH as well as the composition and concentration of salts vary significantly (Ward et al., 2000; Sorokin et al., 2001) thus creating different niches for AOB. Accordingly, different AOB were found in different salt lakes. From a salt lake in Saudi Arabia, a strain of *Nitrosococcus halophilus* was obtained (Koops et al., 1990), and from soda lakes located in Mongolia, alkali tolerant representatives of *Nitrosomonas halophila* have been isolated (Sorokin et al., 2001). In molecular analyses, *N. europaea* -like and *N. eutropha* -like organisms were detected in the hypersaline Mono Lake in California (Ward et al., 2000). However, the PCR primers used in this study (NitA and NitD) were not suited for a general AOB diversity study (Tables 6 and 7). To get a more general view of the AOB community composition in such environments, more investigations must be carried out.

Freshwater Environments

In natural freshwater environments, members of the *Nitrosomonas oligotropha* lineage are generally the dominant AOB representatives. This is indicated by repeated isolation of members of this lineage from high MPN dilutions (Koops and Harms, 1985; Stehr et al., 1995a; Koops and Pommerening-Röser, 2001). Quantitative PCR-independent molecular data on AOB community composition in these systems are not available, but at least 16S rRNA sequences of this lineage were frequently detected in freshwater water bodies and sediments (Speksnijder et al., 1998; Radeva et al., 1999; Bollmann and Laanbroek, 2001; Whitby et al., 2001b; Regan et al., 2002) as well as in the Schelde estuary (Belgium and The Netherlands; de Bie et al., 2001). In addition, 16S rRNA sequences of *Nitrosospira* clusters 0, 2, 3 and 4 were harvested in molecular analyses of these habitats (Kowalchuk et al., 1998; Speksnijder et al., 1998; Whitby et al., 1999; Whitby et al., 2001b) and in a freshwater aquarium (Burrell et al., 2001). Moreover, using immunological methods, nitrosospiras were found in a eutrophic lake sediment (Smorzewski and Schmidt, 1991). However, these results do not necessarily indicate *Nitrosospira* dominance in freshwater systems because quantitative data are lacking, and several of the PCR primers applied in these studies do not sufficiently cover the *N. oligotropha* cluster (Purkhold et al., 2000; Tables 6 and 7).

Occasionally, strains of *N. europaea*, *N. eutropha* and *N. nitrosa* have been isolated from eutrophicated rivers and eutrophic lakes (Koops and Pommerening-Röser, 2001). Furthermore, 16S rRNA sequences of *N. europaea*/*Nc. mobilis*, of *Nitrosomonas* environmental lineage 5 and of the *N. marina* lineage were retrieved from freshwater systems (Speksnijder et al., 1998; Whitby et al., 1999; Bollmann and Laanbroek, 2001; Burrell et al., 2001), but the significance of these findings remains unclear.

Artificial Aquatic Environments

Urea and ammonia are the most frequently found nitrogen compounds in sewage. In wastewater treatment plants, AOB oxidize ammonia to nitrite, which is subsequently converted to nitrate by the nitrite-oxidizing bacteria. Nitrate is then removed from the sewage by denitrifying bacteria via anaerobic respiration. The slow growth rate of AOB and their susceptibility to pH- and temperature swings as well as to several sewage compounds is responsible for frequent failure of the nitrification in municipal and industrial wwtps.

Isolation techniques indicate that in standard municipal wwtps, *N. eutropha* seems to be the dominant representative (Watson and Mandel, 1971b; Koops and Harms, 1985), but *N. europaea* and *Nc. mobilis* have also been repeatedly cultivated (Juretschko et al., 1998; Koops and Pommerening-Röser, 2001). All these species belong to the same *Nitrosomonas* lineage (Fig. 7)

In industrial wwtps, the cultivation of representatives of members of the *N. oligotropha* lineage as well as *N. nitrosa* has been regularly reported (Koops and Harms, 1985; Suwa et al., 1997). In laboratory experiments, a remarkable high tolerance of members of the *N. oligotropha* lineage to heavy metals was observed, and the production of significant amounts of exopolymeric materials by these species was suggested to be the major reason for this tolerance (Stehr et al., 1995b). This resistance to heavy metals may be responsible for the presence of members of this lineage in special wwtps.

During the last decade, molecular techniques were extensively applied to characterize AOB community structure and activity in activated sludge and biofilm from various wwtps. FISH revealed that AOB generally occur in tight microcolonies formed of up to several thousand individual cells (Fig. 14). These microcolonies cannot be easily dispersed using ultrasonic or Ultraturrax homogenizer treatments and thus contribute to the underestimation of AOB numbers in MPN assays. Typically, the AOB are detected in situ in close proximity to nitrite oxidizers reflecting the mutualistic relationship between both guilds (Mobarry et al., 1996; Schramm et al., 1996; Schramm et al., 1998; Juretschko et al., 1998; Okabe et al., 1999). Experiments using a combination of FISH with ^{14}C-bicarbonate microautoradiography demonstrated that almost all AOB microcolonies in activated sludge are physiologically active. Furthermore, AOB in wwtps can incorporate pyruvate (Daims et al., 2001a). Using quantitative FISH data, the specific activity of a single AOB cell in activated sludge has been estimated to be 2.3 ± 0.4 fmol of ammonia per hour (Daims et al., 2001c). According to FISH analyses, nitrosomonads are predominant over nitrosospiras in most activated sludge and biofilm systems (Mobarry et al., 1996; Schramm et al., 1996; Schramm et al., 2000; Juretschko et al., 1998; Okabe et al., 1999; Daims et al., 2001b; Gieseke et al., 2001). With the exception of *Nitrosomonas cryotolerans*, *Nitrosomonas halophila* and *Nitrosomonas* sp. Nm143, relatives of all other *Nitrosomonas* species occur in wwtps (Purkhold et al., 2000). *Nitrosococcus mobilis*, originally thought to be restricted to brackish water, is abundant in many wwtps (Juretschko et al., 1998; Daims et al., 2001b). Some nitrifying wwtps are dominated by a single AOB species (Juretschko et al., 1998), while other plants harbor a high diversity of AOB (Purkhold et al., 2000; Daims et al., 2001b; Gieseke et al., 2001). Whether and how differences in diversity of ammonia oxidizers are linked to the stability of the nitrification process is a yet unanswered question. Investigations of AOB populations in the raw sewage most probably also could give important insight into AOB diversity in sewage disposal plants.

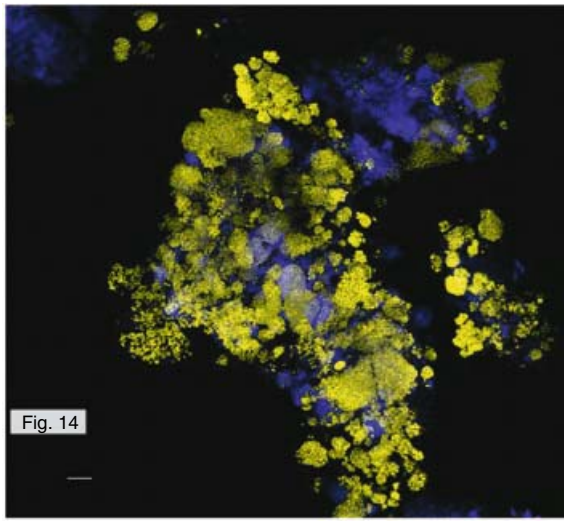


Fig. 14. FISH detection of an AOB microcolony in a nitrifying biofilm from a sequencing batch biofilm reactor. AOB were stained by probe NEU, and the fluorescence signals were recorded by confocal laser scanning microscopy. Probe-conferred fluorescence is shown in yellow, while autofluorescence of the biofilm is depicted in blue. Bar = 10 µm. The picture was kindly provided by Holger Daims.

Soils

In acidic soils (heathlands, grasslands, forest soils, and marshy lands), where sufficient amounts of free ammonia are missing, urease-positive species seem to be the exclusive representatives of the AOB. This has become obvious

via isolations as well as from molecular investigations (Kowalchuk and Stephen, 2001). Exclusively, species of the genera *Nitrosospira* and *Nitrosovibrio* could be detected. Molecular studies confirmed the predominance of nitrosospiras in acidic soils (Stephen et al., 1996; Smith et al., 2001) and indicated that, in particular, members of the *Nitrosospira* cluster 2 are well adapted to low pH terrestrial habitats (Kowalchuk et al., 1997; Stephen et al., 1998). A more detailed up-to-date summary of these findings can be found in Kowalchuk and Stephen (2001). However, urease-positive strains of *Nitrosolobus multiformis* and of the genus *Nitrosomonas*, respectively, are missing in acidic soils.

In more neutral soils, especially if agriculturally used, members of the *N. communis* lineage together with strains of *Nitrosolobus multiformis* are absolutely dominant in high MPN dilutions (MacDonald, 1986; Koops and Pommerening-Röser, 2001). In oligotrophic natural soils, especially if moderately acidic (pH around 6.0), strains of the *N. oligotropha* lineage as well as strains of the genera *Nitrosospira* or *Nitrosovibrio* have also been isolated (Koops and Pommerening-Röser, 2001).

Rocks and Building Stones

AOB are also common on surfaces of rocks or building stones. Many strains have been isolated, and all were found to be members of the genera *Nitrosospira* or *Nitrosovibrio* (Spieck et al., 1992). Neither the closely related genus *Nitrosolobus* nor any of the lineages of the genus *Nitrosomonas* seem to be represented in these environments. Currently, the selective factors leading to these distribution patterns are speculative. Most likely, the acidic pH caused by the nitrification process is a crucial factor. Periodically appearing dryness or drastic change in temperature also may be selective for members of the genera *Nitrosospira* or *Nitrosovibrio*.

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Prokaryotes (2006) 5:812–827
DOI: 10.1007/0-387-30745-1_37

CHAPTER 3.2.13

The Genus *Thiobacillus*

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Introduction

Ever since the genus *Thiobacillus* was first described in 1904, the ability to grow while using a reduced sulfur compound as a source of energy has been considered sufficiently important taxonomically to merit classifying all Gram-negative, sulfur-oxidizing, nonphototrophic rods in this genus. However, as studies using modern taxonomic methods began to reveal that some of the species are only superficially related, virtually every paper describing a new *Thiobacillus* species in the last decade has mentioned the need to reorganize the genus. Most of this reorganization has now been done (Kelly and Wood, 2000a), and the authors are grateful to D.P. Kelly and A. Wood for permission to see and use their resulting manuscripts prior to publication.

The effect of the reorganization is dramatic (Table 1). In the last edition of *The Prokaryotes*, the genus *Thiobacillus* included 17 species. There are now only three species left—all autotrophs, and all members of the β-subclass of the Proteobacteria. The 4 or 5 facultative autotrophs that fall into the β-subclass have now been assigned to a new genus—*Thiomonas*. Most of the other former *Thiobacillus* species fall into the γ-subclass, together with the members of the genus *Thiomicrospira*. This includes both the well-known acidiphilic and recently discovered alkaliphilic "*Thiobacillus*" species, as well as the marine species. However, because the reorganization is still so new and most researchers will look for these organisms in one group, many of them will be dealt with in this chapter, with the exception of the γ group. *Thiobacillus acidophilus* and *Thiobacillus versutus* have both been transferred to existing genera (see Table 1). Similarly, *Thiosphaera pantotropha* has now been reclassified as *Paracoccus pantotrophus* and will be described in the chapter on that genus. The last member of the γ group, *Thiobacillus novellus*, is now *Starkeya novella*. Within this chapter, the generic terms "thiobacilli" and "colorless sulfur bacteria" refer to the whole group, regardless of their new classification.

Habitats

In nature, the distribution of reduced inorganic sulfur compounds is only one of the factors governing the presence and activity of the colorless sulfur bacteria, and a complex of chemical, physical, and microbial interactions is almost as important a controlling force. One crucial factor is the requirement for the simultaneous presence of an electron donor (the reduced sulfur compound) and electron acceptor (i.e., oxygen or nitrogen oxides). Sulfide is one of the most important natural substrates, and many of the colorless sulfur bacteria grow in the narrow zones and gradients where sulfide and oxygen coexist (Nelson and Jannasch, 1983). Such gradients can be found, for example, in stratified lakes and at the interface between aerobic water and an anaerobic sediment, but may be even more common in microniches in environments that contain anaerobic pockets (e.g., sediments and wet soils). It must be realized that extremely high turnover rates of sulfide can be observed at very low levels of sulfide and oxygen ($<10^{-6}$ mM; Nelson et al., 1986). In fact, it is thanks to these low oxygen concentrations that the sulfide-oxidizing bacteria can successfully compete with the spontaneous oxidation of sulfide. This implies that even where environmental sulfide concentrations are very low, the actual sulfide flux may be very high. Similarly, other gradients can influence the growth and selection of sulfur oxidizers. Thus, it has been shown that thiobacilli respond differently to varying redox potentials (Sokolova and Karavaiko, 1968; Timmer ten Hoor, 1975). The occurrence of acidophilic bacteria in apparently neutral environments indicates the existence of pH gradients associated with acidic microniches. Sulfur oxidizers have been enriched and isolated from natural sites chosen by the obvious presence of inorganic sulfur or iron compounds (e.g., sediments from rivers, canals, estuaries and tidal flats, acid sulfate soil, [hot] acid springs, mine drainage effluent, hydrothermal vents, hyperalkaline soda lakes, and wastewater treatment systems). The thiobacilli, especially the specialized chemolithotrophs, have long

Table 1. Summary of the reorganization of the colorless sulfur bacteria, particularly the status of species previously placed in the genus *Thiobacillus*.

Sub-class	Current name	Synonyms
α	*Acidiphilium acidophilum*	*Thiobacillus acidophilus, Thiobacillus organoparus*
α	*Paracoccus pantotrophus*	*Thiosphaera pantotropha*
α	*Paracoccus versutus*	*Thiobacillus versutus, Thiobacillus rapidicrescens, Thiobacillus A2*
α	*Starkeya novella*	*Thiobacillus novellus*
β	*Thiobacillus aquaesulis*	
β	*Thiobacillus denitrificans*	
β	*Thiobacillus thioparus*^{T}	*Thiobacillus thiocyanoxidans, Bacterium thioparum*
β	*Thiomonas cuprina*	*Thiobacillus cuprinus*
β	*Thiomonas intermedia*^{T}	*Thiobacillus intermedius*
β	*Thiomonas perometabolis*	*Thiobacillus perometabolis, Thiobacillus rubellus*
β	*Thiomonas thermosulfata*	*Thiobacillus thermosulfatus, Thiomonas thiosulfata*
β	Unknown	*Thiobacillus plumbophilus*
γ	*Acidithiobacillus albertensis*	*Thiobacillus albertis*
γ	*Acidithiobacillus caldus*	*Thiobacillus caldus*
γ	*Acidithiobacillus ferrooxidans*	*Thiobacillus ferrooxidans, Ferrobacillus ferrooxidans*
γ	*Acidithiobacillus thiooxidans*^{T}	*Thiobacillus thioxidans, Thiobacillus concretivorans, Thiobacillus kabobis, Thiobacillus thermitanus, Thiobacillus lobatus, Thiobacillus cretanus, Thiobacillus umbonatus*
γ	*Halothiobacillus halophilus*	*Thiobacillus halophilus*
γ	*Halothiobacillus hydrothermalis*	
γ	*Halothiobacillus kellyi*	
γ	*Halothiobacillus neapolitanus*^{T}	*Thiobacillus neapolitanus, Thiobacillus X*
γ	*Thermithiobacillus tepidarius*^{T}	*Thiobacillus tepidarius*
γ	*Thioalcalivibrio denitrificans*	
γ	*Thioalcalivibrio nitratus*	
γ	*Thioalcalivibrio versutus*	
γ	*Thioalcalimicrobium aerophilum*	
γ	*Thioalcalimicrobium sibericum*	
γ	*Thiomicrospira chilensis*	
γ	*Thiomicrospira crunogena*	
γ	*Thiomicrospira frisia*	
γ	*Thiomicrospira kuenenii*	
γ	*Thiomicrospira pelophila*^{T}	
γ	*Thiomicrospira thyasirae*	*Thiobacillus thyasiris*
γ	Unknown	*Thiobacillus prosperus*
γ	Unknown	*Thiomicrospira denitrificans*
Unknown		*Thiobacillus delicatus*
Unknown		*Thiobacillus capsulatus*

been viewed as one of the most important groups of organisms contributing to the transformation of sulfur in soils and sediments, although only a few systematic studies on their occurrence and distribution have been carried out. However, there is evidence that, depending on the particular environmental conditions, the mixotrophic and heterotrophic sulfur oxidizers may be at least equally important in the overall conversion of reduced sulfur compounds to a more oxidized state (Guitoneau and Keiling, 1932; Kuenen, 1975; Mason and Kelly, 1988; Robertson et al., 1989). In general, sulfur and iron oxidizers are ubiquitous in soil, sediments, and freshwater and marine environments. They grow over a wide range of pH values and temperatures (see Tables 2 and 3). Thus, it is relatively easy to find them in nature. An obvious factor that limits their growth is the availability of reduced inorganic sulfur compounds, a limitation that is especially important for the obligate autotrophs that completely depend on these compounds for growth. A special case is the spontaneous oxidation of sulfide by tetrathionate, which is controlled by thiosulfate-recycling bacteria such as *Catenococcus thiocyclus*. This organism oxidizes thiosulfate to tetrathionate at concentrations as low as 1–10 μmol/liter. The tetrathionate is then chemically reduced to thiosulfate by sulfide, making it available to *C. thiocyclus* again. Thus, even where sulfide or thiosulfate levels appear to be very low, it has been shown that such thiosulfate-recycling heterotrophs can contribute significantly to the turnover within the local sulfur cycle (Sorokin et al., 1996).

Despite their ability to incorporate some organic compounds into cell material, the contribution of the obligate autotrophs to the

Table 2. Basic characteristics of the species in the β-subgroup.

Species	GC content Ubiquinone (mol%)	Motility	Carboxysomes[a]	pH[b]	Product of denitrification NO_2^-	Product of denitrification N_2	Temp (°C)[b]	
Thiobacillus								
T. aquaesulis	66	+	?	7.5–8	+	–	40–50	Q-8
T. denitrificans	63–68	+	–	6–8	+	+	25–30	Q-8
T. thioparus	61–66	+	+	6–8	+	–	25–30	Q-8
Thiomonas								
T. cuprina	66–69	+	?	3–4	–	–	30–36	Q-8
T. intermedia	65–67	+	+	5.5–6	–	–	30–35	Q-8
T. perometabolis	65–68	+	–	5.5–6	–	–	30–35	Q-10
T. thermosulfata	61	+	?	5.2–5.6	–	–	50–52.5	Q-8
Unknown								
T. plumbophilus	66	+		4–6.5	–	–	21–34	Q-8

Abbreviations: + = present; – = absent; ? = unknown; [a] = under some growth conditions; [b] = for best growth.

Table 3. Basic characteristics of the species in the γ subgroup.

Species	GC content (mol%)	Motility	Carboxysomes[a]	pH[b]	Product of denitrification NO_2^-	Product of denitrification N_2	Temp (°C)[b]	Ubiquinone
Acidithiobacillus								
A. albertensis	61.5	+	+	2–4	–	–	30–35	?
A. caldus	63.1–63.9	+	?	1–3.5	?	?	32–52	Q-8
A. ferrooxidans	55–65	+	+[c]	2–4	–	–	30–35	Q-8
A. thiooxidans	51–53	+	+	2–4	–	–	25–30	Q-8
Halothiobacillus								
H. halophilus	64.2	?	?	7	–	–	30–32	Q-8
H. hydrothermalis	67.1–67.4	+	?	7.5–8	–	–	35–40	Q-8
H. kellyi	62	+	?	6.5	–	–	37–42	Q-8
H. neapolitanus	52–56	+	+	6–8	–	–	25–30	Q-8
Thermithiobacillus								
T. tepidarius	66.6	+	+	6–8	+	–	40–45	Q-8
Thioalcalivibrio								
T. denitrificans	62.3–65	+	+	10	–	+[d]	25–30	Q-8
T. nitratus	61.3–62.1	+	+	10	+	–	25–30	Q-8
T. versutus	63–65.6	+	+	10–10.2	–	–	25–30	Q-8
T. jannaschii	63.7	+	+	10	–	–	30	?
T. paradoxus	65.6–66.2	–	–	10	–	–	30	?
T. nitratireducens	64.8	–	–	10	+	–	30	?
T. thioyanoxidans	66.2–66.9	+	+	10	–	–	30	?
Thioalcalimicrobium								
T. aerophilum	49.5	+	+	9–10	–	–	25–30	Q-8
T. sibericum	48.9	+	+	10	–	–	25–30	Q-8
T. cyclicum								
Thioalcalispira								
T. microaerophilum	58.9	+	–	10	–	–	30	?
Thiomicrospira								
T. chilensis	49.9	+	?	7	–	–	32–37	Q-8
T. crunogena	42–43	+	–	7–8	–	–	28–32	?
T. frisia	39.6	+	?	6.5	–	–	32–35	Q-8
T. kuenenii	42.4	+	?	6	–	–	29–33	Q-8
T. pelophila	44	+	–	6–8	–	–	25–30	?
T. thyasirae	52	–	–	7–8	+	+	35–40	Q-10
Unknown *"Thiobacillus"*								
"T. prosperus"	61–64	+	+	1–4	?	?	30–35	Q-8

Abbreviations: + = present; – = absent; ? = unknown; [a] = under some growth conditions; [b] = for best growth; [c] = under CO_2 limitation; [d] = best denitrification from N_2O, none from NO_3^-.

breakdown of organic compounds in nature is probably negligible. Gottschal and Kuenen (1980) showed that the versatile species are generally favored in freshwater environments when the turnovers of available inorganic and organic substrates are approximately equivalent. When the inorganic substrates are dominant, obligate autotrophs should be favored, and similarly, heterotrophs will be favored when organic compounds make up the bulk of the available substrate (Fig. 1). However, this does not necessarily hold true for marine situations (Kuenen et al., 1985). Further work remains to be done to discover what other selective pressures are operative in the marine milieu. It should also be remembered that groups of benthic sulfur-oxidizing bacteria such as *Beggiatoa* and *Thiovulum*, described elsewhere, are especially important in such situations (Otte et al., 1999).

Because many of the colorless sulfur bacteria produce sulfuric acid or ferric iron, they are often associated with the oxidative corrosion of concrete and pipes and have been implicated in the corrosion of buildings and ancient monuments. Acid and metal pollution can also be a result of the activities of thiobacilli in mine wastes (Tuovinen and Kelly, 1972). On the more positive side, the production of acid contributes, for example, to the leaching of metals from poor ores that are unsuitable for extraction by conventional metallurgical methods (Boon, 1996). In addition, the potential application of the pyrite-oxidizing capacity of *Acidithiobacillus ferrooxidans* and related organisms for the desulfurization of coal has been successfully explored (Bos et al., 1988; Bos and Kuenen, 1990). However, the problem of the acid waste stream from this process, which contains ferric hydroxysulfate has not yet been solved.

In soils, thiobacilli may sometimes be responsible for the solubilization of sulfur compounds such as pyrite and other metal sulfides, thus making sulfur available (as sulfate) for assimilation by other microorganisms and plants. In soil reclaimed from the sea, the oxidation of pyrite may lead to the formation of "cat clay." The sulfur budget and the role of microorganisms in the sulfur cycle of cultivated soils are, of course, important for assessing fertility. In Australia, for example, thiobacilli are scarce in sulfur-deficient areas. Under suitable climatic conditions (e.g., in the tropics), rock phosphate pelleted with sulfur and seeded with thiobacilli has been shown to be a useful, slow release source of phosphate and sulfate for soil fertilization (Swaby, 1975).

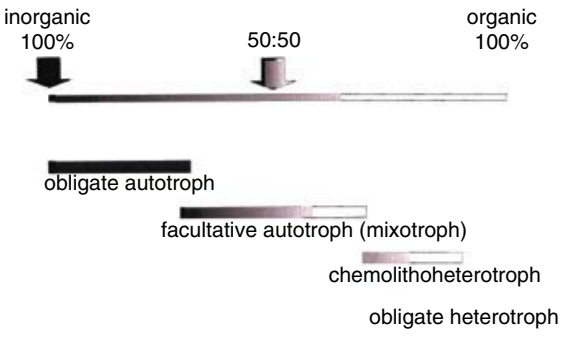

Fig. 1. A "spectrum" showing the ratios of inorganic to organic substrates most likely to favor the four physiological types of colorless sulfur bacteria.

Marine thiobacilli have been isolated from oceans, soda lakes, hydrothermal vents, and coastal and tidal areas and classified by reference to nonmarine cultures. Characteristically, some (but not all) marine forms have a requirement for (sodium) chloride levels comparable with the salinity of their natural environment. It is difficult to explain their presence in the open oceans; perhaps they are able to scavenge volatile sulfur compounds released from bottom sediments during the process of decomposition. At the hydrothermal vents, they are believed to be important producers of organic compounds at the start of the geochemically based food chain (Jannasch, 1985a). However, as they are, on the whole, dependent on oxygen generated by photosynthesis for electron acceptors, they cannot therefore be considered "primary producers" in the strict sense of the term.

Many *Thiomicrospira* species were isolated from estuarine mud rich in sulfides and from the waters of hydrothermal vents (Jannasch et al., 1985b; Brinkhoff et al., 1999a; Brinkhoff et al., 1999b). Some, but not all, require NaCl for growth.

Moderately thermophilic thiobacilli (optimum growth around 50°C) have been isolated from hot springs in geothermal areas (Williams and Hoare, 1972; Le Roux et al., 1977; Wood and Kelly, 1985), and from copper-leaching operations (Brierley and Lockwood, 1977; Brierley et al., 1978). The sulfur transformations in hot springs involve some unique microbiological features. Sulfur oxidizers found at high temperatures (up to 85°C) are typically acidophilic and lack a rigid cell wall structure because of a lack of peptidoglycan. These organisms are all classified as belonging to the genus *Sulfolobus*. One sulfur-oxidizing member of the genus *Acidianus* has been reported to grow at temperatures up to 100–105°C (Stetter, 1988).

At the other end of the pH scale, the range of extremely alkaliphilic bacteria present in environments such as soda lakes has only recently become apparent, and sulfur-oxidizing bacteria (Table 3) make up a significant population within these communities (Sorokin et al., 2001).

Another source of colorless sulfur bacteria is the rich mixture of microbial populations found in wastewater treatment systems. Indeed, *Paracoccus pantotrophus* was isolated from a denitrifying, sulfide-oxidizing, fluidized bed reactor (Robertson and Kuenen, 1983a; Robertson and Kuenen, 1983b), and it has been claimed that *Thiobacillus denitrificans* has been isolated from others (e.g., Batchelor and Lawrence, 1978). As these reactors can, in many ways, be regarded as enrichment cultures on the large scale, they may be a rich source of other strains with novel physiological traits.

Physiology

By definition, all of the species described in this chapter can derive energy for growth from the oxidation of reduced sulfur compounds if provided with a terminal electron acceptor, but the conditions required (e.g., low pH) may vary somewhat. Moreover, the ability of all of the colorless sulfur bacteria to oxidize certain sulfur compounds has not been rigorously tested. Until this is done, caution should be exercised when basing differentiation of the species on which sulfur compounds they can oxidize or accumulate. Some of the reactions can be summarized as follows:

$$H_2S + 2O_2 \rightarrow H_2SO_4 \quad (1)$$

$$2H_2S + O_2 \rightarrow 2S^\circ + 2H_2O \quad (2)$$

$$2S^\circ + 3O_2 + 2H_2O \rightarrow 2H_2SO_4 \quad (3)$$

$$Na_2S_2O_3 + 2O_2 + H_2O \rightarrow Na_2SO_4 + H_2SO_4 \quad (4)$$

$$4Na_2S_2O_3 + O_2 + 2H_2O \rightarrow 2Na_2S_4O_6 + 4NaOH \quad (5)$$

$$2Na_2S_4O_6 + 7O_2 + 6H_2O \rightarrow Na_2SO_4 + 6H_2SO_4 \quad (6)$$

$$2KSCN + 4O_2 + 4H_2O \rightarrow (NH_4)_2SO_4 + K_2SO_4 + 2CO_2 \quad (7)$$

$$5H_2S + 8KNO_3 \rightarrow 4K_2SO_4 + H_2SO_4 + 4N_2 + 4H_2O \quad (8)$$

$$5S^\circ + 6KNO_3 + 2H_2O \rightarrow 3K_3SO_4 + 2H_2SO_4 + 3N_2 \quad (9)$$

$$2FeS_2 + 2H_2O + 7O_2 \rightarrow 2FeSO_4 + 2H_2SO_4 \quad (10)$$

$$4FeSO_4 + O_2 + 2H_2SO_4 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O \quad (11)$$

The complete oxidation of reduced sulfur compounds to sulfate is common to all of the obligate and facultatively autotrophic sulfur oxidizers, and the ability of a culture to lower its pH while growing on thiosulfate is one of the criteria that has been used for the detection of these bacteria. The pH often rises in cultures of heterotrophs able to partially oxidize inorganic sulfur compounds because of the formation of polythionates (e.g., tetrathionate from thiosulfate). It should be noted, however, that a lack of acid production is not a decisive criterion for the differentiation between the chemolithotrophic and chemoorganotrophic sulfur oxidizers because under unfavorable cultural conditions, even the chemolithotrophs accumulate intermediates in significant amounts. For example, it has been observed that *Acidithiobacillus ferrooxidans*, when growing on thiosulfate, trithionate or sulfide, produces sulfur which is detectable not only outside the cells, but also as a finely dispersed precipitate outside the cell membrane (Hazeu et al., 1988). *Halothiobacillus neapolitanus* and its probable relatives known as "*Thiobacillus* strains O and W" produce sulfur during growth on reduced sulfur compounds under various environmental stresses (e.g., oxygen tension), but do not appear to accumulate sulfur internally (Stefess and Kuenen, 1989; Visser et al., 1997).

Enrichment, Isolation, and Cultivation Techniques

Within certain limits (e.g., NaCl requirement), most of the colorless sulfur bacteria discussed in this chapter appear to have similar requirements for inorganic salts. All of the known species can be cultivated in chemically defined media. However, many of their nutritional needs have not been quantitatively established, and the chemical composition of the basal media used in different laboratories varies enormously. For example, their requirements for trace elements have not been studied in detail and many recipes are variations of the trace element solution described by Vishniac and Santer (1957). Small quantities of yeast extract have sometimes been used as a source of trace elements and vitamins, but it should be remembered that yeast extract does not contain vitamin B_{12}. Marine enrichment cultures are best made in artificial seawater or by supplementing a mineral medium with sodium chloride (20–40 g/liter). Nitrogen assimilation and dissimilation have received relatively little attention, but ammonium (NH_4^+) and/or nitrate (NO_3^-) ions can usually serve as nitrogen sources, and some species have more than one pathway for nitrogen assimilation (e.g., *Halothiobacillus neapolitanus* has both the glutamate synthase (GOGAT) and alanine dehydrogenase pathways; Beudeker, 1982). However, most species (*Thermothiobacillus tepidarius*, *Acidithiobacillus thiooxidans* and *A. albertis* are exceptions) can

also use organic nitrogen compounds such as urea and glutamate. Several species are capable of dissimilatory nitrate reduction to nitrite, but only a few can denitrify completely to nitrogen gas (see Tables 2 and 3; see also the chapter on the genus *Paracoccus*). With the exception of *Paracoccus pantotrophus*, the known mixotrophic colorless sulfur bacteria are all unable to grow on reduced sulfur compounds when they denitrify (i.e., they can only denitrify heterotrophically or, as with *Paracoccus denitrificans*, with hydrogen as the electron donor). Because of this, a combination of denitrifying conditions with reduced sulfur compounds as substrate can be employed for the selective enrichment of the obligately autotrophic species and strains with a physiological resemblance to *Pa. pantotrophus*. Care should be taken, however, as *Thiobacillus thioparus* (which can only reduce NO_3^- to nitrite [NO_2^-]) sometimes appears in denitrifying enrichments in coculture with bacteria that can only denitrify from NO_2^-.

The enrichment and isolation procedures for the colorless sulfur bacteria are based on their chemolithotrophic properties and their ability to grow autotrophically or mixotrophically. Thiosulfate is the most commonly used substrate for their growth because it is soluble and reasonably stable over much of the required pH range. However, chemical degradation of thiosulfate results in the formation of (colloidal) sulfur. This is enhanced in acid media (Roy and Trudinger, 1970). An important factor in the selection of sulfur oxidizers is the presence of organic material that can support the growth of mixotrophic or heterotrophic organisms (Fig. 1). All other factors being equal, the specialized chemolithoautotrophs are generally dominant in batch enrichment cultures, even if organic compounds have also been added. This dominance is due to the fact that these chemolithoautotrophs have higher maximum specific growth rates on reduced sulfur compounds than do their facultative counterparts, which in batch culture will also exhibit diauxy. In addition to the truly facultative autotrophs (or mixotrophs), many other strains are able to oxidize reduced sulfur compounds, frequently carrying out incomplete oxidations (e.g., thiosulfate to tetrathionate; Trudinger, 1967; Vitolins and Swaby, 1969; Tuttle and Jannasch, 1972; Tuttle and Jannasch, 1973; Sorokin et al, 1996). Many such strains do not seem to be able to gain energy from the reaction, and there is probably a range of heterotrophs with varying efficiencies for deriving energy from the oxidation of the various reduced sulfur compounds (Trudinger, 1967; Tuttle et al., 1974; Kuenen, 1975; Kuenen, 1989a; Kelly and Harrison, 1989). *Thiomonas perometabolis* was previously believed to be the only validly named chemolithoheterotroph, but it has now been shown to grow autotrophically under specialized conditions. *Catenococcus thiocyclus* is thus one of the few named chemolithotrophs that has been shown to gain energy from thiosulfate oxidation (Sorokin, 1992; Sorokin et al, 1996), although other well-documented (albeit unnamed) chemolithoheterotrophs exist (Suylen et al., 1986; Gommers and Kuenen, 1988). An alternative benefit to energy generation might be gained if the sulfide is used to detoxify hydrogen peroxide generated during aerobic growth (Kuenen et al., 1985).

A successful method of enriching for facultatively autotrophic sulfur bacteria from freshwater sources is to use chemostat cultures under double substrate (e.g., acetate and thiosulfate) limitation (Gottschal and Kuenen, 1980). In these cultures, because of the limitation, the concentrations of the two substrates are very low and diauxy does not occur. The facultative chemolithotrophs can therefore grow mixotrophically and can successfully compete with the specialists for thiosulfate. Depending on the ratio of inorganic to organic substrates used, enrichments for chemolithoheterotrophs can also be made (Fig. 1). However, as mentioned above, marine species do not always follow this pattern, and denitrifiers can also behave differently. Growth within a biofilm may also produce anomalous results, with versatile species appearing where obligate chemolithotrophs might be expected (M.Verbeek et al., unpublished observations).

The media shown for individual species are generally intended for batch cultures and the preparation of agar plates. Chemostat media tend to be less strongly buffered because the pH of the culture can be controlled by automatic titration. This can be an advantage in the cultivation of some species. For example, *Thiobacillus* strain Q tends to accumulate large deposits of polyphosphate when grown even at the phosphate levels found in normal chemostat media (Gommers and Kuenen, 1988). The chemical constitution of chemostat medium varies depending on which nutrient is intended to be limiting. Care should be taken that the limiting factor is indeed that which was intended, since bacterial requirements for different nutrients vary. The putative limiting factor can be confirmed by altering (e.g., doubling or halving) its concentration and then checking whether the biomass yield indeed changes proportionately.

Many of the colorless sulfur bacteria have not yet been studied in the chemostat. However, the technique presents several advantages, including defined conditions and growth rates and the possibility of using potentially toxic substrates (e.g., sulfide) by keeping their concentrations low. In

addition, the sulfur-oxidizing capacity of some mixotrophs and chemolithoheterotrophs can be inhibited by the amounts of sulfite present in batch culture media supplied with reduced sulfur compounds (especially if heat-sterilized), and chemostat cultures may provide the only option for discovering whether the oxidation of these compounds does indeed generate energy (Gommers and Kuenen, 1988). Examples of media that have been used with success for growing neutrophilic, alkaliphilic and acidiphilic colorless sulfur bacteria in continuous culture are therefore given here. They can be adapted to specific needs by a careful selection of electron donors and electron acceptors and by controlling (slightly changing) the pH of the culture with an autotitrator. Titration can be done with bicarbonate (especially where obligate autotrophs are concerned) as it can serve as a source of CO_2, NaOH, and H_2SO_4 (where it will not interfere with the making of sulfur balances) or dilute HCl. The disadvantage of using HCl is its corrosive nature.

For neutrophilic bacteria, the following basic mineral salts medium can be used (concentrations in g/liter): K_2HPO_4, 0.8; KH_2PO_4, 0.3; NH_4Cl, 0.4; $MgSO_4 \cdot 7\ H_2O$, 0.4; and 2 ml of trace element solution (see below). To avoid precipitation during autoclaving at 110–120°C, the $MgSO_4$ and trace element solution must be sterilized separately. Alternatively, the medium can be made up in two parts, supplied to the chemostat by separate pumps, and mixed in the culture vessel. If this is done, the mineral salts medium listed above is made up double strength, and concentrated H_2SO_4 (1.25 ml/liter) is added to prevent precipitation during autoclaving. The substrates are then supplied, also double strength, in a second bottle to which NaOH (0.15 g/liter) has been added.

A range of substrates can be used. Concentrations in the range of 20 mM acetate or 10 mM succinate are suggested for heterotrophic cultures. Forty mM thiosulfate or sulfide can be used for autotrophic cultures. It should be noted that if sulfide is to be used, it must be sterilized separately and then aseptically added to a medium that has been cooled under N_2 or argon to exclude oxygen. The medium bottle should then be kept under a slight N_2 or argon over-pressure, even when in use, to prevent chemical oxidation of the sulfide. For denitrifying cultures, KNO_3 or KNO_2 can be used at concentrations calculated from the stoichiometry of the oxidations (e.g., for 20 mM thiosulfate, 32 mM nitrate would be more than sufficient as 10–20% of the electrons would be used for CO_2 fixation and thus are not available for electron acceptor reduction). Where organic substrates must be included in the calculation, allowance should be made for assimilation. It is necessary to add bicarbonate (to serve as a carbon source) to the substrate bottle when growing autotrophic cultures under anaerobic conditions, unless CO_2 is supplied in another way, perhaps in the gas stream.

For growing acidiphiles, a modified 9K medium (Silverman and Lundgren, 1959) has been used successfully with *Acidithiobacillus ferrooxidans* (Hazeu et al., 1986; Hazeu et al., 1988). This medium contains (in g/liter): $(NH_4)_2SO_4$, 1.0; KCl, 0.1; $Ca(NO_3)_2$, 0.01; $MgSO_4 \cdot 7\ H_2O$, 0.2; K_2HPO_4, 0.5, and 1 ml/liter of trace element solution. As with the neutrophilic medium, a range of substrates including 20 mM thiosulfate or 10 mM tetrathionate can be added. If the medium is to contain reduced sulfur compounds, they must either be sterilized separately, or the medium should be autoclaved at pH 7.0 to avoid chemical reactions. The pH of the culture is then adjusted by autotitration with 2N H_2SO_4.

The trace element solution (Vishniac and Santer, 1957) used with these chemostat media contains (in g/liter): ethylene diamine tetraacetate (EDTA), 50; $ZnSO_4$, 2.2; $CaCl_2$, 5.5; $MnCl_2 \cdot 4\ H_2O$, 5.06; $FeSO_4 \cdot 7\ H_2O$, 5.0; $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, 1.1; $CuSO_4 \cdot 5\ H_2O$, 1.57; and $CoCl_2 \cdot 6H_2O$, 1.61. It should be noted that the original description contains 10 times the concentration of $ZnSO_4$ or 22 g/liter.

Both obligately and facultatively chemolithotrophic strains are generally purified by streaking on thiosulfate or sulfur agar. Thus far, there is no specific isolation technique for the facultative chemolithotrophs. They are generally isolated from enrichment cultures by streaking on inorganic thiosulfate agar and subsequent isolation and testing for heterotrophic potential or by plating on media with a mixture of thiosulfate and an organic substrate such as acetate. Chemolithoheterotrophs must, of course, be purified on medium containing both thiosulfate and an organic substrate. The inclusion of a suitable indicator in the agar permits the detection of colonies actually using the thiosulfate (and where neutrophilic bacteria are concerned, can indicate sulfate or polythionate production). Solid media generally contain thiosulfate, but sulfur plates may sometimes be appropriate. Alternatively, plates can be incubated in an atmosphere of H_2S gas. A crude method of generating the H_2S is to mix Na_2S crystals with acid, but remember that H_2S is 10 times more toxic than HCN. As H_2S oxidizes spontaneously in air, a more sophisticated method is to continuously generate an air, nitrogen and H_2S mixture, which is then passed over the cultures (Visser et al., 1997). If desired, organic compounds can be included, as they are not toxic for the obligate

chemolithotrophs. Some amino acids may, however, inhibit growth when added as the sole organic substrate in an inorganic medium. It is likely that the inhibition is caused by an imbalance in the amino acid synthesis in the organism (Kelly, 1969; Lu et al., 1971). Obviously, when acid-producing bacteria are being grown in batch cultures, it is necessary to control the pH. This can be done in several ways including strong buffers, the addition of solid calcium carbonate, and periodic or automatic adjustment with carbonate solution. Solid calcium carbonate dissolves at pH values below 6.0–6.5, and it is therefore only useful for the neutrophiles. It can also be used in solid media. Suitable pH indicators, such as bromocresol purple (pH 5.2–6.8), bromthymol blue (pH 6.0–7.6) and phenol red (pH 6.8–8.7), can be used.

One of the most common problems in obtaining pure cultures of chemolithotrophs is the extreme persistence of heterotrophic contaminants in association with the chemolithotrophic colonies on agar plates (e.g., Taylor et al., 1971; Harrison, 1984). This is partly due to the presence of trace amounts of soluble organic material in virtually all agar brands and partly due to the fact that many obligate chemolithotrophs excrete organic compounds (Schnaitman and Lundgren, 1965; Borichewski, 1967; Cohen and Kuenen, 1976). An example of this frequently occurs during enrichment for *Thiobacillus denitrificans*. Anaerobic batch cultures containing nitrate and thiosulfate often yield mixed populations of *T. thioparus* and heterotrophic denitrifiers, rather than *T. denitrificans* (Taylor et al., 1971). *Thiobacillus thioparus* can only reduce nitrate to nitrite, but it also excretes organic material that may then be used by heterotrophic, nitrite-reducing bacteria. An analogous problem has been found in the enrichment and isolation of *Acidithiobacillus ferrooxidans*, which may occur in close association with the facultatively chemolithotrophic *Acidiphilium acidophilum* (Guay and Silver, 1975; Tuovinen et al., 1978; Harrison, 1984). The low pH required by these acidophilic bacteria can result in partial hydrolysis of agar and the release of organic compounds into the medium. Critical endpoint dilutions to obtain pure cultures of obligate chemolithotrophs should only be used with extreme caution, as often only the highest dilution that contains both the chemolithotroph and heterotrophic contaminant will show growth. A new technique that avoids organic support media has been successfully used for the isolation of acidophilic and neutrophilic obligate autotrophs (De Bruyn et al., 1990). This method involves passing the inoculum through a sterile, inert, polycarbonate membrane filter. The filter is then floated on the surface of a suitable mineral medium (see below) and incubated at an appropriate temperature. Substrate can be supplied as dissolved thiosulfate or as H_2S. By eliminating the use of agar, the presence of all organic compounds can be avoided and overgrowth by heterotrophs thus prevented. This method is both faster and simpler than other wholly inorganic methods such as silica gel plates. It should also be remembered that some microorganisms may not be able to tolerate the high concentrations of substrate (or possible contaminants such as sulfite in some grades of thiosulfate) generally required in batch cultures, and a continuous culture or fed batch culture might be more appropriate.

It is important to note that the enrichment or isolation technique will often define the type of isolate obtained. For example, in a study of the microbial community in a sulfide-oxidizing wastewater treatment system (Visser et al., 1997), it was found that direct streaking of samples onto agar gave a number of colonies equivalent to only 0.2% of the number obtained from direct counts. Even though a mineral salts agar with thiosulfate had been used, 80% of these colonies proved to be heterotrophic. Serial dilution methods gave an even lower count, equivalent to 0.06% of the direct count. When isolations were made using an adaptation of the floating filter technique and sulfide, however, the number of autotrophic colonies obtained was equivalent to 15% of the direct count. Different autotrophs dominated the cultures obtained from the serial dilutions and the floating filters. These organisms, known as "*Thiobacillus* strains W and W5" (and on the basis of 16S RNA sequences, related to *Halothiobacillus neapolitanus*) behaved differently to the extent that the original isolate W5 would not grow on agar plates. Comparison of these isolates with the original microbial community, using denaturing polyacrylamide gel electrophoresis of the total protein of these cultures, showed that isolate W5 was most probably the dominant organism in the original bioreactor. Because the results from the filter technique are so dramatically different from those of conventional methods, it is worth saying more about it here. For the work described above, 0.2-mm polycarbonate filters (Poretics Corporation, Livermore, CA) were used. Twenty-five mm diameter filters were incubated in sterile tissue culture clusters (Costar, Cambridge, MA) with wells (35 mm in diameter) filled with a medium suitable for autotrophic growth. The clusters were placed in dessicators, but any vessel allowing the controlled flushing of the headspace would do. Also, H_2S was continually circulated through the headspace by sparging an acid solution of sodium sulfide with air.

Identification

By definition, none of the colorless sulfur bacteria are phototrophic, and bacteria possessed of photopigments belong in other genera (e.g., *Chromatium* or *Thiocapsa*) even if they can grow lithoautotrophically on reduced sulfur compounds in the dark. The basic taxonomic features of the members of this group are shown in Tables 2 and 3. For detailed descriptions of most of the species, the reader is referred to Kelly and Wood (2000b), where the currently known physiological and taxonomic features of most of the members of these genera are shown.

It cannot be stressed too strongly that before identification, cultures should be rigorously checked for purity, since heterotrophic contaminants are both common and persistent in autotrophic cultures. "Normal" heterotrophic contaminants should be sought not only in rich media at neutral pH values, but also using a range of environmental conditions (e.g., pH, dissolved oxygen, and denitrification) as close to those conditions under which the culture is normally grown. Bearing in mind that even some of the contaminants may be fastidious, several different organic substrates should be checked with the mineral salts media suggested for use with colorless sulfur bacteria.

Enrichment, isolation, and enumeration procedures for sulfur-oxidizing bacteria were at one time almost invariably based on the use of either thiosulfate or elemental sulfur as substrates. As a result, potential isolates may not have been found, as some colorless sulfur bacteria preferentially or even exclusively use sulfide (W. J. Bijleveld et al., unpublished observations). Equally, the use of agar plates selects for bacteria able to cope with the organic substrates and with those substrates supplied in relatively high concentrations. Diffusion through the gel is the only way by which oxidation products including H_2SO_4 are removed. As already mentioned (see the discussion of "*Thiobacillus* W5" above), a very different isolation pattern emerged when the bacterial community in a wastewater treatment reactor was cultivated on inorganic filters floating on mineral medium (used as a support). The ability to oxidize reduced sulfur compounds is not routinely included in standard taxonomic tests, and species, which might otherwise be considered hydrogen bacteria (e.g., *Pa. denitrificans*) or methylotrophs (e.g., *Hyphomicrobium* EG), are now found capable of oxidizing (and gaining energy from) reduced sulfur compounds (Friedrich and Mitrenga, 1981; Suylen et al., 1986). Thus the ability to oxidize reduced sulfur compounds, especially sulfides, may be much more widespread in nature than indicated by currently employed methodology.

Some commonly used media are described in the references given. It must be emphasized that for most thiobacilli, these media do not ensure selective enrichments. Isolation procedures generally involve colony development. However, because the morphological characteristics of the thiobacilli are similar, and because the colony shape and color are, to some extent, subject to the medium and growth conditions, their appearance is of little identification value. Identification procedures should involve reference to relevant previously described strains and studies at the physiological and molecular levels. Further details can be found below, in the original descriptions of the different species, and in Kelly and Harrison (1989), Kuenen and Robertson (Kuenen and Robertson, 1989b; Kuenen and Robertson, 1989c), and Kelly and Wood (2000a).

As discussed in the introduction, the taxonomy of the colorless sulfur bacteria was originally based on the classical taxonomic tests that are largely founded on physiological reactions. The most critical requirements for inclusion in the genus *Thiobacillus* have been that the organism is a Gram-negative rod with the ability to use reduced sulfur compounds as an energy source for growth. In recent years, the heterogeneity of the genus became apparent, (Kuenen and Robertson, 1987; Mason et al., 1987; Robertson et al., 1989), and a need for some form of reorganization became apparent (Kelly and Harrison, 1989; Kuenen, 1989a). Writing for the previous edition of *The Prokaryotes*, we said "More data are necessary before the taxonomic world is turned upside down." As can be seen from Table 1, this was not an understatement; the genus *Thiobacillus* alone was split into five new genera once the molecular taxonomy was done. However, while it is tempting to rely only on molecular analysis, this reliance would lead to an equally inaccurate or uninformative picture; thus, it is far better to use a mixture of classical and new methods. After enrichment and isolation on the basis of their ability to derive energy from the oxidation of reduced sulfur compounds, physiological factors such as the ability to fix CO_2, pH range, temperature range and denitrification capability should all be checked in addition to the 16S mRNA analysis, %G+C content, and DNA:DNA hybridization with possibly related organisms. It is also desirable to check such isolates on substrates such as H_2, methane and methanol as well as more traditional heterotrophic substrates such as acetate or glucose and alternative reduced sulfur compounds. Electron microscopy is important not only to establish the presence and number of flagellae, but also to identify intracellular inclusions such as sulfur particles, carboxysomes, and stored carbohydrates. It should be noted that suspected

chemolithoheterotrophic bacteria should really be checked in continuous cultures under electron donor limitation to confirm that they can gain energy from the oxidation and that they can't fix CO_2. Sometimes bacteria do not respond well to the relatively high substrate concentrations in batch cultures, or to the presence of contaminants (e.g., SO_3^{-2} frequently contaminates thiosulfate, and can be produced in thiosulfate solutions during heat sterilization).

The Bacteria

The γ-Subclass of the Proteobacteria

These four species were all listed as members of this physiological grouping in earlier editions of *The Prokaryotes* and *Bergey's Manual*. They have now all been transferred to other genera and will be described in the chapter on their respective genera.

Acidiphilium acidophilum synonyms: *Thiobacillus acidophilus* and *Thiobacillus organoparus.*

Paracoccus versutus synonyms: *Thiobacillus versutus*, *Thiobacillus rapidicrescens* and *Thiobacillus* A2.

It is worth noting that *Pa. versutus* was originally isolated from enrichment cultures inoculated from freshwater sediments and designed to produce *Thiobacillus denitrificans*. Anyone aiming to make such enrichments should be aware of the possibility.

Paracoccus pantotrophus synonym: *Thiosphaera pantotropha*.

Starkeya novella synonym: *Thiobacillus novellus*.

The β-Subclass of the Proteobacteria

THE GENUS *THIOBACILLUS* All members of this genus (for synonyms, see Table 1) are Gram-negative rods, some of which are motile. They are also obligately or facultatively autotrophic. Detailed taxonomic descriptions can be found in Kelly and Wood (2000a). However, see Table 2 for general characteristics.

Thiobacillus aquaesulis *Type Strain ATCC 43788 (Wood and Kelly, 1988)* This moderately thermophilic species was isolated from the thermal spring at Bath, Avon, Great Britain. In batch culture containing thiosulfate, this species accumulates sulfur. However, accumulation is not observed under thiosulfate limitation in the chemostat. Although *T. aquaesulis* can grow heterotrophically on complex substrates such as yeast extract, it cannot grow if supplied sugars, organic acids, or methylamine as its sole substrate. NH_4^+ can be used as a source of nitrogen.

Thiobacillus denitrificans *Neotype Strain NCIB 8327 (Beijerinck, 1904; Kelly and Harrison, 1989) Thiobacillus denitrificans* is an obligate autotroph and depends on the oxidation of inorganic reduced sulfur compounds and carbon dioxide fixation. It can assimilate a limited amount of organic carbon. Under anaerobic conditions, these bacteria couple the oxidation of sulfur compounds to the respiratory reduction of nitrogen oxides (such as nitrate or nitrite) to nitrogen gas (denitrification). Their enrichment and isolation can thus be made selective by the use of anaerobic media and cultural conditions. This species contains ubiquinone Q8.

The taxonomic status of *Thiobacillus denitrificans* has recently been evaluated by Kelly and Wood (2000b).

Thiobacillus thioparus *Type Strain ATCC 8158 (Beijerinck, 1904) Thiobacillus thioparus* is the type species of the genus *Thiobacillus*. Like all obligately chemolithotrophic thiobacilli, it has a limited capacity for incorporating organic carbon (e.g., acetate and succinate) into cellular material, but its energy generation for growth obligately depends on the oxidation of inorganic, reduced sulfur compounds. It is differentiated from the similar *T. neapolitanus* by 1) its ability to grow anaerobically in the presence of nitrate (which is only reduced to nitrite), 2) its ability to grow on thiocyanate, and 3) the immediate formation of sulfur in the batch culture medium described for *T. novellus*. Some newly isolated strains can grow chemolithoautotrophically on dimethyl disulfide, dimethyl sulfide, carbon disulfide, and carbonyl sulfide (Smith and Kelly, 1988a; Smith and Kelly, 1988b; Smith and Kelly, 1988c). NH_4^+ and NO_3^- can both be used as nitrogen sources. *Thiobacillus thioparus* contains ubiquinone Q8.

Thiobacillus thioparus can be isolated from fresh water and estuarine and marine sediments (Vishniac and Santer, 1957) a suitable inorganic medium and the enrichment and isolation procedures discussed above.

THE GENUS *THIOMONAS* (MOREIRA AND AMILS, 1997) This newly described genus contains many of the facultatively autotrophic members of the old genus, *Thiobacillus*. They are thus Gram-negative rods, some of which might be motile under suitable grow conditions. For synonyms, see Table 1.

THIOMONAS CUPRINA TYPE STRAIN DSM 5495 (HUBER AND STETTER, 1989) This is a facultatively chemolithotropic, mesophilic acidophile (pH optimum for growth, 3–4) capable of growth on a variety of sulfidic ores and sulfur, but not

on ferrous iron. It can also grow on yeast extract, peptone and pyruvate.

Thiomonas intermedia Type Strain ATCC 15466 (London, 1963) Autotrophic growth of *Ts. intermedia* is slow, and mixtures of glucose and thiosulfate give a much faster growth rate (London and Rittenberg, 1966; Matin and Rittenberg, 1970). While able to grow heterotrophically, *Ts. intermedia* requires a reduced sulfur compound as a source of sulfur. Thiosulfate can be replaced by yeast extract or by a mixture of thiamin pyrophosphate, reduced glutathione, lipoic acid, methionine, cysteine (each at a final concentration of 0.15 mM) together with biotin and coenzyme A (both at final concentrations of 0.015 mM; Smith and Rittenberg, 1974). This organism contains ubiquinone Q8.

Although many thiobacilli will grow in media designed for *Ts. intermedia*, the enrichment of *Ts. intermedia* may be promoted if the pH is allowed to fall below 4.0, as it is relatively acid tolerant. In addition to NH_4^+ and NO_3^-, organic nitrogen compounds can be used as a source of nitrogen. Marine and freshwater strains are known. *Thiomonas intermedia* has been isolated from freshwater sediments. For further details, see London (1963) and London and Rittenberg (1966).

Thiomonas perometabolis Type Strain ATCC 23370 (London and Rittenberg, 1966) It was formerly believed that *Ts. perometabolis* was unable to fix carbon dioxide and was thus unable to grow autotrophically. Its ability to gain energy from the oxidation of reduced sulfur compounds was undisputed, and *Ts. perometabolis* was therefore considered to be a chemolithoheterotroph. However, Harrison (1983) and Katayama-Fujimura et al. (Katayama-Fujimura et al., 1983; Katayama-Fujimura et al., 1984) showed that if relatively large inocula were used, *Ts. perometabolis* could grow chemolithoautotrophically in batch cultures on thiosulfate, and the first enzyme of the Calvin cycle, ribulose-bisphosphate carboxylase, was detected in these cultures.

Thus far, chemostat cultures with a carefully chosen mixture of inorganic and organic components in the medium (Fig. 1) appear to be the most successful selective technique for the cultivation of this type of organism, and at least one chemolithoheterotroph, "*Thiobacillus*" strain Q, has been isolated from this type of culture (Gommers and Kuenen, 1988). Since great care is required to grow this type of organism chemolithoautotrophically, the use of substrate ratios expected to favor chemolithoheterotrophs might more successfully enrich for *Ts. perometabolis* than those expected to favor mixotrophs. For batch culture, the basic medium described for *Ts. intermedia* may be used, with yeast extract or casein hydrolysate supplements.

"*Thiobacillus rubellus*," a strain which appears to differ from *Ts. perometabolis* only in its colony color, was described by Mizoguchi et al. (1976).

Thiomonas thermosulfata Type Strain ATCC 51520 (Schooner et al., 1996) This organism grows autotrophically on thiosulfate, tetrathionate and sulfur, heterotrophically on yeast extract, glutamate and succinate, and mixotrophically. It does not grow on ferrous iron or formate. As its name suggests, it is a moderate thermophile. In thiosulfate medium, the pH can fall as low as 2.8. *Thiomonas thermosulfata* contains ubiquinone Q8.

"*Thiobacillus plumbophilus*" Type Strain DSM 6690 (Drobner et al., 1992) This organism was isolated from a German uranium mine. It is obligately autotrophic, able to grow on H_2S, PbS or H_2, but not sulfur, thiosulfate or many other metal sulfides. It is strictly aerobic. It contains 96.5% ubiquinone Q8. As it has little or no DNA hybridization with *Ts. cuprina*, this species is probably not a member of the genus *Thiomonas*. Physiologically, it resembles *Acidithiobacillus ferrooxidans*, which is a member of the γ-subclass. Moreover, there is no DNA hybridization between the two. The status of *T. plumbophilus* therefore requires further consideration.

The γ-Subclass of the Proteobacteria

The range of environmental optima shown by the species listed here serves to emphasize the fact that the ability to metabolize reduced sulfur compounds is a fairly loose taxonomic criterion. In fact, it is in the γ-subclass that the extremophilic colorless sulfur bacteria are found.

The genus *Acidithiobacillus* All members of this genus (for synonyms see Table 1) are Gram-negative rods, some of which are motile. They are obligate acidiphiles. The full description of this genus is given in Kelly and Wood (2000a), but the basic characteristics are summarized in Table 3. These are generally the species previously known as the autotrophic, acidiphilic *Thiobacilli*.

Acidithiobacillus albertensis *Type Strain ATCC 35403 (Bryant et al., 1983; Kelly and Wood, 2000a)* Although physiologically similar to *A. thiooxidans*, the G+C content of this strain differs significantly (Table 3), and it must therefore be considered a separate species. *Acidithiobacil-*

lus albertensis was isolated from the acid soil adjacent to a sulfur stockpile. The cells have a condensed glycocalyx and, like *A. ferrooxidans*, have been observed to accumulate internal sulfur deposits.

As the two species are so similar, *A. albertensis* can be cultured on the media described for *A. thiooxidans*. However, it should be noted that the pH range of *A. albertensis* does not extend to the low level associated with *A. thiooxidans*, and its lower limit is pH 2.0. NH_4^+ is used as a source of nitrogen. Further details can be found in Bryant et al. (1983).

Acidithiobacillus caldus *Type Strain: Strain KU; DSM 8584; ATCC 51756 (Hallberg and Lindström, 1994; Kelly and Wood, 2000a)* Short, motile, rods that can grow on reduced sulfur compounds and hydrogen, but not on ferrous iron or metal sulfides. They can grow mixotrophically if provided with tetrathionate and glucose or yeast extract.

Acidithiobacillus ferrooxidans *Type Strain ATCC 23270 (Temple and Colmer, 1951; Kelly and Wood, 2000a) Acidithiobacillus ferrooxidans* is an obligate autotroph that is able to derive energy from the oxidation of ferrous iron as well as reduced sulfur compounds. During growth on thiosulfate, trithionate, tetrathionate and sulfide, *A. ferrooxidans* has been observed to accumulate fine sulfur deposits, which are predominantly associated with the cell wall (Hazeu et al., 1988). Early reports of facultatively autotrophic *A. ferrooxidans* strains are now believed to have been due to the presence of facultatively autotrophic (see *Acidiphilium acidophilum*) or heterotrophic contaminants, some of which have been difficult to remove, as their presence can stimulate the growth of the autotroph (Harrison, 1984). The ability to oxidize Fe^{2+} is the key characteristic that is generally employed in isolation procedures. Initial enrichments with reduced sulfur compounds frequently produce mixtures of various acidophilic thiobacilli, whereas enrichment on Fe^{2+} can result in almost pure cultures of *A. ferrooxidans*.

Ferrous Sulfate Medium for the Isolation and Batch Culture of *A. ferrooxidans* (Temple and Colmer, 1951; Tuovinen and Kelly, 1973)

Solution I

$K_2HPO_4 \cdot 7\ H_2O$	0.5 g
$(NH_4)_2SO_4$	0.5 g
$MgSO_4 \cdot 7\ H_2O$	0.5 g
H_2SO_4, 15 N solution	5.0 ml

Dissolve in 1 liter of deionized water and sterilize by autoclaving.

Solution II

$FeSO_4 \cdot 7\ H_2O$	167 g
H_2SO_4, 15 N solution	50 ml

Dissolve in 1 liter of deionized water and sterilize by filtration. Four parts of solution I and 1 part of solution II are mixed to give a medium containing 120 mM Fe^{2+}. Should it be necessary to prevent iron precipitates from forming in the medium, the pH of the cultures can be lowered in successive subcultures to a value of 1.3 with H_2SO_4.

To prepare solid media, high purity agar or agarose must be used at low concentrations. Most brands solidify sufficiently at concentrations of 0.3–0.4% (w/v). Better results are obtained if the agar is previously washed with distilled water to remove inhibitory substances. Membrane filters that have been previously washed in distilled water can be used to support the bacteria on solid or liquid media, as described in the section on enrichment and isolation (De Bruyn et al., 1990).

Medium for the Growth of *A. ferrooxidans* on Reduced Sulfur Compounds in Batch Culture

Solution III (per liter):

KH_2PO_4	3.0 g
$(NH_4)_2SO_4$	3.0 g
$MgSO_4 \cdot 7\ H_2O$	0.5 g
$CaCl_2 \cdot 2\ H_2O$	0.5 g

Dissolve in 1 liter of deionized water and sterilize by autoclaving. After sterilizing, 0.15 ml of solution II can be added, together with one of the following substrates: $Na_2S_2O_3 \cdot 7\ H_2O$, 10 g; $K_2S_4O_6$, 3.0 g; or flowers of sulfur, 5.0 g.

For growth on reduced sulfur compounds, the initial pH should be around 3.8–4.4. *Thiobacillus ferrooxidans* can use NH_4^+ and NO_3^- as nitrogen source and has been reported to be able to fix N_2 (McKintosh, 1978).

Acidithiobacillus thiooxidans *Type Strain ATCC 19377 (Waksman and Joffe, 1922; Kelly and Wood, 2000a)* These obligately autotrophic bacteria are typically found in acidic environments where they account for the production of copious amounts of sulfuric acid from reduced sulfur compounds. They have been isolated from soil, sulfur deposits, and mine refuse. *Acidithiobacillus thiooxidans* may be easily enriched in acid media with elemental sulfur as substrate, but this is not a specific medium, and the other acidophiles may also appear. *Acidithiobacillus thiooxidans* can grow at lower pH values (down to pH 0.5) than any other known species in this group.

Medium for the Isolation and Batch Culture of *A. thiooxidans* (Waksman and Joffe, 1922; Starkey, 1935)

K_2HPO_4	3.5 g
$(NH_4)_2SO_4$	0.3 g
$MgSO_4 \cdot 7\ H_2O$	0.5 g
$FeSO_4 \cdot 7\ H_2O$	0.018 g
CaCl	0.25 g
Flowers of sulfur	5.0 g

Dissolve in 1 liter of deionized water, adjust to pH 4.5 with HCl, and sterilize by autoclaving.

Solid media can be prepared for *A. thiooxidans* using thiosulfate (10 g/liter) or colloidal sulfur (Wieringa, 1966):

Mix 25–30 ml of molten, sterile mineral salts medium (containing 15 g/liter agar) with 1–1.5 ml of 0.15 N HCl in a 10-cm Petri dish and allow to set. Then pour 10 ml of mineral medium supplemented with polysulfide solution on top (2 ml/liter). The HCl diffuses upwards and precipitates sulfur as a fine suspension. The H_2S is removed and the surface of the agar is dried by warming the plate. Polysulfide solution can be prepared by mixing a saturated H_2S solution in water with a saturating amount of sulfur, followed by autoclaving.

These plates can be prepared with different mineral salts media for the growth of other sulfur bacteria at different pH values. NH_4^+ can be used as a nitrogen source.

"Thiobacillus prosperus" *Type Strain DSM 5130 (Huber and Stetter, 1989)* This organism is a member of the γ-subclass and therefore not a true member of the genus *Thiobacillus*, but requires further study before it can be assigned to its new genus. However, because it is an acidophile, it is most appropriately listed with the other acidophiles until such time as its correct genus is established.

"*Thiobacillus prosperus*" was first isolated from a marine hydrothermal area off Italy (Huber and Stetter, 1989). It is an obligate autotroph that can grow aerobically on pyrite, sphalerite, chalcopyrite, arsenopyrite, and galena as well as on H_2S. Growth on Fe^{2+} and sulfur is poor, and thiosulfate, tetrathionate, and synthetic versions of the different ores do not generally support growth. This species is markedly less sensitive to salt (grows at NaCl concentrations up to 6%) than *A. ferrooxidans* is (does not grow at NaCl concentrations above 1%; Huber and Stetter, 1989), but it should be regarded as halotolerant rather than halophilic because a concentration of 1% NaCl nearly doubled its doubling time. As it is physiologically similar to *A. ferrooxidans* (although the amount of DNA hybridization between them was negligible), "*T. prosperus*" can be grown in the media described for *A. ferrooxidans*.

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Prokaryotes (2006) 5:828–839
DOI: 10.1007/0-387-30745-1_38

CHAPTER 3.2.14

The Genera *Simonsiella* and *Alysiella*

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Introduction

The genera *Simonsiella* and *Alysiella* are filamentous, aerobic, chemoorganotrophic members of the Neisseriaceae in the Betaproteobacteria. *Simonsiella* is part of the normal flora of the oral cavity of a variety of phylogenetically and ecologically diverse warm-blooded vertebrates (Table 1). *Simonsiella* is distinguished from other bacteria on the basis of morphology (Fig. 1). Individual cells are wide (1.9–6.4 μm), short (0.5–1.3 μm), and relatively flat (0.5–1.3 μm) and attach to form monoseriate filaments that are 8 or more cells long. Cells at either end of the filament are often narrower than cells in the center, giving the filament a tapered appearance. In some strains, filaments of 8 or more cells remain attached, giving rise to "super" filaments that reach 50 or more μm in length. *Simonsiella* cells are crescent-shaped, which causes filaments to bend, yielding a watchband shaped filament with convex-dorsal and concave-ventral asymmetry. This dorsal-ventral asymmetry is functional since *Simonsiella* attach to squamous epithelial cells exclusively via their ventral surface and glide along the long axis of the filament (Fig. 1e). Chemotaxonomic and phylogenetic studies of *Simonsiella* isolates from the mouths of different mammals suggest that each mammal may host a unique species of *Simonsiella*.

Alysiella is similar to *Simonsiella* except filaments are composed of oblong disk-shaped cells that are neither dorsoventrally differentiated nor tapered toward the ends (Fig. 1f). Currently there is only one confirmed isolate of *Alysiella* and this isolate is related to *Simonsiella* on the basis of 16S rDNA sequence analysis. Other strains previously referred to as *Alysiella* belong to the genus *Moraxella* in the Gammaproteobacteria.

Phylogeny

Rossau et al. (1989) and Dewhirst et al. (1989) showed that isolates of *Simonsiella* and the type strain of *Alysiella filiformis* are closely related to the genera *Neisseria*, *Kingella* and *Eikenella* by DNA-DNA hybridization (Rossau et al., 1989), rRNA-DNA hybridization (Rossau et al., 1989), and 16S rRNA sequencing and phylogenetic analysis (Dewhirst et al., 1989); therefore, the family Neisseriaceae was emended to include these genera (Dewhirst et al., 1989). Rossau et al. also showed that two other strains deposited in culture collections as *Alysiella*, American Type Culture Collection (ATCC 29468) and Hygiene Institut, Universitä Marburg, Marburg, Germany (HIM 1018-2), actually belong to the Gammaproteobacteria (Rossau et al., 1989; Rossau et al., 1991), and this led to an erroneous report that *Alysiella* is a member of the Gammaproteobacteria (Stackebrandt et al., 1988). More recently the 16S rRNA gene has been sequenced from ATCC 29468 and this sequence confirms that this strain is a member of the genus *Moraxella* (C. Xie and A. Yokota, unpublished data). Apparently certain filamentous morphotypes of *Moraxella* are morphologically and chemotaxonomically very similar to the type strain of *Alysiella*.

The first study of the phylogeny within the genus *Simonsiella* was by Hedlund and Staley (2002), who sequenced the 16S rRNA gene from four isolates from each of four different animal hosts: humans, sheep, dogs and cats. The resulting phylogeny showed that the genus *Simonsiella* is paraphyletic within the Neisseriaceae, a result that could be considered surprising given the distinctive morphology of the genus. There are many plausible explanations for the paraphyly of *Simonsiella* (see Funk and Omland, 2003). One possible explanation for the paraphyly of the genus is that horizontal transfer of 16S rRNA genes has occurred within the Neisseriaceae through transformation and recombination, partly obscuring the record of the vertical evolution of the group. Indeed, members of the genus *Neisseria* are well known for their natural competence, and this genus cohabits the oral cavity with *Simonsiella* (see The Genus Neisseria in this Volume). Alternatively, it is possible that *Simonsiella* morphology is unstable on an evolutionary scale.

Table 1. Known occurrence of *Simonsiella* in the oral cavities of warm-blooded vertebrates.

	References to	
Commensal	Observations	Axenic cultivation
Humans	Miller, 1911	Richardson et al., 1966
	Simons, 1922	Kuhn et al., 1974
	Langeron, 1923	Kuhn et al., 1978
	Fellinger, 1924	Whitehouse et al., 1987
	Berger, 1963	
	Hoffman and Frank, 1966	
	Bruckner and Fahey, 1969	
	Carandina et al., 1984	
Chickens	Miller, 1911	
	Simons, 1922	
Sheep	Simons, 1922	Steed, 1962
	Richardson et al., 1966	Kuhn et al., 1978
Horses	Simons, 1922	
Pigs	Simons, 1922	
Cows	Simons 1922	
Goats	Simons, 1922	
Rabbits		Steed, 1962
Guinea pigs		Berger, 1963
Dogs	Beust, 1929	Berger, 1963
	Hoffman and Frank, 1966	
	Richardson et al., 1966	Saphir and Carter, 1976
	Hodgin, 1988	Nyby et al., 1977
		Bailie et al., 1978
		Kuhn et al., 1978
Cats		Berger, 1963
		Richardson et al., 1966
		Kuhn et al., 1978

Despite the apparent paraphyly of the genus, *Simonsiella* isolates from each host animal form a well-supported monophyletic group (Hedlund and Staley, 2002), suggesting that each species of mammal hosts a unique species of *Simonsiella*, as originally suggested by Kuhn and coworkers (Jenkins et al., 1977; Kuhn et al., 1978) and supported by chemotaxonomic studies (Kuhn et al., 1978), distinct ranges of DNA G+C content (Kuhn et al., 1977), distinct fatty acid content (Jenkins et al., 1977), and distinct cellular carbohydrate content (Heiske and Mutters, 1994; see the section Identification in this Chapter). These host-specific *Simonsiella* groups correspond to the three validly described *Simonsiella* species: *S. muelleri* from humans, *S. crassa* from domestic sheep, and *S. steedae* from domestic dogs. Although the *Simonsiella* strains isolated from domestic cats form a well-supported monophyletic group, they have not been assigned a species since this group is more phylogenetically diverse than the described *Simonsiella* species and since these strains are phenotypically similar to *S. steedae*. Furthermore, *S. steedae* and the isolates from cats have not been tested by DNA-DNA hybridization.

If the pattern of host animal-*Simonsiella* specificity is confirmed with studies of *Simonsiella* isolates from other hosts, this would indicate that the barriers to *Simonsiella* transfer between hosts of different animal species are much higher than the barriers to *Simonsiella* transfer among members of a single host species. Kuhn (1981) suggested that these host-specific *Simonsiella* groups could be considered ecospecies and that the *Simonsiella* that normally inhabit the oral cavity of a particular animal might evolve to thrive within the mouth of its host through adaptations to the nutrition provided by the host animal, to the microbial community within the mouth of the host animal, or to other host-specific factors.

It has been suggested that the divergence of *Simonsiella* into host-specific groups may have been forced by the divergence of the host animals (Hedlund and Staley, 2002). Evidence in support of this hypothesis is that the *Simonsiella* isolates from carnivores—domestic dogs and cats—are more closely related to each other than they are to *Simonsiella* strains from humans and sheep. This pattern is predicted by the co-divergence hypothesis because dogs and cats are together in the order Carnivora and are thought to have diverged about 45 million years ago, whereas the carnivores are thought to have diverged from primates and artiodactyls 80–100

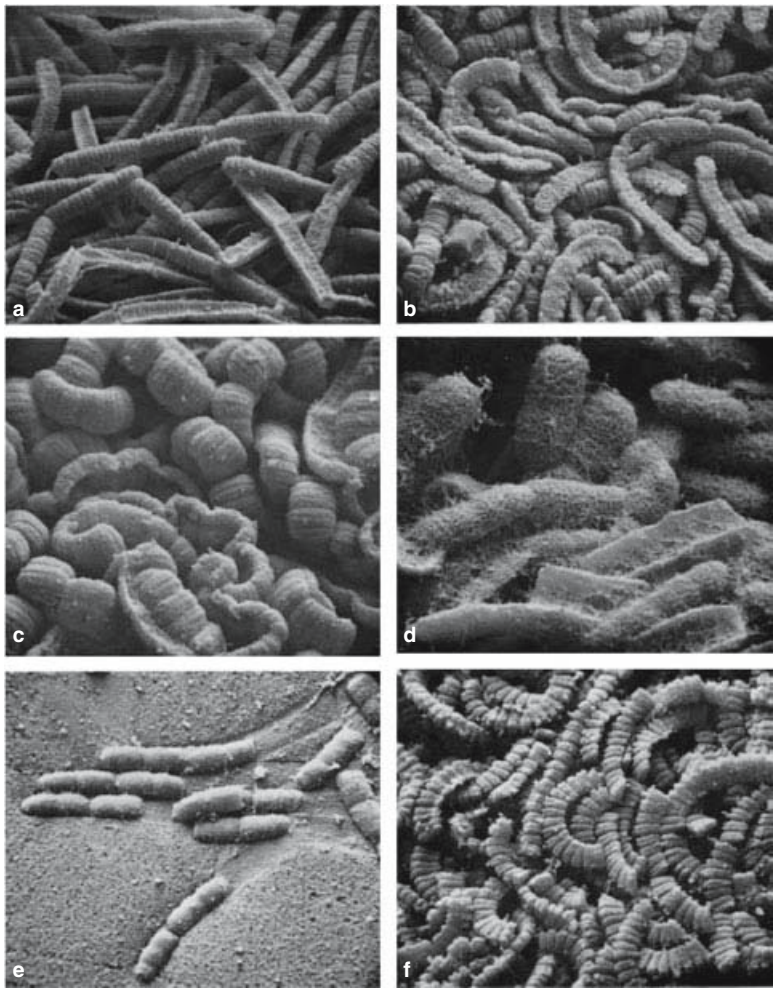

Fig. 1. Scanning electron micrographs illustrating the morphology of four *Simonsiella* strains and one *Alysiella* strain cultivated on BSTSY agar at 37°C for 10 h (a, b, c, d and f) and 4 days (e). a) *Simonsiella crassa* neotype ATCC 27504 of sheep origin forms straight, relatively slender filaments with rounded ends; the individual crescent-shaped cells can be recognized on both the convex dorsal and the concave ventral sides of the filaments; compare with Fig. 7a. b) *Simonsiella* sp. ATCC 27381 of cat origin shows lateral curvature of the filaments with large amounts of irregularly structured ("fuzzy") extracellular material obscuring the cells on the concave ventral side of the filaments; compare with Fig. 7g. c) *Simonsiella steedae* ATCC 27396 of dog origin forms broad filaments with dorsal curvature that causes the filaments to lie on edge. Some filaments are apposed to each other on their ventral sides. d) *Simonsiella steedae* ATCC 27411 of dog origin possesses copious amounts of extracellular capsular material obscuring entirely the multicellular nature of the *Simonsiella* filaments; compare with Fig. 7f. e) *Simonsiella steedae* ATCC 27411 of dog origin showing gliding tracks impressed in the agar surface. In the hydrated state the tracks are filled with liquid and can be seen with a light microscope at low magnification. The dorsal capsular material obscures the multicellular structure of the filaments. f) *Alysiella filiformis* neotype ATCC 29469 of sheep origin. The multicellular filaments are flat and are composed of closely apposed pairs of oblong disk-shaped cells; *Alysiella* filaments lack the dorsal-ventral and the terminal differentiations characteristic of the genus *Simonsiella*. Compare with Fig. 7b. × 2250 (a, b, c, d and f). × 1125 (e). (a, b, c, d and e from Pangborn et al., 1977.)

million years ago (Kumar and Hedges, 1998). We believe that the co-divergence hypothesis should be tested with *Simonsiella* strains isolated from wild animals since it seems that host switching is more likely to occur in domestic and zoo animals than in natural populations and since presumed transient *Simonsiella* strains have occasionally been isolated from domestic animals (Kuhn et al., 1978; D. A. Kuhn, unpublished observations).

The 16S rRNA gene of type strain of *Alysiella filiformis* was recently sequenced by two groups (K. K. Garborg, et al., unpublished data; C. Xie and A. Yokota, unpublished data) and shown to form a well-supported monophyletic group with the sequences from *S. crassa* (Fig. 2). It is interesting that both *A. filiformis* and *S. crassa* are of sheep origin. Given the close phylogenetic position of the type strain of *A. filiformis*

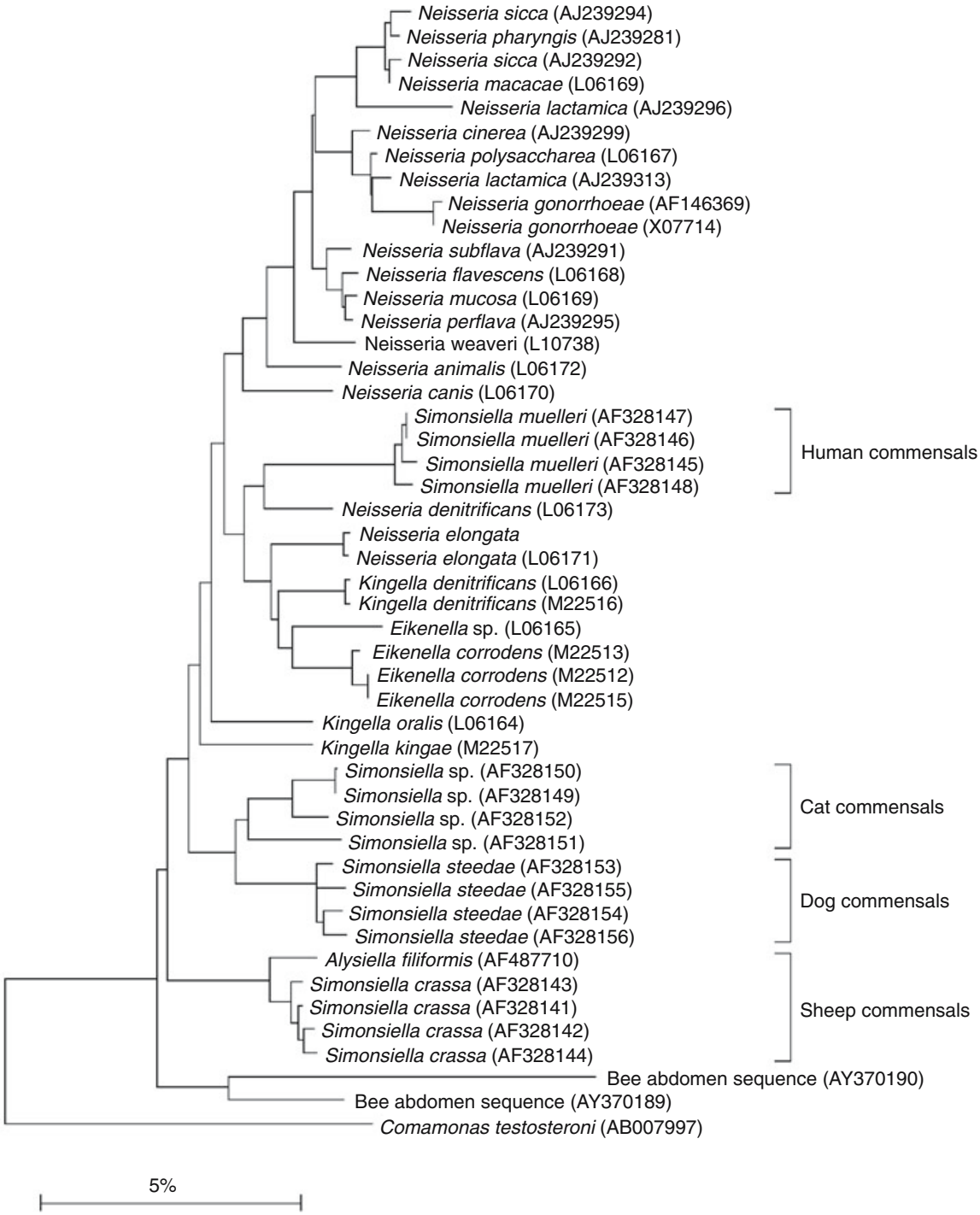

Fig. 2. 16S rRNA gene phylogenetic tree showing the position of *Simonsiella* and *Alysiella* among some other members of the Neisseriaceae. The tree was created using Treecon (Van de Peer and de Wachter, 1994) with a Kimura 2-parameter correction and the neighbor-joining algorithm of tree construction. Similar phylogenies were obtained with parsimony and maximum-likelihood methods (Hedlund and Staley, 2002).

with *Simonsiella crassa*, along with the confusion in identifying *Alysiella*, it may be prudent to dissolve the genus *Alysiella* and emend the description of *Simonsiella* and *S. crassa* to include the type strain of *A. filiformis*. However, we believe further study is warranted to address this issue.

Given the paraphyly of *Simonsiella* and the poor representation of *Alysiella* 16S rRNA gene sequences in public databases, caution should be

used when identifying members of these genera via culture-independent 16S rRNA gene surveys. *Simonsiella*-like sequences have recently been recovered from material from the abdomens of worker bees (Jeyaprakash et al., 2003); however, these two sequences group together at the base of the Neisseriaceae and there are no data on whether these sequences come from bacteria phenotypically similar to *Simonsiella*; thus, these bacteria are unlikely to belong to the genus *Simonsiella* sensu stricto.

Taxonomy

Currently, three species of *Simonsiella* have been validly described: *S. muelleri* from humans (Kuhn and Gregory, 1978), *S. crassa* from domestic sheep (Steed, 1962), and *S. steedae* from domestic dogs (Kuhn and Gregory, 1978). These species are each monophyletic (see the section Phylogeny in this Chapter) and can be distinguished chemotaxonomically (Kuhn et al., 1978; Heiske and Mutters, 1994; Table 2). A fourth monophyletic group from domestic cats has not been assigned a species designation due to its phenotypic similarity with *S. steedae* and the fact that the cat *Simonsiella* isolates have not been compared with *S. steedae* by DNA-DNA hybridization.

Only a single species of *Alysiella* has been described, *A. filiformis* (Steed, 1962). It seems likely that study of *Simonsiella* and *Alysiella* from more diverse hosts could greatly expand the taxonomy of the group.

Habitat

To date, representatives of the genera *Simonsiella* and *Alysiella* have been found exclusively in the oral cavities of warm-blooded vertebrates. The earliest recorded observation of *Simonsiella* was made by Miller in 1906 (Simons, 1922) in the throat mucus of a diphtheritic chicken and in plaque and saliva of humans. Since then, many of these organisms have been discovered inhabiting the mouths of a wide variety of mammals (Table 1) and Simons (1922) mentions that Schmid may have seen such organisms in the mouths of donkeys, elephants, hippopotami, monkeys, and other animals. *Simonsiella* has also been observed in an oral smear from an orangu-

Table 2. Differential traits of the species of the genera *Simonsiella* and *Alysiella*.

Trait	*S. crassa*	*A. filiformis*	*S. muelleri*	*S. steedae*	*S.* sp.
Acid produced from					
D-Glucose, maltose, and D-trehalose	+	+	+	–	–
D-Ribose	+	–	–	–	–
D-Fructose and sucrose	+	+	–	–	–
Salicin	–	–	NG	–	–
Degradation of					
Gelatin	+	(+)	–	–	–
Casein	+	–	–	–	(+)
Inspissated serum	+	–	NG	–	–
Litmus milk	P	N	N	N	N
Growth tolerance ranges					
27°C	+	–	+	–	–
43°C	+	+	–	–	–
pH 6	+	–	+	–	–
pH 8	+	+	–	+	+
NaCl 1.5%	+	–	–	+	+
Cellular carbohydrates[a]					
Meso-erythrol (no. of strains)	+ (2)	+ (1)	– (2)	– (3)	– (3)
Rhamnose (O)	–	–	+	+	+
Arabinose (O)	+	+	–	+	+ (2/3)
Heptose (O)	–	–	–	+	–
Nitrate reduction					
NO_2^-	–	NR	+ (9/18)	+	–
N_2	+	–	+ (7/18)	–	–
G+C content (mol%) ± SE (no. of strains)	44.3 ± 0.29 (5)	45.4 (1)	41.8 ± 0.52 (18)	50.1 ± 0.16 (19)	51.1 ± 1.9 (8)
Source	Sheep	Sheep	Humans	Dogs	Cats

Symbols and abbreviations: +, positive; (+), weakly positive; –, negative; NG, no growth; P, peptonization; N, no change; and NR, not clearly reported.

[a](O) Peracylated O-methyloxime. Representative data from Heiske and Mutters (1994).

Data generally from Kuhn et al. (1978).

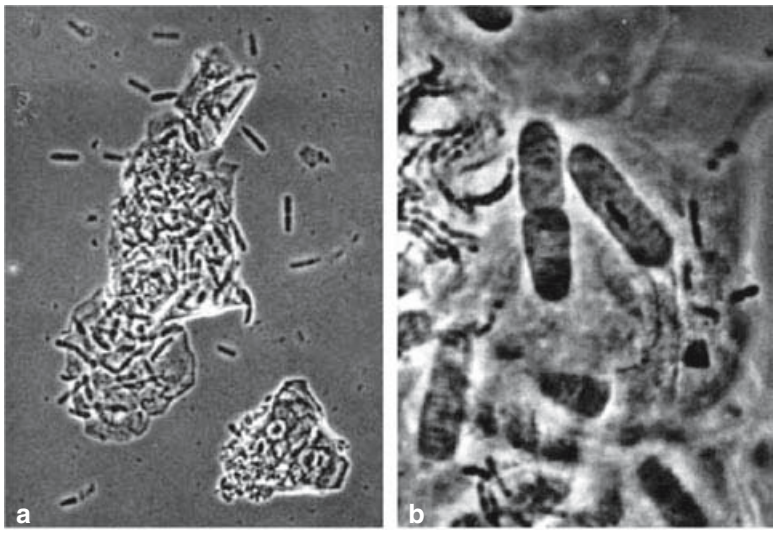

Fig. 3. Phase-contrast photomicrographs of a wet mount in saliva of an oral swabbing from the hard palate of a youth. a) At low magnification (×260) with a 10× objective lens, numerous rod-shaped *Simonsiella* filaments are seen on and near the epithelial cells. b) At high magnification (×1825) with an oil-immersion 100× objective lens, the multicellular (striated) structure of the *Simonsiella* filaments can be recognized. Their enormous size is apparent by comparison with the adjacent streptococci.

tan at the Los Angeles Zoo (R. Bledsoe, unpublished observations). Simons (1922) found *Simonsiella* in every oral specimen from every animal species he examined.

Simonsiella and *Alysiella* colonize the mouth by adhering to the mucosal squamous epithelium. In wet mounts or stained smears of oral swabbings, the multicellular filaments are seen attached to desquamated cells, often in great numbers; generally, there are few free filaments (Fig. 3). Stereoscopic scanning electron micrographs show the flat *Simonsiella* filaments oriented with their concave ventral side toward the surface of the epithelial cells (Nyby et al., 1977). The ventral capsule with its fibrillar components (the dorsal capsule of *Simonsiella* filaments lacks these structures) is probably involved in the adhesion to and the gliding locomotion on the cells of the oral mucosa (Figs. 1a–d and 4). Such structures are also present on the type strain of *A. filiformis* (Kaiser and Starzyk, 1973; J. Pangborn et al., unpublished observations). These organisms likely maintain themselves in the oral cavity by gliding and adhering to new epithelial cells during the ongoing desquamation of these cells and their elimination in the flow of saliva.

The preferred site of colonization in the mouth appears to be the palate, although *Simonsiella* has also been found in samples from the buccal cavity, gingival margin, tongue, throat, and base of the mouth (Miller, 1911; Simons, 1922; Fellinger, 1924; Berger, 1963; Richardson et al., 1966; Saphir and Carter, 1976; Nyby et al., 1977; Kuhn et al., 1978). *Simonsiella* has been seen neither in tubercular sputum nor on teeth (Fellinger, 1924). Fellinger (1924) attributed the abundant occurrence of *Simonsiella* in the mouth to the ready access of air to that site. Today, the strict aerobic nature of *Simonsiella* has been established in axenic culture.

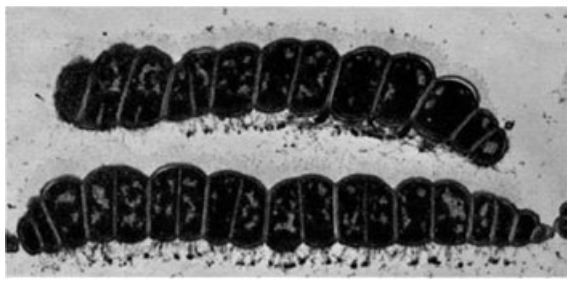

Fig. 4. Transmission electron micrograph of a longitudinal thin section of multicellular filaments of *Simonsiella steedae* ATCC 27411 situated within a colony showing the dorsal-ventral differentiation of *Simonsiella* at the cytological level. The prokaryotic cells possess Gram-negative-type cell walls that form asymmetrically from the ventral side. The dorsal capsule is undifferentiated; the ventral capsule contains fibrils emerging from the cells at right angles; within the cytoplasm there are fewer ribosomes and more membranes in the ventral region. Compare with Fig. 1. ×19,400. (From Pangborn et al., 1977.)

In healthy human populations, the incidence of *Simonsiella* is in the range of 30–40% (Simons, 1922; Kuhn et al., 1974; Gregory et al., 1985), while it is 4% or less in individuals with oral pathologies (Simons, 1922; Bruckner and Fahey, 1969). A study comparing the diets of *Simonsiella* carriers versus noncarriers indicated that carriers have a significantly higher daily intake for 13 dietary variables, including four fat components; however, the carbohydrate intakes were not statistically different between carriers and noncarriers (Gregory et al., 1985). Children possibly have a higher incidence than adults (Fellinger, 1924; Richardson et al., 1966). Some people consistently harbor large numbers—literally masses—of *Simonsiella*, especially after waking up in the morning; others carry few or none. Antibiotic (ampicillin) treatment for a res-

piratory ailment has been known to eliminate *Simonsiella* from the mouth of a long-term carrier (Gregory, 1975).

In dogs and cats, the incidence and abundance of *Simonsiella* are very high, the incidence approaching 100% in specimens from the palate or from the buccal cavity (Nyby et al., 1977). *Simonsiella* is less likely to be found in specimens from oral fluids and gingival margins (about 20% incidence; Saphir and Carter, 1976; Bailie et al., 1978).

Note, however, that the preceding survey provides only a rough estimate of the occurrence of *Simonsiella*. The figures vary not only with the site in the mouth from which the specimens are obtained, but also with the method of observation. Direct microscopic observation of oral specimens on plates (see Kuhn et al. [1974], Nyby et al. [1977], Kuhn et al. [1978] and the section Isolation) yields higher percentages than the detection of macroscopic colonies after prolonged incubation (Richardson et al., 1966; Saphir and Carter, 1976; Bailie et al., 1978). As previously mentioned, the sampling of animals for these bacteria has been limited to a phylogenetically and ecologically limited group of animals so the breadth of habitats for *Simonsiella* and *Alysiella* may not be currently appreciated.

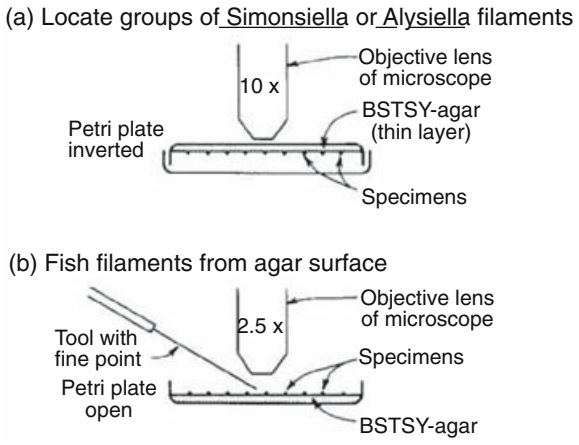

Fig. 5. Procedure for the isolation of *Simonsiella* and *Alysiella*. a) After a brief incubation period, the oral swabbings on the agar surface can be surveyed microscopically for suitable specimens with the Petri plates covered (to prevent microbial contamination from the air) and inverted. b) The use of a 2.5× objective lens facilitates the "fishing" process because it affords a greater working distance and a wider field of view with the microscope. Figure 5 shows oral specimens at low magnification.

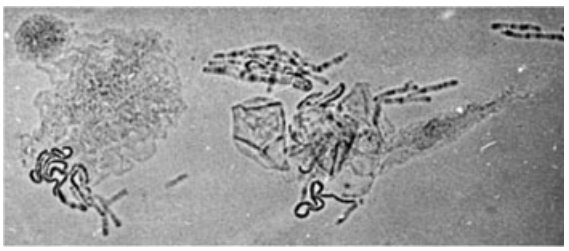

Fig. 6. General appearance of oral specimens after 6 h of incubation at 37°C on a thin layer of BSTSY agar in a Petri plate viewed microscopically with a 10× objective lens. Several groups of *Simonsiella* filaments, seen on broad side and on edge, are located near epithelial cells (center) and microcolonies of "ordinary-sized" bacteria (upper left and right). The group at top center is located in a suitable position for isolation of axenic cultures of *Simonsiella*. ×200.

Isolation

Enrichment culture techniques to facilitate the isolation of *Simonsiella* and *Alysiella* have as yet not been developed. Preliminary attempts to design selective media with antibiotics and dyes (e.g., crystal violet) in the hope of encouraging the growth of these Gram-negative organisms while suppressing or inhibiting the numerous Gram-positive members of the oral flora have not met with success (Nyby, 1974). Therefore, the most direct and least time-consuming approach for isolating axenic cultures relies on the microscopic recognition of the morphologically conspicuous *Simonsiella* and *Alysiella* organisms amid the great diversity of other oral bacteria (Steed, 1962; Kuhn et al., 1978). This approach has also been used to determine the incidence of *Simonsiella* in humans (Kuhn et al., 1974) and in dogs (Nyby et al., 1977).

Oral samples are obtained by rubbing sterile cotton swabs over the animals' palates. The swabs are then rolled directly and without delay (without the use of a transport medium in laboratory as well as in field situations) over the surface of thin layers of BSTSY agar in plastic Petri plates. The plates are then incubated aerobically at 37°C for 6–10 h. This brief incubation period allows the *Simonsiella* and *Alysiella* filaments to multiply and glide from the oral squamous epithelial cells onto the agar surface so that they can be more readily detected. Longer incubation causes the filaments to be overgrown, especially by streptococci. The agar surface is then scanned microscopically with a 2.5× or 10× objective in combination with a 10× or 12.5× ocular lens. Well-isolated microcolonies are transferred to BSTSY agar plates of normal thickness with a flame-sterilized dissection needle or other tool with a fine point (Figs. 5 and 6). Successful transfers result in macroscopic colonies after 16–20 h of aerobic incubation at 37°C.

Simonsiella and putative *Alysiella* can be readily recognized microscopically at low magnification. The filaments are generally attached to

or located near epithelial cells; they are broad, 2–10 μm wide, and the intercellular septations give the filaments the appearance of cross-striation (Fig. 3). After a few hours of incubation, a second microscopic examination generally reveals more numerous filaments and evidence of gliding locomotion from the epithelial cells onto the agar surface. Researchers must take care in identifying putative *Alysiella* isolates. The identity of such cultures should be confirmed with some genetic approach, preferably by 16S rRNA gene analysis, to distinguish this genus from morphologically and phenotypically similar *Moraxella* strains which also inhabit the oral cavity (see the section Phylogenetics in this Chapter).

The tool with a fine point that is used for isolation of filaments has to fit the manual dexterity of the individual operator while the plates are being viewed under the microscope. Individual preferences have called for tools differing in weight and shape (e.g., a dissecting needle, a dental probe, or an inoculating needle with or without a holder).

The incubation temperature of 37°C is an important factor, because newly isolated strains tend to have a narrow temperature tolerance range. However, in attempts to isolate *Simonsiella* or *Alysiella* from a new animal host, the incubation temperature should probably match the oral temperature of the host. An aerobic atmosphere (air without the addition of, e.g., 5% CO_2) is advantageous in selecting the strictly aerobic *Simonsiella* and *Alysiella*. Also, newly isolated strains need to be transferred at 2- to 3-day intervals. Refrigeration is not recommended.

Great abundance of filaments in a given oral cavity aids isolation. However, it is not necessarily an indicator of probable success. Some filaments are difficult to "fish" and transfer. Furthermore, filaments from a known carrier have been known to lyse after a mere 2–3 h of incubation on BSTSY agar (Kuhn et al., 1978). The cultural requirements are evidently not yet known for some members of the genera *Simonsiella* and *Alysiella*.

Another approach for isolating axenic cultures of *Simonsiella* and *Alysiella* depends on picking macroscopic colonies that develop from oral samples. This is, however, a more random process because it is difficult to identify the correct colonies among the many colonies of other oral organisms. Also, if the original sample consisted of a relative minority of *Simonsiella* or *Alysiella*, these bacteria may become crowded out or overgrown by other bacteria. On blood agar, small (0.2–0.3 mm diameter), oxidase-positive colonies with a narrow zone of hemolysis may on microscopic examination be *Simonsiella*, although hemolysis is not necessarily a reliable guideline for detecting these colonies. As the colonies expand, they cover the area of hemolysis and thus obscure it (Berger, 1963). Various types of blood (e.g., sheep, rabbit and horse) may not be hemolyzed equally by all *Simonsiella* strains. Finally, some strains, especially from dogs and cats (Berger, 1963; Bailie et al., 1978), may not be hemolytic.

BSTSY Agar for Isolation of *Simonsiella* and *Alysiella* (Kuhn et al., 1978)

Tryptic soy broth without dextrose (Difco)	2.75% w/v
Yeast extract (Difco)	0.4% w/v
Agar (Difco)	1.5% w/v
Distilled water	90% v/v
Sterile bovine serum	10% v/v

Add the sterile bovine serum aseptically after autoclaving the rest of the medium and cooling it to 45°C in a water bath. Pour the medium into plastic Petri plates in thin layers to facilitate microscopic examination during the isolation procedure or in layers of normal thickness for cultivation of axenic strains.

Identification

Identification of the genus *Simonsiella* can be made by careful microscopic examination; axenic cultures are not necessary for generic identification. The filaments of *Simonsiella* possess distinctive dimensions for bacteria: Cells are ≥2 μm × ≥10 μm, and filaments as wide as 10 μm or as long as 90 μm have been observed (Fig. 7). The scanning electron micrographs in Fig. 1 clearly depict the morphology of *Simonsiella* and some of its variations. With these images in mind, one can readily recognize the distinctive features of these bacteria via light microscopic examination, preferably with a phase-contrast microscope, of wet mounts or agar surfaces (e.g., to photograph the bacteria; Kuhn and Starr, 1972). In contrast to *Simonsiella*, putative *Alysiella* strains identified by microscopy must be confirmed by some genetic technique, such as 16S rRNA gene analysis.

Caution should be practiced in identifying *Simonsiella* cells stained with routine bacteriological stains, especially the Gram stain. Firstly, the filaments fall flat onto the microscope slide when a smear is dried, and their three-dimensional ribbon-like structure can be overlooked. Secondly, the Gram stain procedure may give erroneous results on this large organism because of incomplete decolorization. Thus, the flat, Gram-negative *Simonsiella* may be mistakenly identified as the cylindrical, Gram-positive *Caryophanon* (see Smith and Trentini [1973] for discussion of Gram staining in *Caryophanon*).

While *Simonsiella* and *Alysiella* have been classified on the basis of their gliding capacity

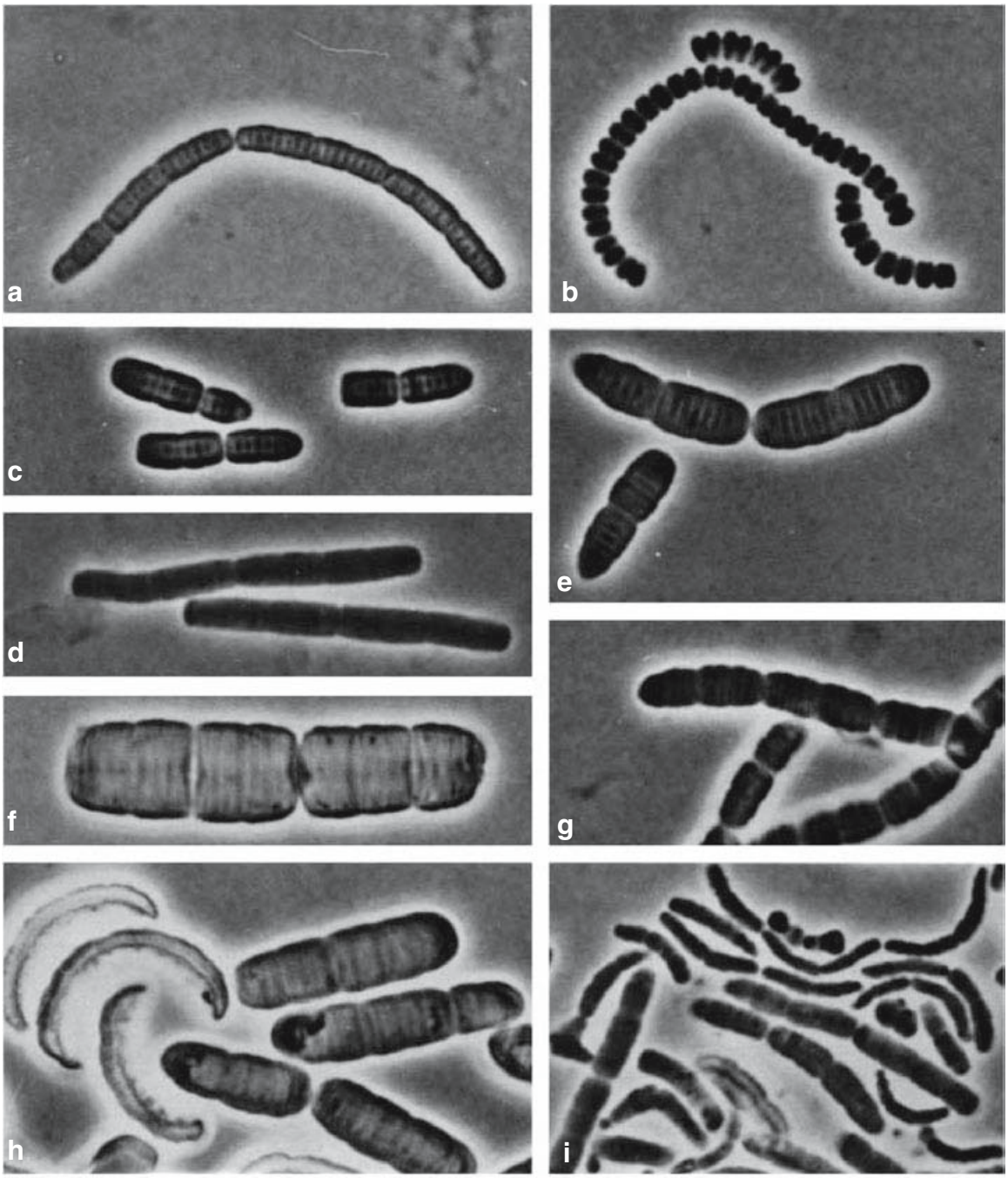

Fig. 7. Comparative morphology of *Simonsiella* and *Alysiella* seen in phase-contrast microscopy with a 100× Neofluar objective on a Zeiss photomicroscope. All strains had been cultured on BSTSY agar at 37°C. Magnification: ×1850. a) *Simonsiella crassa* neotype ATCC 27504 of sheep origin forms straight, relatively slender filaments with rounded ends; compare with Fig. 1a. b) *Alysiella filiformis* neotype ATCC 29469 of sheep origin forms long, flat filaments of closely apposed pairs of cells; compare with Fig. 1b. c) *Simonsiella muelleri* neotype ATCC 29453 of human origin. d) *Simonsiella muelleri* ATCC 29432 of human origin forms long, slender filaments. e) *Simonsiella* strain ATCC 29437 of cat origin forms bullet-shaped filaments because the terminal cells diminish in size. f) *Simonsiella steedae* ATCC 27411 of dog origin possessed broadest filaments yet observed when recently isolated. The filaments became narrower after many transfers of the strain in vitro; compare with Fig. 1d and e. g) *Simonsiella* sp. ATCC 27381 of cat origin shows long, more frequently segmented filaments with less regular contours. h) *Simonsiella steedae* ATCC 29429 of dog origin shows dorsal curvature with edgewise position of filaments and large amounts of irregularly structured extracellular material on the ventral side; compare with Figs. 1c and 3. i) *Simonsiella muelleri* ATCC 29458 of human origin develops many involution forms; it grows poorly under the environmental conditions provided for all strains displayed in this figure.

(Steed-Glaister, 1974), the actual gliding locomotion may not always be readily detectable in the process of identification. *Simonsiella* glides only when the concave ventral side of its filaments is in contact with a solid surface (e.g., epithelial cell, agar or glass). It cannot glide when its filaments are curled and on edge or when they are suspended in liquid media, as in wet mounts (Steed, 1962; Buchanan and Kuhn, 1978). Also, the rate of gliding locomotion is relatively slow (2–28 μm/min) and demands expertise and patience in microscopy. Some axenic strains of *Simonsiella* have never been observed to glide, and others have lost their gliding capacity in vitro with time (Buchanan and Kuhn, 1978).

In addition to habitat, morphology, Gram-stain behavior, and motility, the genera *Simonsiella* and *Alysiella* possess the following traits: They are strict aerobes that decompose H_2O_2 and possess cytochrome-oxidase. Nutritionally, they are chemoorganotrophs that require complex media and may require serum.

On BSTSY agar, their surface colonies are opaque, pale-yellow, and have entire, low-convex form, diameters from ≤1 mm to ≥2 mm, and a smooth, glistening, butyrous texture. On blood agar, they are generally hemolytic. They grow well between 33°C and 40°C, at 1% NaCl, pH 7.2, and 5% sucrose. They do not grow at 45°C and 2% NaCl.

The DNA G+C content of *Simonsiella* and *Alysiella* is 40–55 mol%. The genome sizes of several of these bacteria have been determined by renaturation rate experiments of DNA: *S. crassa* ATCC 15533^T, 2.4×10^9; *S. muelleri* ATCC 29453^T, 2.3×10^9; *S. steedae* ATCC 27409^T, 2.2×10^9; *A. filiformis* ATCC 15532^T, 2.7×10^9 (Rossau et al., 1989).

The fatty acid contents of *Simonsiella* are typical of Gram-negative Proteobacteria, in that even numbered saturated and monounsaturated fatty acids predominate. In all 48 strains studied, the predominant fatty acids are tetradecanoic acid, 9-hexadecenoic acid, an unidentified fatty acid with equivalent chain length of 20, and dodecanoic acid. Certain fatty acids are elevated in certain species of *Simonsiella* including hexadecanoic acid in the *Simonsiella* isolates from cats and 9-octadecenoic acid in *S. crassa*, allowing the species of *Simonsiella* to be identified by fatty acid profiles (Jenkins et al., 1977).

The cell wall ultrastructures also appear to be typical of Proteobacteria, and electron microscopy studies show that the cell wall forms asymmetrically from the ventral side of the cell (Pangborn et al., 1977; Fig. 4).

The species of the genera *Simonsiella* and *Alysiella* can be identified by the physiological and biochemical properties in Table 2, preferably coupled with 16S rRNA gene sequencing. They cannot be identified unequivocally by the dimensions of their filaments (Kuhn et al., 1978). Detailed descriptions of the *Simonsiella* species, *S. crassa*, *S. muelleri* and *S. steedae*, are presented in the papers by Jenkins et al. (1977), Kuhn et al. (1977) and Kuhn et al. (1978). The physiological data of *Alysiella filiformis* are for its neotype strain; they have been determined by Steed (1962) and concurrently with 50 strains of *Simonsiella* by Kuhn et al. (1978). The media compositions and procedures of the diagnostic tests are described in the paper by Kuhn et al. (1978). In addition, the cellular carbohydrates of *Simonsiella* and *Alysiella* have been described by Heiske and Mutters (1994), and have been found to be unique among the Neisseriaceae.

Preservation

Strains of *Simonsiella* and *A. filiformis* have been lyophilized at the ATCC. In addition, *Simonsiella* strains have been preserved by freezing a liquid culture with glycerol as a cryoprotectant (35% [v/v] final concentration).

Ecology

The ecological niche or role of *Simonsiella* and *Alysiella* in their habitat, the oral cavity, is not known at the present time. They appear to be residents of the healthy, normal oral cavity and not transient inhabitants, as has been suggested (Bruckner and Fahey, 1969). The reports of *Simonsiella* in mouths with obvious pathologies are relatively rare (Carandina et al., 1984; Whitehouse et al., 1987; Hodgin, 1988), and a causal relationship between *Simonsiella* and the pathologies is not obvious. In fact, the incidence of *Simonsiella* in human populations with obvious oral pathologies appears to be considerably lower than in groups of healthy people (Simons, 1922; Bruckner and Fahey, 1969). Thus, the presence of *Simonsiella* and *Alysiella* might be considered an indication of a normal flora and good health.

The mode of transmission of *Simonsiella* and *Alysiella* to new hosts has never been studied, though the most likely possibility would seem to be direct mouth-to-mouth contact, either vertically from parents to offspring or horizontally between mates. These bacteria have never been observed in the intestines or in stool, so a fecal-oral route of transmission seems unlikely. Although the mouth is usually considered sterile at birth, Whitehouse et al. (1987) reported isolating a *Simonsiella* strain from the gastric aspirate or oral cavity of a human neonate, 15 min postpartum. The mother's membranes had burst

24 h before delivery and it was speculated that this was the source of the *Simonsiella* inoculum; however, *Simonsiella* has never been reported from the urogenital tract.

Acknowledgment. We sincerely thank Jim Staley for critically reviewing drafts of the manuscript.

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DOI: 10.1007/0-387-30745-1_39

CHAPTER 3.2.15

Eikenella corrodens and Closely Related Bacteria

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Introduction

Eikenella corrodens was first isolated by Henriksen (1948), who described the organism as a slow-growing, anaerobic, Gram-negative rod. Because the organism appeared to pit or corrode the agar surface, producing colonies that grew in depressions, the organism was called "the corroding bacillus" (Holm, 1950) and was classified by Eiken (1958) as *Bacteroides corrodens*. Subsequent studies by Henriksen (1969) showed that the organism described by Eiken as *B. corrodens* was not an obligate anaerobe. In fact, careful genetic, biochemical, and serologic studies by Jackson and Goodman (1972) demonstrated that the term *B. corrodens* had been applied to two genotypically dissimilar groups of organisms, each of which warranted separate taxonomic classification even though both groups shared the common characteristic of pitting the surface of agar media. They, therefore, proposed the name "*Eikenella corrodens*" for the facultatively anaerobic Gram-negative rod and placed it within the family Brucellaceae. *Bacteroides ureolyticus* became the new species designation for the Gram-negative rods that grew as obligate anaerobes and were previously known as *B. corrodens* (Jackson and Goodman, 1978). Furthermore, *Eikenella corrodens* also became the accepted designation for the "HB-1" group of organisms described by King and Tatum (1962), since these isolates were shown to be biochemically and serologically identical (Riley et al., 1973). As now established, the genus *Eikenella* consists of a single, genetically uniform species, *E. corrodens*, with a G+C content of 56–58 mol%, (Coykendall and Kaczmarek, 1980) and has been placed within the Neisseriaceae family.

Habitat

Eikenella corrodens is part of the resident microflora of mucous membrane surfaces in humans. The organism is associated most commonly with the normal flora of the respiratory tract but also is recognized as a mucous membrane commensal of the gastrointestinal and urogenital tracts. Apparently no animal or environmental reservoirs exist for the microorganism (Decker, 1986a).

The predominance of *E. corrodens* in the mouth and gingival surfaces appears to be associated with the adherence of the organisms to human buccal epithelial cells. Yamazaki et al. have shown that the adherence of *E. corrodens* to human buccal epithelial cells may require the interaction of lectin-like proteins on the bacterial cell surface with galactose-like receptors on the surface of epithelial cells (Yamazaki et al., 1981; Yamazaki et al., 1988). Coaggregation of *E. corrodens* with other bacterial species in the oral cavity is also lectin mediated (Ebisu et al., 1988).

Isolation

Media for Selection

Eikenella corrodens is a facultatively anaerobic (microaerophilic), Gram-negative rod, which usually fails to grow on most selective media, such as MacConkey or eosin methylene blue (EMB) agars. When incubated aerobically, *E. corrodens* appears to have a definitive requirement for hemin upon primary isolation, with growth developing slowly on media such as Mueller-Hinton or Brucella agar supplemented with 5% blood or on chocolate agar (Goldstein et al., 1981). Upon subculture of the primary isolate, however, variants may be selected that do not require hemin for growth. Growth of *Eikenella* may be stimulated by incubating the culture plates in a 3–10% CO_2 environment, even though the organism does not require CO_2. Interestingly, when incubated anaerobically, *E. corrodens* does not have an obligate requirement for hemin. The anaerobic growth that occurs on non-heme-containing media, such as brain heart infusion agar, is comparable to that observed with growth on blood or chocolate agar plates incubated in air and/or a CO_2 environment.

Because *E. corrodens* is a slow-growing organism and is usually recovered from polymicrobic samples, its growth may be obscured by faster-

growing bacteria, which makes its detection difficult, if not impossible. Recovery of *Eikenella* from such polymicrobic specimens may be improved significantly by adding clindamycin (5 μg/ml) to the blood-supplemented isolation medium (Slee and Tanzer, 1978).

Growth Characterization

Within 19–24 hours of incubation at 35–37°C in 5% CO_2, tiny, pinpoint colonies (0.2–0.5 mm) may be detected on a blood or chocolate agar plate. Occasionally, prolonged incubation for 2–3 days may be required before colony size increases sufficiently for the colonies to become readily apparent (1.0–3 mm). Colonies of *E. corrodens* may appear either smooth or rough when grown on blood or chocolate agar media. The ability of the organism to pit or corrode the agar surface is an important distinguishing feature and is a useful identifying characteristic. However, this colonial characteristic is not universally present since only 45% of clinical isolates exhibit this corroding phenomenon on primary isolation. When present, pitting of the agar surface is usually detected by observing the agar surface at different angles with oblique lighting. Occasionally, however, some colonies may have to be moved to detect the underlying depression caused by their growth. Optimum pH for growth is 7.3.

In addition to pitting the agar surface, when grown on blood agar, *E. corrodens* produces a distinctive colonial morphology consisting of a dry, flat, radially spreading, pale yellow-pigmented colony with an irregular periphery and a moist central core. When observed under a stereoscopic microscope, three distinct zones of colonial growth are evident: 1) a clear, moist, glistening center zone; 2) a highly refractile, speckled, pearl-like circle of growth resembling mercury droplets; and, 3) an outer nonrefractile perimeter of spreading growth (Bottone et al., 1973). The typical colonial morphology is shown in Figs. 1 and 2. Microscopic (500×) observation of radially spreading colonies shows bacilli translocating for short distances from a central inoculum. Termed "twitching motility" (Henrichsen, 1975a; Henrichsen and Blom, 1975b; Schröter, 1975), this form of surface translocation takes place by intermittent "jerks" as the bacilli migrate away from a point inoculum. This phenomenon is not flagellum-mediated but is dependent on a thin film of water provided by cultures incubated in a humid atmosphere. Penetration into the agar media by *E. corrodens* during translocation may account for the corroding colonial characteristics, as well as the colony imprint when surface growth is removed. *Eikenella corrodens* strains lacking twitching motility produce translucent, dome-shaped, nonspreading colonies 0.5–1.0 mm in diameter. As noted above, such colony morphology may be encountered on primary isolation or by careful selection from corroding colony types.

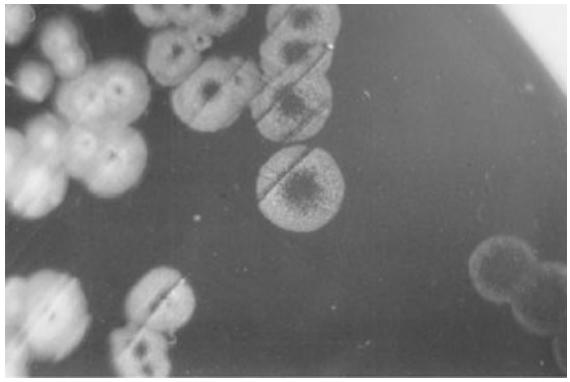

Fig. 1. Characteristic appearance of colonies of *E. corrodens* after growth on sheep blood agar for 48 h. From Bottone et al. (1973).

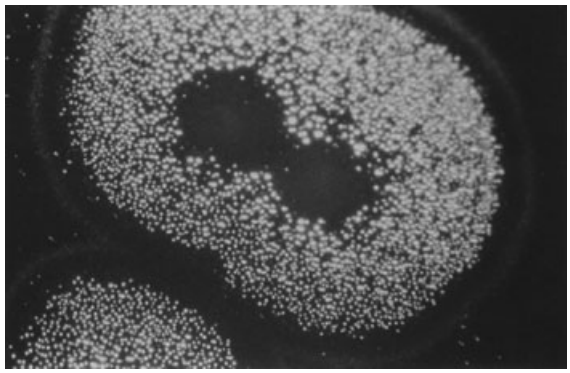

Fig. 2. Appearance of an *E. corrodens* colony at higher magnification showing the three characteristic zones of growth. From Bottone (1973).

When grown on chocolate agar, the characteristic three zones of growth are less pronounced. Additionally, growth of *E. corrodens* on blood or chocolate agar is accompanied by either an odor similar to hypochlorite bleach, or a musty odor reminiscent to that produced by *Pasteurella* or *Haemophilus* species. Upon prolonged incubation on blood agar, the medium surrounding the colonies becomes slightly green.

In liquid media, growth rates of individual strains may vary, and growth may be improved by the addition of 0.1–0.2% agar or cholesterol (10 μg/ml) to the broth medium. In thioglycolate broth supplemented with yeast extract, a band of growth usually develops a few centimeters below the surface of the medium after 3 days of incubation. When grown in a glucose broth with yeast extract, growth may develop as a uniform tur-

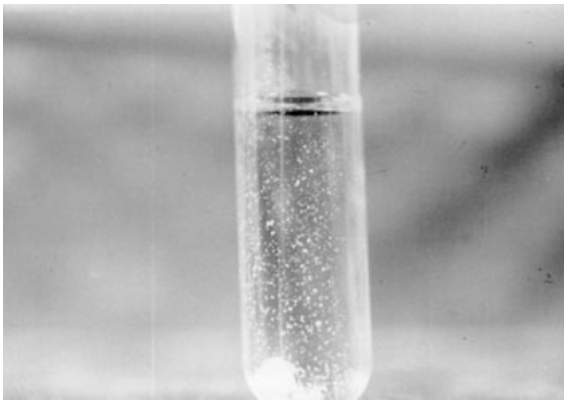

Fig. 3. Granular growth of *E. corrodens* in supplemented glucose broth, showing adherence to the side of the tube.

bidity or, as shown in Fig. 3, as discrete granules adhering to the side of the tube. Using scanning electron microscopy of 7-day-old cultures, Progulske and Holt (1980) showed that a fibrillar material connects *E. corrodens* cells, and this material may account for a surface hydrophobicity resulting in granule formation in liquid media. *Eikenella corrodens* also produces pili composed of type IV pilin (Hood and Hirschberg, 1995) and possesses an enteric type of lipopolysaccharide (Progulske and Holt, 1984a). Piliated isolates form corroding colonies, whereas nonpiliated variants form large smooth noncorroding colonies (Villar et al., 2001).

Microscopic Morphology

Eikenella corrodens is a small (0.3–0.5 by 1.0–3.0 μm), straight, nonsporeforming, nonencapsulated, nonmotile, Gram-negative coccobacillus. The organism has a uniform morphology with rounded ends, although occasional short filaments of up to 12 μm may be observed (Jackson et al., 1971). In direct smears of clinical material, however, *E. corrodens* is more coccobacillary, resembling *Haemophilus* species, and may even possess a capsule (Decker et al., 1986b).

Identification

The biochemical characteristics that distinguish *E. corrodens* are listed in Table 1. Generally, the organism is biochemically inactive, lacking oxidative and fermentative capabilities and failing to produce urease, indole, or hydrogen sulfide (H_2S). All isolates are oxidase positive and most isolates are capable of reducing nitrate to nitrite. Catalase is usually negative, although rare catalase-positive isolates may be encountered (Kasten et al., 1998). Tests separating *E. corrodens* from other corroding bacilli are shown in Table 2.

Table 1. Biochemical characteristics of *Eikenella corrodens*.

Characteristics	% Positive[a]
Oxidase	100
Catalase	9[b]
Growth on	
MacConkey agar	0.8
SS agar	0
Cetrimide agar	0
Hydrogen sulfide on	
TSI	0[c]
Lead acetate paper	0
Oxidation—fermentation	0
Urease	0
Indole	0
Methyl red/Voges-Proskauer	0
Citrate (Simmons), alkaline reaction	0
Motility	0
Gelatin	0
Acid from	
Glucose	0
Xylose	0
Mannitol	0
Lactose	0
Sucrose	0
Maltose	0
Esculin	0
Nitrate to nitrite only	99.7
Pigment (pale yellow)	100

Abbreviations: SS, *Salmonella-Shigella*; and TSI, triple sugar iron.
[a]Test reactions are read after 1 to 2 days of incubation.
[b]Weakly positive reaction.
[c]64% give a positive.

Antigenic Structure

Eikenella corrodens has been found to possess four major antigenic components (Jackson et al., 1971). All strains of *E. corrodens* appear to be antigenically related, but differences in the amounts of each antigen type may exist among strains. In fact, some strains may even lack one or two of the four major antigenic components, as detected by immunodiffusion studies (Wong and Jackson, 1971). A type-specific antigen (Maliszewski et al., 1983) and a group antigen with endotoxin activity (LPS), and containing 0.5% ketodeoxyoctonate, have been found in *E. corrodens*, and the latter is distinct from outer-membrane, type-specific protein antigens (Progulske et al., 1984b).

Pathogenicity

Even though *E. corrodens* is generally regarded as an organism of low virulence and lacks animal

Table 2. Differentiation of *E. corrodens* from similar species showing gliding motility and/or producing colonies that "pit" the agar surface.

Species	Oxidase[a]	Catalase	Nitrate reduction	Nitrite reduction	Indole	Glucose	Lactose	Ornithine decarboxylase	V-factor dependence
Eikenella corrodens	+	–[b]	+	+	–	–	–	+	–
Actinobacillus actinomycetemcomitans	v	[+]	+	–	–	+	–	–	–
Haemophilus aphrophilus	v	–	+	–	–	+	+	–	–
Haemophilus paraphrophilus	v	–	+	–	–	+	+	–	+
Capnocytophaga species[c]	–	–	v	–	–	[+]	v	–	–
Cardiobacterium hominis	+	–	–	–	+	+	–	–	–
Kingella denitrificans	+	–	+	v	0	+	–	–	–
Kingella kingae	+	–	–	–	–	+	–	–	–
Kingella indologenes	+	–	–	–	+	+	–	–	–
Moraxella atlantae	+	+	–	–	–	–	–	–	–

Symbols and abbreviations: +, positive; [+], positive delayed; –, negative; and v, variable.
[a]Using tetra-methyl-*p*-phenylenediamine.
[b]Rare positive strains reported by Kasten et al. (1998).
[c]*Capnocytophaga ochracea*, *gingivalis*, and *sputigena* group.
Adapted from Weyant et al. (1966).

pathogenicity (Klairat, 1967; Bottone et al., 1973), it has become increasingly recognized as a cause of human infection (Bottone et al., 1973; Brooks et al., 1974; Dorff et al., 1974; DeMello and Leonard, 1979; Suwanagool et al., 1983; Tami and Parker, 1984; Drake et al., 1986; Tveteras et al., 1987). Human infections usually result from some predisposing factor, such as trauma to a mucous membrane surface that compromises normal host defense mechanisms and allows the organism to gain access to surrounding tissue. Once primary infection occurs, hematologic dissemination may establish foci of infection in other parts of the body.

In intravenous drug abusers, *E. corrodens* endocarditis, osteomyelitis, and septicemia have been reported in association with the practice of licking needles or injection sites prior to drug inoculation (Angus et al., 1994; Olopoenia et al., 1994; Swisher et al., 1994). *Eikenella corrodens* osteomyelitis has been reported subsequent to a toothpick puncture to a foot (Robinson and Kourtis, 2000); vertebral osteomyelitis and a liver abscess occurred secondary to a fishbone piercing the patient's pharynx (Lehman et al., 2000) and liver (Kessler and Kourtis, 2001).

In normal human hosts, *Eikenella* is usually involved in mixed bacterial infections, often with viridans group streptococci, and less frequently with various members of the Enterobacteriaceae (Bottone et al., 1973; Brooks et al., 1974; Badger et al., 1979). Infections frequently involve the head, neck (Goodman, 1977; Jones and Romig, 1979; Knudsen and Simko, 1995), or abdominal area (Tami and Parker, 1984; Drake et al., 1986; Tveteras et al., 1987). Severe *E. corrodens* and viridans streptococcal cellulitis and septic arthritis of the knee subsequent to dental manipulation also has been reported in a patient with a history of hemarthrosis (Flesher and Bottone, 1989). Jacobs et al. (1993) offer compelling evidence that most concomitant streptococcal isolates are members of the *S. anginosis* group, which presently consists of three species, *S. anginous*, *S. constellatus* and *S. intermedius* (Whiley and Beighton, 1991). *Eikenella* are also responsible for 7–29% (Bilos et al., 1978; Goldstein et al., 1978a; Peeples et al., 1980; Faralli and Sullivan, 1986; Brook, 1987) of human hand-bite wound infections, as well as clenched-fist injuries, which are frequently complicated by bone resorption and osteomyelitis. Additionally, a severe *E. corrodens* genital ulcer occurred after a human bite to the penis (Rosen and Conrad, 1999). Other studies have reported that *Eikenella* may be an important periodontal pathogen with resultant severe alveolar bone loss (Socransky, 1977; Irving et al., 1978; Johnson et al., 1978; Listgarten et al., 1978; Behling et al., 1979). This bacterial species is often recovered from subgingival plaque in patients with severe periodontitis (Mueller et al., 1997); *E. corrodens* coaggregates with other bacterial species in plaque and elaborates a toxin in gingival sulcus, which contributes to periodontal disease (Levine and Miller, 1996; Young, 1996). Production of

hydrolytic enzymes also may contribute to periodontal disease (Allaker et al., 1994).

Eikenella corrodens also may be the sole infecting pathogen in cases of endocarditis, meningitis, brain abscesses, subdural empyema, septic arthritis, pneumonia, postsurgical infections, and soft-tissue diseases (Johnson and Pankey, 1976; Sheng et al., 2001). Several reports (Suwanagool et al., 1983; Tami and Parker, 1984; Tveteras et al., 1987; Sheng et al., 2001) have shown that *E. corrodens* may have an accentuated potential to cause disease in immunocompromised patients and, as such, may serve as an opportunistic pathogen in that patient population.

In a review of *E. corrodens* pleuropulmonary infections, Joshi et al. (1991) reported that this infectious complication occurs in two groups of patients: adults 44 years old with underlying medical conditions (immunosuppression, lung disease, alcoholism, intravenous drug use, or propensity to pulmonary aspiration) and in children ≥ 14 years of age. *Eikenella corrodens* septic pulmonary embolization secondary to internal jugular vein phlebitis after oropharyngeal infection also has been described (Celikel and Muthuswamy, 1984).

Antimicrobial Susceptibility

In vitro antimicrobial susceptibility studies (Goldstein et al., 1978b; Goldstein, 1986; Sheng et al., 2001) indicate that *E. corrodens* infections can be successfully treated with a wide variety of agents including penicillin, ampicillin, amoxicillin, but not cephalothin or cefuroxime. *Eikenella corrodens* is susceptible in vitro to cefoxitin, ceftriaxone, cefotetan, ciprofloxacin, moxalactam, imipenem and tetracycline. Resistance to clindamycin, lincomycin, and metronizadole is constant, but variable with regard to aminoglycosides. Two β-lactamase-producing strains have been isolated from patients with infectious complications in Spain (Trallero et al., 1986). *Eikenella corrodens* in vitro antibiotic susceptibility may be determined by disk-agar diffusion, agar dilution, and E-test methods (Luong et al., 2001). Alcala and colleagues (1998) found a microdilution method using *Haemophilis* test medium correlated well with the agar dilution method using Mueller-Hinton sheep blood agar.

Gram-Negative Species Capable of Forming Corroding Colonies

Species placed in this category are for the most part neither genotypically, morphologically, nor biochemically related to *E. corrodens*. They are enjoined with *E. corrodens* mainly because they, on occasion, may produce colonies that pit the agar surface. By and large, they comprise the HACEK group of organisms (*Haemophilis aphrophilus*, *H. paraphrophilus*, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens* and *Kingella* species). Although not a HACEK group microorganism, oral *Capnogtophaga* species also may give rise to corroding colonies and demonstrate twitching motility.

Haemophilis paraphrophilus was first described by Zinnemann et al. (1968) as a V-dependent *Haemophilus* species that grows preferentially under 10% CO_2 tension. In air without CO_2 supplementation, two types of colonies develop: a small, smooth colony and a larger colony with an irregular contour and a dry, crinkled surface resembling breadcrumbs. Microscopically, analogous to *E. corrodens*, *H. paraphrophilus* may present as either a small coccobacillus after growth in CO_2, or as a more bacillary form in ambient air. Part of the normal oral flora, on occasion, these species have caused endocarditis and brain abscesses and may be isolated from vaginal specimens. Distinguishing characteristics of *H. paraphrophilus* are shown in Table 2. Although generally regarded as catalase negative in several references (Weyant et al., 1996), Zinneman (1968) originally reported all 16 of his strains studied as catalase positive "though rather slow to develop."

Cardiobacterium hominis was the name proposed by Slotnick and Dougherty (1964) for a pleomorphic, teardrop-shaped, Gram-negative bacterium isolated from patients with endocarditis. *Cardiobacterium hominis*, in addition to demonstrating a swollen terminus, may occur also as rosettes comprising clusters of bacilli, as well as long serpentine filaments, and may retain some crystal violet during Gram staining. Stick-like bacillary forms with rounded ends similar to those observed with *E. corrodens* may occur after growth on media containing yeast extract.

Colonies of *C. hominis* when viewed microscopically show a roughened surface comprised of an intertwining network of bacilli extending from the colony core to its periphery. From the colony edge, a streaming of slender bacilli may be observed, which often articulate with other bacillary forms from proximal colonies (Wormser and Bottone, 1983). Such surface translocation of bacillary forms may be attributed to a gliding motility of individual cells, especially evident after growth under CO_2 enrichment and in a humid atmosphere.

Biochemically, *C. hominis* anaerogenically ferments glucose, maltose, mannitol and sucrose, usually within 48 h, especially after

serum or hemin enrichment of the carbohydrate-containing medium. Lactose is not degraded (Table 2). Indole production is a major distinguishing feature of *C. hominis* (Table 2) and is best demonstrated by the addition of Kovac's reagent to a 48-hour-old peptone broth culture supplemented with fetal bovine serum (Wormser and Bottone, 1983). Hydrogen sulfide production may be detected by suspending a lead acetate strip over inoculated triple sugar or Kliger iron agar slants.

Via cryptogenic bacteremia, *Cardiobacterium hominis* (part of the normal nasopharyngeal flora) causes mainly endocarditis in individuals with prior cardiac disease or in patients with prosthetic heart valves. A rare β-lactamase-producing strain with high-level resistance (minimal inhibitory concentration [MIC]: ≥256 μg/ml) to penicillin and reduced susceptibility to vancomycin (MIC: 8 μg/ml) has recently been reported isolated from a patient with endocarditis (Lu et al., 2000).

The genus *Kingella* within the family Neisseriaceae is comprised of three species (*K. kingae*, *K. denitrificans* and *K. indologenes*). All three species show similar microscopic morphology, namely short, stout, Gram-negative rods with rounded or square ends. This morphologic characteristic is shared with *Moraxella* species, as is the tendency to resist decolorization and appear partially Gram positive. On blood agar incubated at 35–37°C, two colonial morphotypes may develop: one a spreading, corroding colony associated with twitching motility and fimbriation, and in the absence of fimbriation and twitching motility, a smooth, convex colony. *Kingella kingae* produces colonies surrounded by a zone of β hemolysis. *Kingella* species colonize mucous membranes of the upper respiratory tract from where they may become blood-borne and gain access to various body sites, especially joints of young children (Abuamara et al., 2000).

In 1962, King and Tatum reported on the isolation of small Gram-negative rods with fastidious growth requirements, which they categorized as members of the HB group because they morphologically and culturally fell between *Haemophilus* and *Brucella* species (King and Tatum, 1962). Designated HB-1, HB-2, HB-3 and HB-4, members were subsequently accorded genus status with, as noted earlier, HB-1 becoming *E. corrodens* and HB-2 and HB-3 being designated "*Haemophilus aphrophilus*" and "*Actinobacillus actinomycetemcomitans*," respectively. Because these latter two species are closely related genetically and phenotypically, they are discussed conjointly.

Actinobacillus actinomycetemcomitans (as the name implies) has long been recognized in conjunction with *Actinomyces* infections, since both are derived from the oral flora. Furthermore, both *H. aphrophilus* and *E. corrodens* also have been documented in association with actinomycotic lesions (Page and King, 1966; Blake and Haburchak, 1982).

Microbiologically, both *A. actinomycetemocomitans* and *H. aphrophilus* are small, Gram-negative coccobacilli that bear a remarkable resemblance to *Brucella* species, with which they may be confused on initial isolation. On 5% sheep blood agar, colonies are initially smooth after a 24-h incubation but may become rough and adherent with prolonged incubation; such colonies, when viewed microscopically, may show a star-shaped formation in the center of the colony (Page and King, 1966; Meyers et al., 1971; Bottone et al., 1973). In liquid media, compact colonies will develop adherent to the sides of the tube, while the remainder of the liquid media is clear. Biochemical tests that distinguish these two species from each other and from *E. corrodens* are shown in Table 2. Clinically, both species have been associated with a variety of human infectious complications, most notably endocarditis and abscesses (Page and King, 1966).

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Prokaryotes (2006) 5:848–860
DOI: 10.1007/0-387-30745-1_40

CHAPTER 3.2.16

The Genus *Burkholderia*

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Introduction

The genus *Burkholderia* contains organisms that are important causes of human, animal and plant disease, as well as organisms useful in promoting plant growth and bioremediation. The type species, *Burkholderia cepacia*, is important in all of these activities. Originally identified as a plant pathogen that caused soft rot in onions (Burkholder, 1950), *B. cepacia* has emerged in the last 20 years as an opportunistic pathogen in nosocomial infections, particularly in individuals with cystic fibrosis (CF) or chronic granulomatous disease (Gilligan, 1991; Govan et al., 1996b; LiPuma, 1998; Speert et al., 1994). Other significant human and animal pathogens include *B. pseudomallei*, the cause of melioidosis, and *B. mallei*, the cause of glanders (Sanford, 1995). A newly described nonpathogenic species, *B. thailandensis* (Brett et al., 1998), has been useful in defining the pathogenesis of diseases caused by *B. pseudomallei* and *B. mallei* (Brett et al., 1997).

Burkholderia cepacia is a serious pathogen in CF patients because many strains have been determined to be easily transmitted from person to person (Govan et al., 1993; LiPuma et al., 1990) and intrinsically resistant to many antibiotics (Goldman and Klinger, 1986; Prince, 1986). Once acquired, *B. cepacia* infection rarely is eradicated from the lungs of CF patients. In addition, a proportion of patients who acquire *B. cepacia* infection develop the so-called "cepacia syndrome," a rapidly fatal pneumonia and sepsis, with a high mortality (Govan and Deretic, 1996a; Isles et al., 1984). The pathogenic mechanisms involved in this syndrome remain unclear. Indeed, the nature of the virulence factors that contribute to the pathogenicity of *B. cepacia* in CF remains largely unknown.

Aside from its potential as a human pathogen, *B. cepacia* and the related species in the *B. cepacia* complex have tremendous potential for agricultural and environmental use. Because it has anti-fungal and anti-nematodal properties, *B. cepacia* is very attractive as a biocontrol agent for the enhancement of crop yields (Govan et al., 1996b). *Burkholderia cepacia* also is a natural colonizer of the rhizophere and therefore considered by many to be preferable to the toxic compounds currently used as fertilizers. Because of its nutritional versatility, *B. cepacia* also has applications for bioremediation of contaminated soils.

Burkholderia pseudomallei is a common cause of human pneumonia and fatal bacteremias in endemic areas (Chaowagul et al., 1989). The organism is an opportunistic pathogen, and those individuals with underlying conditions such as diabetes or renal disease are particularly susceptible to *B. pseudomallei* infection (Chaowagul et al., 1989; Woods et al., 1993). Clinical manifestations of *B. pseudomallei* infection, a disease known as melioidosis, vary from an asymptomatic state, to benign pneumonitis, to acute or chronic pneumonia, to overwhelming septicemia (Smith et al., 1987). Treatment of melioidosis can involve up to 9 months of antibiotic therapy (Tanphaichitra and Srimuang, 1984), and relapse is common. Additionally, the latency period of the organism ranges from 2 days to 26 years (Smith et al., 1987; Mays and Ricketts, 1975).

Burkholderia pseudomallei occurs as a soil organism in Southeast Asia and northern Australia. Incidence of melioidosis generally is confined to these endemic areas (Chaowagul et al., 1989). Recent surveys, however, show that the organism is much more prevalent worldwide than previously believed. Isolation of *B. pseudomallei* from the environment and in clinical situations in parts of Africa, the Middle East, Europe, and in Central and South America has been documented (Dance, 1991). Subclinical and clinical disease is evident in humans, as well as wild and domestic animals, residing in the areas subtended by latitude 20 degree north to 20 degrees south (Dance, 1991), and as many as 10 to 30% of Southeast Asian population have serum antibodies to the organism. Likewise, individuals who travel to, through, and reside in endemic areas are susceptible to infection. One to two percent of healthy soldiers and up to 9% of wounded individuals who served in Vietnam are seropositive. Thus, the approximately 3 million American soldiers who traveled and lived in that

endemic region include a significant number who have latent infection, often recrudescing after a latent period of months to years (Sanford, 1995). More recently, a report from India indicates *B. pseudomallei* is present and is responsible for a significant level of disease in that country (Bharadwaj et al., 1994).

Burkholderia mallei is the causative agent of glanders, a disease which primarily affects horses, mules or donkeys (Sanford, 1995). The mode of infection in animals remains controversial; considerations include inhalation, ingestion or inoculation through breaks in the skin. Glanders in humans has never been common, but its importance is tremendous due to the serious nature of the infection. The pathogenesis of this disease is unknown. It is recognized from earlier studies that *B. mallei* (an organism with tremendous infectivity) poses a significant hazard to humans exposed to aerosols containing this organism (Howe, 1949). At present, no effective vaccines are available against this organism, and information on the treatment with antibiotic therapy also is not available.

Reports in the literature suggest that *B. pseudomallei*-like species exist that are nonpathogens in animals (Brett et al., 1997; Smith et al., 1995; Wuthiekanum et al., 1996a; Wuthiekanum et al., 1996b). To definitively assess relatedness, more than 95% of the 16S rDNA from *B. pseudomallei* 1026b (a virulent clinical isolate) and *B. pseudomallei-like* E264 (an avirulent environmental isolate) has been cloned and sequenced. The results of these studies, based upon a 16S rDNA phylogenetic analysis, confirmed the presence of a new *Burkholderia* for which the name *Burkholderia thailandensis* has been proposed (Brett et al., 1998).

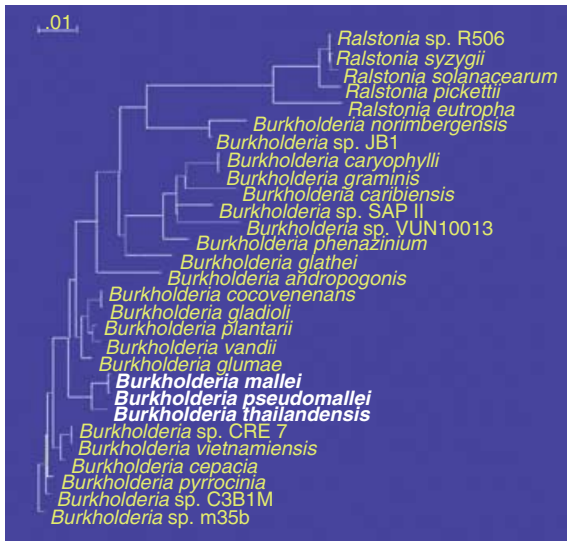

Fig. 1.

Taxonomy

In 1992, Yabuuchi and colleagues (Yabuuchi et al., 1992) established the genus *Burkholderia* and transferred seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species designated as *Burkholderia cepacia* (Palleroni and Holmes, 1981). The rationale for the new genus was based on the 16S rRNA sequences, DNA-DNA homology values, cellular lipid and fatty acid composition, and phenotypic characteristics. Thus, seven new species were created: *Burkholderia cepacia* (Palleroni and Holmes, 1981), *Burkholderia mallei* (Zopf, 1885), *Burkholderia pseudomallei* (Whitmore, 1913), *Burkholderia caryophilli* (Burkholder, 1942), *Burkholderia gladioli* (Severini, 1913), *Burkholderia picketii* (Ralston et al., 1973), and *Burkholderia solanaceareum* (Smith, 1896). Based on biochemical, immunological and genetic data, *B. pseudomallei*, *B. mallei*, *B. cepacia* and *B. thailandensis* are relatively closely related (Fig. 1). Two *Burkholderia* species and an *Alcaligenes* species have been subsequently reclassified in a new genus *Ralstonia* on the basis of 16S rRNA analysis, DNA homology, and cellular lipid composition (Yabuuchi et al., 1995). *Burkholderia pickettii* and *B. solanacearum* are now members of the genus *Ralstonia*.

During the last 15–20 years, *B. cepacia* has been isolated frequently from respiratory cultures of CF patients with a variety of clinical outcomes. Some patients infected with *B. cepacia* suffer a necrotizing pneumonia followed by a rapid and often fatal clinical deterioration. Other patients are colonized chronically and experience a slow deterioration of lung function (Govan and Deretic, 1996a; Govan et al., 1996b; Isles et al., 1984). During investigations to identify properties that might account for differences in clinical outcomes, it was discovered that strains classified as *B. cepacia* were very heterogeneous. A detailed genotypical and phenotypical analysis of properties including whole-cell protein profiles, DNA-rRNA hybridization, DNA-DNA hybridization, fatty acid analysis and biochemical tests resulted in the determination that *B. cepacia* was actually made up of five closely related but distinct genomic species. This group of five phenotypically related species often is referred to as the *B. cepacia* complex (Vandamme et al., 1997). These species were originally classified as genomovars I–V. Genomovars are phenotypically similar, genotypically distinct groups of strains that share a low level of DNA hybridization and are considered distinct species,

but are not given an official species name until differential diagnostic tests have been established. The *B. cepacia* complex consists of *B. cepacia* genomovar I, which contains the type strain of *B. cepacia*, *B. cepacia* genomovar III, the recently described species *B. multivorans* (formerly genomovar II), *B. stabilis* (formerly genomovar IV), and *B. vietnamensis* (formerly genomovar V; Vandamme et al., 1997).

Habitat

B. cepacia and the related species are soil bacteria that are found most commonly on plant roots or the immediately adjacent area (the rhizosphere) and in moist environments (Nijhuis et al., 1993). Although considered opportunistic pathogens, they rarely are isolated from the hospital environment in CF centers and are not commonly recovered from respiratory equipment used for CF patients (Govan et al., 1996b; Mortensen et al., 1995). In non-CF patients, however, outbreaks have occurred due to the presence of *B. cepacia* in contaminated nebulizers, disinfectants and drinking water (Pegues et al., 1996; Riboli et al., 1996). All five species of the *B. cepacia* complex have been isolated from patients (Vandamme et al., 1997). *Burkholderia cepacia* genomovar I is more frequently isolated from soils and plants, and *B. multivorans* primarily has been isolated from the sputum of CF patients. *Burkholderia vietnamiensis* originally was identified as a nitrogen-fixing organism isolated from the rice rhizosphere (Gillis et al., 1995).

Burkholderia pseudomallei is predominantly found in Southeast Asia and northern Australia (Chaowagul et al., 1989). Because *B. pseudomallei* is nutritionally diverse and is capable of resisting a variety of environmental extremes, it is puzzling that the global distribution of *B. pseudomallei* is not more uniform (Smith et al., 1987). The organism can be readily isolated from environmental niches such as rice paddies, still or stagnant waters, and moist soils that predominate in the tropics, and it is believed these habitats are the primary reservoirs from which susceptible hosts acquire infections (Ellison et al., 1969; Leelarasamee and Bovornkitti, 1989).

Isolation

Three different selective media have been developed and are currently used to isolate organisms belonging to the *B. cepacia* complex. These media are designed for the isolation of organisms (e.g., *B. cepacia* complex) that use lactose or sucrose and are resistant to antibiotics such as polymyxin, gentamycin and vancomycin. These antibiotics inhibit the growth of *Pseudomonas aeruginosa*. *Pseudomonas cepacia* agar (PCA) is composed of DeCicco holding medium with 300 U of polymyxin per ml and 100 mg ticarcillin per ml and has been used in clinical laboratories since approximately 1985 (Gilligan et al., 1985). Oxidation-fermentation polymyxin bacitracin lactose agar (OFPBL) is composed of oxidation-fermentation agar supplemented with lactose, 300 U of polymyxin per ml, and 0.2 U bacitracin per ml and has been widely used since approximately 1987 (Welch et al., 1987). Recently a new selective medium, *Burkholderia cepacia* selective agar (BCSA), has been developed that is more enriched for the growth of the *B. cepacia* complex and is more selective against the growth of other organisms than either PCA or OFPBL (Henry et al., 1997; Henry et al., 1999). Commonly misidentified organisms on OFPBL or PCA include *Stenotrophomonas maltophilia*, *Alcaligenes xylosoxidans* and *Comamonas acidovorans*. The selective medium BCSA contains a base of casein and yeast extract supplemented with 1% lactose, 1% sucrose, 600 U polymyxin per ml, 10 mg gentamycin per ml, and 2.5 mg vancomycin per ml. *Burkholderia cepacia* complex strains grow more quickly on BCSA and are readily visible in 24 hours. This medium is more effective at suppressing the growth of non-*B. cepacia* complex organisms than either PCA or BCSA or OFPBL (Henry et al., 1999). Addition of vancomycin to this medium reduced the rate of false positives, particularly due to *Staphylococcus* sp. and *Pseudomonas aeruginosa*.

Isolation and identification of *B. pseudomallei* by the culture method is still the method of choice for definitive diagnosis of melioidosis. It is relatively simple and economical to perform. The use of a modified Ashdown medium containing colistin further helps to increase the efficiency of isolation (Dance et al., 1989).

Identification

Burkholderia cepacia colonies appear smooth and slightly raised on blood agar or selective medium. Owing to the oxidation of lactose on MacConkey agar, colonies will become dark red or pink after extended incubation. Key biochemical tests include a positive oxidase reaction, lysine decarboxylase activity, oxidation of glucose and xylose, and usually oxidation of maltose, lactose, and/or sucrose. Members of this complex also have multitrichous polar flagella and are motile (Gilligan and Whittier, 1999). *Burkholderia multivorans* can be differentiated from *B. cepacia* genomovars I and III by its lack of sucrose utilization and variable

lysine decarboxylase activity (Vandamme et al., 1997). *Burkholderia multivorans* can be differentiated from *B. stabilis*, which also does not oxidase sucrose, by its ability to grow at 42°C. *Burkholderia stabilis* does not grow at 42°C and can be differentiated from all the other genomovars by the absence of β-galactosidase activity (Vandamme et al., 1997; Vandamme et al., 2000).

DNA-based assays also have been developed to facilitate identification and discrimination between members of the *B. cepacia* complex (Bauernfeind et al., 1999; Segonds et al., 1999). Polymerase chain reaction (PCR) primers based on 16S and 23S ribosomal RNA sequences are able to differentiate *B. multivorans* and *B. vietnamiensis* from *B. cepacia* genomovars I and III and *B. stabilis* (Bauernfeind et al., 1999). The PCR primers, based on *recA* gene sequences, can differentiate between all five members of the *B. cepacia* complex (Vandamme et al., 2000).

Availability of an API 20NE test panel has considerably simplified the identification of *B. pseudomallei*. However, this API panel of tests has been reported to misidentify *B. pseudomallei* for *Chromobacter violaceum* and others (Inglis et al., 1998). A PCR procedure for the discrimination of *B. mallei* and *B. pseudomallei* has been developed. Identificaton is based on a single nucleotide difference T 2143 C (T versus C at position 2143) in the 23S rDNA sequence of *B. malle* and *B. pseudomallei*. In comparison with conventional methods, the procedure allows more rapid identification (Bauernfeind et al., 1998). *Burkholderia mallei* has no unusual nutritional requirements, grows slower on laboratory media than *B. pseudomallei*, and can be differentiated from *B. pseudomallei* by being nonmotile.

Epidemiology

Acquisition of *B. cepacia* infections in CF patients can occur either from the environment or via patient-to-patient transmission. Transmission between patients may depend on several factors including properties of the strain, the patient, and the treatment center. Several molecular typing methods, which include ribotyping (LiPuma et al., 1988), pulse-field gel electrophoresis (Govan et al., 1993), and PCR-based random amplified polymorphic DNA (RAPD) typing (Mahenthiralingam et al., 1996), have facilitated epidemiological analysis of *B. cepacia* infections. The RAPD typing method has proven to be reproducible, discriminatory and versatile, allowing both clinical and research laboratories to perform fingerprint analysis of *B. cepacia* strains (Mahenthiralingam et al., 1996). These molecular typing methods have led to the observation that some *B. cepacia* strains infect multiple patients and appear to be transmissible; other strains appear to be unique isolates that may be acquired from the environment. It is not known what factors account for the transmissibility of epidemic *B. cepacia* isolates, although two properties have been shown to correlate with at least some epidemic strains. Isolates from one epidemic strain prevalent in eastern Canada and the United Kingdom were shown to possess the cable pilin subunit gene *cblA*, although other epidemic strains do not carry this marker (Mahenthiralingam et al., 1997; Sun et al., 1995). A DNA marker (BCESM = *B. cepacia* epidemic strain marker), identified by RAPD typing analysis, has been shown to be conserved among epidemic isolates of *B. cepacia* and only occasionally is present in unique clinical or environmental isolates (Mahenthiralingam et al., 1997). All of the isolates that contain the BCESM marker belong to *B. cepacia* genomovar III (Mahenthiralingam et al., 2000). The functions of any genes associated with the BCESM marker have not yet been determined, although this region contains an open reading frame with homology to negative transcriptional regulators (Mahenthiralingam et al., 1997). Most of the strains that infect multiple patients belong to genomovar III, although one strain of *B. multivorans* also has been reported to be an epidemic strain. Most strains of *B. multivorans* appear to be unique isolates that may have been acquired from the environment (Vandamme et al., 1997). Most strains that infect non-CF patients belong to *B. cepacia* genomovar I. *Burkholderia stabilis* has been isolated from CF sputum, blood and ear and from non-CF respiratory infections, as well as from the hospital environment (Vandamme et al., 2000).

Though epidemiological surveys have demonstrated that *B. pseudomallei* is endemic to regions that typically border 20° north and south of the equator, the incidence of disease is particularly high in South-East Asia and northern Australia (Chaowagul et al., 1989; Leelarasamee and Bovornkitti, 1989; Dance, 1991). In northeastern Thailand alone, an estimated 20% of community acquired septicemia and approximately 40% of deaths due to the complications of bacterial sepsis can be attributed to *B. pseudomallei* (Chaowagul et al., 1989). Although the organism is not strictly confined to the equatorial regions, the probability of acquiring melioidosis outside of these geographic domains is exceedingly low (Howe et al., 1971).

Burkholderia mallei is an obligate mammalian pathogen (Pitt, 1990; Sanford, 1990). Glanders is endemic in the Far and Middle East, Northern Africa, eastern Mediterranean and southeastern

Europe (Benenson, 1995; Kovalev, 1971). The Western Hemisphere is currently free of the disease (Benenson, 1995; Howe et al., 1971; Parker, 1990), and the United States has been glanders-free since 1937.

Disease

B. cepacia was originally identified as a plant pathogen that caused soft rot in onions (Burkholder, 1950). In the last 20 years it has emerged as an opportunistic pathogen in nosocomial infections, particularly in individuals with cystic fibrosis (CF) or chronic granulomatous disease (Gilligan, 1991; Govan et al., 1996b; LiPuma, 1998; Speert et al., 1994). The incidence of *B. cepacia* infections in CF patients varies geographically, but prevalence has been reported as high as 40% in some North American centers. Approximately 20% of CF patients colonized with *B. cepacia* experience a rapid and often fatal pulmonary decline, sometimes associated with septicemia, even in patients with previously mild disease (Govan and Deretic, 1996a; Govan et al., 1996b). Other patients are chronically colonized with a slow deterioration of lung function; however, some patients appear to experience no adverse affects following colonization with *B. cepacia* (Govan et al., 1996b; Isles et al., 1984). A 20-year study of Canadian CF patients concluded that colonization with *B. cepacia* significantly increased the risk of mortality at all levels of pulmonary function and that the risk was significantly higher in children (Corey and Farewell, 1996). A recent study comparing two-year and long-term survival rates in patients colonized with *B. cepacia* also concluded that colonization is associated with reduced long-term survival (Frangolias et al., 1999). *Burkholderia cepacia* infections are difficult to treat effectively due to the organism's multidrug resistance to potent antibiotics (Gilligan and Whittier, 1999; Simpson et al., 1994). There is considerable evidence, which has led to great concern in the CF community, that *B. cepacia* can be transmitted from person to person through nosocomial or social contacts (Govan et al., 1993; Johnson et al., 1994; Pegues et al., 1994; Smith et al., 1993). A recent study in the United Kingdom reported a hospital outbreak with an epidemic strain of *B. cepacia* that infected both CF and non-CF patients (Holmes et al., 1999), suggesting that transmission is possible between these patient groups. Acquisition of this strain in the CF patient group was associated with significantly increased mortality rates (Holmes et al., 1999).

Diagnosis of *B. cepacia* is normally based on culture and identification on selective medium (including BCSA, OFPBL and PCA) of the organism from respiratory secretions or sputum from CF patients. In other patient populations, diagnosis also depends on culture of *B. cepacia* from blood, respiratory secretions, indwelling catheters or urine.

All five genomovars and species of the *B. cepacia* complex have been isolated from the sputum of CF patients (Vandamme et al., 1997). The majority of isolates from CF patients are *B. multivorans, B. cepacia* genomovar III and *B. stabilis*. The majority of the epidemic or transmissible strains belong to genomovar III, although one epidemic clone has been identified as *B. multivorans*. Most environmental and soil isolates are either *B. cepacia* genomovar I or *B. vietnamiensis* (Vandamme et al., 2000). The recent differentiation of the *B. cepacia* complex into different species has not yet been followed by clinical data that might predict outcomes of infections by various species or genomovars. Currently, it also is not known if these *Burkholderia* species possess different virulence factors or regulate virulence factors differently and subsequently vary in their potential pathogenicity in CF or immunocompromised individuals.

The manifestations of melioidosis commonly are represented by acute, subacute and chronic illnesses, with the clinical indications of some forms of the disease often being mistaken for malaria, plague, pneumonia and miliary tuberculosis (Howe et al., 1971; Smith et al., 1987; Leelarasamee and Bovornkitti, 1989). Thus, melioidosis should be considered in any febrile patient with a history of residence in a major endemic region. If Gram-negative bipolar staining bacilli are observed in sputum, the organism can be readily cultured and identified (Sanford, 1990). In acute cases, blood and urine cultures are frequently positive; whereas, if chronic or subacute forms of the disease are suspected, biopsy may be required (Sanford, 1990). Serological studies can be helpful for diagnosing active and recrudescent disease, and an immunoglobulin M (IgM) immunofluorescence test is often positive in recent infections (Dance, 1991). Also, indirect hemagglutination and complement fixation tests are available but require the testing of paired sera over several weeks to confirm the presence of an active infection (Smith et al., 1987; Chaowagul et al., 1989).

Melioidosis is primarily acquired via the inoculation of compromised surface tissues by soil and water contaminated with *B. pseudomallei*, the highest incidence of disease occurring during the monsoon and rainy seasons (Chaowagul et al., 1989; Leelarasamee and Bovornkitti, 1989). It is believed that during these periods, rising water tables percolate the organism up through the underlying soil to the surface, thus enhancing their potential for exposure to humans and ani-

mals. This route of transmission tends to explain the prevalence of disease amongst rice farmers and their families who labor in the rice paddies without the benefit of protective clothing (Chaowagul et al., 1989; Leelarasamee and Bovornkitti, 1989). Another important route of infection appears to be the inhalation and aspiration of contaminated fomites. During the Vietnam War, a disproportionate number of helicopter crewmen contracted *B. pseudomallei* infections as compared to other soldiers stationed in the same regions. To explain this phenomenon, it has been proposed that the helicopter rotors acted to disturb infectious dust particles around landing zones and thus facilitated the pulmonary inoculation of the crewmen with *B. pseudomallei* (Howe et al., 1971; Sanford, 1990). Alternatively, ingestion of the organism and human-to-human transmission, although to much lesser extents, have been implicated as routes of inoculation (McCormick et al., 1975). To date, there have been no reports of transmission of disease between animals and humans (Leelarasamee and Bovornkitti, 1989; Dance, 1990).

A number of physiological abnormalities have been correlated with the predisposition of certain populations to *B. pseudomallei* infections. In particular, during a one-year study of patients admitted to a hospital in northeastern Thailand for treatment of septicemic melioidosis, 32% demonstrated preexisting diabetes mellitus (Chaowagul et al., 1989). Similarly, it has been shown in a diabetic, infant-rat model of infection that such animals are far more sensitive to challenge with *B. pseudomallei* isolates than are the healthy, non-diabetic rats (Woods et al., 1993). The reasons for this increased susceptibility, however, are still being investigated. Other health-related factors that appear to increase the probability of acquiring melioidosis include impaired cellular immunity, leukemia, lymphomas, HIV infections, renal disorders, and debilitating afflictions such as alcoholism and parenteral drug abuse (Whitmore and Krishnaswami, 1912; Whitmore, 1913; Leelarasamee and Bovornkitti, 1989; Tanphaichitra, 1989). Although *B. pseudomallei*-related illnesses are documented in apparently healthy individuals, the organism probably is still best described as an opportunistic pathogen.

Glanders is a zoonotic disease primarily of horses, donkeys and mules; however, nearly all mammals are susceptible to the causative agent, *B. mallei*. Natural infections of humans have been sporadic and usually are subclinical. Disease can result from a low infectious dose by aerosol, oral or parenteral routes. The incubation period is short, and nonspecific signs and symptoms confound definitive diagnosis of glanders. There are two major presentations of the disease: the nasal-pulmonary form (glanders) and the cutaneous form (farcy). These two forms may present simultaneously and usually are accompanied by systemic disease (Howe et al., 1971; Steele, 1973; Von Graevenitz, 1973). The route of infection, dose and virulence of *B. mallei* determine the severity of the disease. Clinically evident disease may be acute or chronic (Hornick, 1982; Parker, 1990; Steele, 1973), but subclinical and even latent infections may occur. The acute form of the disease most often afflicts humans (Freeman, 1985) and is characterized by a rapid onset of pneumonia, bacteremia, pustules and death within days. The chronic form of the disease, in contrast, is characterized by intermittent recrudescence and milder signs and symptoms and may last up to 25 years.

Pathogenicity

Despite the severity of *B. cepacia* infections in some patients, the virulence of this organism remains an enigma. Several potential virulence factors have been identified, including siderophores, proteases, hemolysins, lipase, and an extracellular toxic complex (Govan et al., 1996b). Bacterial pathogens must contend with an iron-restricted environment when colonizing mammalian hosts inasmuch as iron is bound to transferrin and lactoferrin and the binding of iron renders it essentially unavailable to microbial invaders (Crosa, 1989; Mietzner and Morse, 1994). Pathogenic bacteria require specialized iron acquisition systems to overcome the iron limitation imposed by the host. The most common mechanism of iron acquisition is the secretion of small chelators called "siderophores," which bind ferric iron and return it to the cell via specific outer membrane receptors (Crosa, 1989). *Burkholderia cepacia* produces four different siderophores (pyochelin, salicylic acid, cepabactin and ornibactins). Salicylic acid and ornibactins are the most prevalent siderophores produced, whereas cepabactin rarely is produced by CF isolates of *B. cepacia* (Darling et al., 1998). Approximately 50% of *B. cepacia* isolates from CF patients produce pyochelin (Sokol, 1986). Pyochelin-positive strains were more frequently isolated from patients with severe pulmonary disease, whereas pyochelin-negative strains were more frequently isolated from patients with moderate or mild infections. Genes have been identified that are required for the biosynthesis of the siderophore ornibactin (Sokol et al., 1999). The *pvdA* gene that codes for the enzyme L-ornithine N^5-oxygenase is required for effective colonization and persistence in acute and chronic *B. cepacia* respiratory infection models (Sokol et al., 1999).

Burkholderia cepacia produces at least one extracellular zinc metalloprotease that is similar to *Pseudomonas aeruginosa* elastase (Kooi et al., 1994; McKevitt et al., 1989). Purified protease has been shown to induce bronchopneumonia in rats (McKevitt et al., 1989). We recently have determined that anti-protease antibodies can reduce lung injury in chronic lung infections in rats (Sokol et al., 2000). Lipase activity has been reported in 67–100% of *B. cepacia* isolates (Gilligan, 1991; McKevitt and Woods, 1984; Nakazawa et al., 1987); however, purified lipase is not toxic to mice or Hela cells (Lonon et al., 1988). We have identified a quorum-sensing system in a *B. cepacia* genomovar III strain (Lewenza et al., 1999). The *cepI* gene directs the synthesis of the autoinducer molecule, *N*-octanoylhomoserine lactone. The *cepR* gene codes for a transcriptional regulator that positively regulates genes required for protease production or secretion and negatively regulates genes involved in ornibactin biosynthesis (Lewenza et al., 1999). Hemolysin production in *B. cepacia* is rare, with most studies reporting a 4% or lower incidence of hemolysin production in clinical isolates (Gilligan, 1991; McKevitt and Woods, 1984; Nakazawa et al., 1987). Recently, a low-molecular–weight (>3,000 Da) hemolytic activity was described in one strain of *B. cepacia* that was able to induce nucleosomal degradation (consistent with apoptosis) in human neutrophils and in a mouse macrophage cell line (Hutchison et al., 1998). There does not appear to be any correlation between production of extracellular virulence factors and transmissibility of epidemic *B. cepacia* isolates. Some strains of the *B. cepacia* complex have been shown to invade and survive in cultured epithelial cells and macrophages (Burns et al., 1996; Martin and Mohr, 2000; Saini et al., 1999). *Burkholderia cepacia* also has been reported to be resistant to growth inhibition by epithelial cell β-defensins and resistant to nonoxidative neutrophil-killing mechanisms compared to *P. aeruginosa* (Baird et al., 1999; Speert et al., 1994).

The ability to acquire iron from host sources is a prerequisite for the successful establishment and maintenance of most bacterial infections. Yang et al. have demonstrated that 84 : 84 *B. pseudomallei* strains examined during their studies tested positive for siderophore production using the chrome azurol S (CAS) assay. A structural and chemical analysis of the siderophore synthesized by *B. pseudomallei* U7 confirmed the molecule was approximately 1,000 Da in size, was water soluble with a yellow-green fluorescence, and belonged to the hydroxamate class (Yang et al., 1991). Furthermore, studies also have demonstrated the siderophore was capable of scavenging iron from both lactoferrin and transferrin in vitro (Yang et al., 1993). The name malleobactin has been proposed for this compound (Yang et al., 1991).

Although *B. pseudomallei* isolates can express an impressive array of both secreted and cell-associated antigens, the role(s) of these products in the pathogenesis of disease to date have been relatively ill defined. One of the primary reasons for this has been the lack of suitable techniques for genetically manipulating the organism. Owing to the recent application of a transposon-based mutagenesis system for use in *B. pseudomalle*, genetic loci, which encode a number of these putative virulence determinants and protective antigens, have been identified and characterized (DeShazer et al., 1997; DeShazer et al., 1998).

It has been shown previously that *B. pseudomallei* isolates can secrete antigens that demonstrate biological activities consistent with proteases, lecithinases, lipases and hemolysins (Esselman and Liu, 1961; Ashdown and Koehler, 1990; Sexton et al., 1994). However, though the importance of these factors has been implicated in the pathogenesis of the disease, only the protease has been characterized to date. Studies conducted by Sexton et al. (1994) have confirmed the presence of a 36-kDa antigen with associated proteolytic activities in *B. pseudomallei* culture supernatants. In particular, a protease expressed by *B. pseudomallei* (isolate 319a) was found to be a metalloenzyme requiring iron for maximal protease activity and to be optimally active at pH 8.0 and 60°C (Sexton et al., 1994). Furthermore, monoclonal antibodies (MAb) raised against a *Pseudomonas aeruginosa* alkaline protease were cross-reactive with this antigen (Sexton et al., 1994).

Most recently, an 11.8-kb chromosomal locus in *B. pseudomallei* has been identified that demonstrates a high degree of homology to operons that encode for the products of the main terminal branch of the general secretory pathway (GSP; Pugsley, 1993). Further characterization of the open-reading frames in this locus have confirmed that their orientation and physical arrangement are virtually identical to the *pul* gene cluster of *Klebsiella oxytoca* (Pugsley, 1993). Not surprisingly, the phenotypic analysis of individual transposon mutants also has confirmed their inability to secrete antigens associated with protease, lipase and lecithinase into the extracellular milieu. Interestingly, although the protease, lipase and lecithinase may play a small role in the pathogenesis of acute melioidosis, mutants deficient in their ability to secrete these particular exoenzymes were not severely attenuated in their ability to cause a fulminating illness (DeShazer et al., 1999).

In the mid-1950s, several studies demonstrated that filter-sterilized *B. pseudomallei* culture

supernatants were lethal for mice and hamsters when administered parenterally (Nigg et al., 1955; Heckly and Nigg, 1958; Heckly, 1964). These results were consistent with the fulminating illnesses observed in animals following inoculation with viable bacteria and suggested that *B. pseudomallei* strains might be capable of secreting a lethal toxin. In studies conducted by Ismail et al. (1987), mouse lethal, thermolabile toxin was presumably purified to homogeneity and characterized as a 31-kDa protein. Haase et al. (1997) have also described the presence of cytotoxic activity in culture filtrates. Their results, however, suggest that the antigen is only 3 kDa in size and that the cytotoxic activity in this instance most likely is due to the presence of a small peptide. Recently it has been reported that a rhamnolipid purified from *B. pseudomallei* culture supernatants demonstrates a cytotoxic effect against HL60 and HeLa cell lines (Haubler et al., 1998). Because this activity can be neutralized by albumin, however, it is unlikely to be of consequence in the pathogenesis of *B. pseudomallei* infections. These results have not been reproduced in animal models, even when using preparations concentrated by lyophilization (Brett et al., 1997; Brett et al., 1998).

Burkholderia pseudomallei strains can synthesize capsular antigens (Smith et al., 1987; Leelarasamee and Bovornkitti, 1989), and they may play an important role in the pathogenesis of melioidosis. Whereas in vitro studies have determined that encapsulated *B. pseudomallei* strains are as susceptible to phagocytic uptake by polymorphonuclear leukocytes (PMNs) as non-encapsulated variants, evidence tends to suggest that the presence of exopolysaccharide confers upon them the ability to resist the bactericidal effects of the phagolysosomal environment (Smith et al., 1987; Pruksachartvuthi et al., 1990). This feature of *B. pseudomallei* strains may help to explain why these organisms are able to remain latent in a host for as long as 26 years.

Recently, Steinmetz et al. isolated and purified a high-molecular-weight capsular antigen (150 kDa) from *B. pseudomallei* NCTC 7431 and succeeded in raising a Mab against it (Steinmetz et al., 1995). They were able to demonstrate the reactivity of both mucoid and non-mucoid strains with the MAb, suggesting that the capsular antigen is constitutively expressed by *B. pseudomallei* strains. Interestingly, temperature appeared to have little effect on the synthesis of the exopolysaccharide inasmuch as *B. pseudomallei* strains grown at both 15 and 37°C were Mab-reactive. Furthermore, an assay utilizing a variety of *Pseudomonas spp.* and *Burkholderia spp.* as controls was able to confirm the specificity of the MAb for *B. pseudomallei* and *B. mallei* strains only (Steinmetz et al., 1995). More recently, Masoud et al. (1997) have been successful at elucidating the chemical and structural characteristics of a capsular polysaccharide isolated from the virulent clinical isolate *B. pseudomallei* 304b. Their results demonstrated that the exopolysaccharide was a linear unbranched polymer of repeating tetrasaccharide units having the structure (-3)-2-*O*-Ac-β-D-Gal*p*-(1-4)-α-D-Gal*p*-(1-3)-β-D-Gal*p*-(1-5)-β-D-KDO*p*-(2-) (where KDO is 3-deoxy-D-manno-2-octulosonic acid). Similarly, Nimtz et al. (1997) have demonstrated that a structurally identical capsular antigen is expressed by *B. pseudomallei* NCTC 7431. Studies by both groups have also shown that patient sera reacted strongly with the purified carbohydrate antigens, indicating that this carbohydrate polymer is most likely expressed in vivo (Steinmetz et al., 1995; Masoud et al., 1997). Previous studies have confirmed that the lipopolysaccharide (LPS) antigens expressed by *B. pseudomallei* strains are highly conserved throughout this species (Pitt et al., 1992). In fact, serological evidence suggests only one serotype of *B. pseudomallei* (Bryan et al., 1994). To investigate this phenomenon, Perry et al. have characterized the LPS antigens isolated from a number of *B. pseudomallei* strains (Perry et al., 1995). Their results demonstrated that *B. pseudomallei* strains coordinately express two distinct somatic *O*-antigens (PS) on their cell surface. The type I antigen consists of a high-molecular-weight unbranched 1,3-linked homopolymer of 2-*O*-acetylated 6-deoxy-β-D-manno-heptopyranosyl residues, while the type II antigen is an unbranched heteropolymer consisting of (-3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranosyl-(1)-disaccharide repeats (L-6dTal*p*: __33% *O*-4 acetylated and *O*-2 methylated; __66% *O*-2 acetylated; Knirel et al., 1992; Perry et al., 1995). Even though the simultaneous expression of two or more LPS moieties is not an uncommon feature associated with Gram-negative bacteria, the degree to which the two PS antigens are conserved amongst *B. pseudomallei* strains is quite remarkable (Perry et al., 1995).

It has been reported that *B. pseudomallei* strains are resistant to the bactericidal effects of normal human serum (Ismail et al., 1988). Type II PS is essential for conferring this resistance phenotype. A 17.5-kb region of the chromosome, which is required for the synthesis of the type II antigen, confers the serum-resistance phenotype (DeShazer et al., 1998). Type II PS is probably a significant determinant in the pathogenesis of melioidosis because the LD50 value associated with a type II PS mutant is approximately 140-

fold higher than that of the wild-type strain (Woods et al., 1993; DeShazer et al., 1998).

Flagella are commonly recognized as important virulence determinants expressed by bacterial pathogens because the motility phenotype imparted by these organelles often correlates with the ability of an organism to cause disease (Penn and Luke, 1992; Moens and Vanderleyden, 1996). A significant degree of size and antigenic homogeneity exists amongst flagellins expressed by *B. pseudomallei* isolates. Furthermore, flagellin-specific antiserum can passively protect diabetic infant rats against a *B. pseudomallei* challenge (Brett et al., 1994). Curiously, there was no significant difference between the virulence capacities associated with a wild-type strain of *B. pseudomallei* and nonmotile mutants in either the diabetic infant rat or Syrian hamster models of infection (DeShazer et al., 1997). These results indicate that though flagella and/or motility may not be major virulence determinants in the pathogenesis of melioidosis, purified flagellin might still be a protective immunogen against *B. pseudomallei* infections.

Although glanders is a serious life-threatening zoonotic disease, relatively little is known about the pathogenesis, virulence factors, strain and the host immunopathological responses to infection (Fritz et al., 1999).

Treatment

Burkholderia cepacia is resistant to most antibiotics, making treatment of CF patients very difficult. It usually is susceptible only to piperacillin, axlocillin, cefoperazone, ceftazidime, chloramphenicol and trimethoprim-sulfamethoxazole (TMP-SMX; Pitkin et al., 1997). Strains isolated from CF sputum from patients treated with several courses of antibiotic therapy are often resistant to all known antimicrobial agents (Gilligan and Whittier, 1999). Resistance to a variety of antimicrobial agents including penicillins, first- and second-generation cephalosporins and many of the aminoglycosides is characteristic of *B. pseudomallei* clinical isolates (Dance et al., 1988; Leelarasamee and Bovornkitti, 1989; Godfrey et al., 1991; Weinberg and Heller, 1997). With this in mind, accurate identification of the organism, evaluation of the severity of the infection and antibiotic susceptibility testing are of paramount importance in devising an effective chemotherapeutic strategy. Whereas the newer therapies that utilize combinations of ceftazidime–cotrimoxazole or amoxicillin–clavulanate for treatment of disease are proving beneficial, the mortality rates associated with the acute septicemic and pulmonary forms of melioidosis are still unacceptably high (Smith et al., 1987; Leelarasamee and Bovornkitti, 1989; Kanai and Kondo, 1994; Weinberg and Heller, 1997; Ho et al., 1997). Typically, prolonged oral therapy also is recommended to assure the full clinical resolution of infections while reducing the potential for recrudescence of disease.

Antibiotic therapy for glanders is complex (Batmanov, 1991); however, there have been reports that ceftazidime, imipenem, doxycycline and ciprofloxacin are active against both *B. mallei* and *B. pseudomallei* (Kenny et al., 1999). Antibiotics clinically proven to be effective in the treatment of melioidosis may therefore be effective for treating glanders.

Acknowledgements. This work was supported by Department of Defense Contract DAMD 17-98-C-8003, the Canadian Bacterial Diseases Network of Centres of Excellence, the Medical Research Council of Canada and the Canadian Cystic Fibrosis Foundation.

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Prokaryotes (2006) 5:861–872
DOI: 10.1007/0-387-30745-1_41

CHAPTER 3.2.17

The Nitrite-Oxidizing Bacteria

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Introduction

Nitrite-oxidizing bacteria are a small group of primarily organo and/or chemoautotrophs that are difficult to grow and work with. For many years they drew very little attention, although it was realized that they provide a key link in the global nitrogen cycle between the ammonia-oxidizing bacteria, which generate nitrite, and the various denitrifying microorganisms that remove nitrate by reducing it to ammonia or molecular nitrogen, thus completing the global nitrogen cycle.

The growing public awareness relating to issues of environmental pollution in recent decades brought renewed interest in this group of bacteria, as their role in the transformation of nitric oxides became apparent. In addition, these bacteria cause economic damage to agriculture and to ground water quality by contributing to the leaching of nitrogen fertilizers (distributed in the form of ammonia) from surface soils, thus polluting aquifers with increasing concentrations of nitrites and nitrates.

Owing to their key role in the global cycling of nitrogen and their activities both as prime contributors to and scavengers of nitric oxides in the biosphere, nitrite-oxidizing bacteria became in recent years the focus of intensive ecophysiological research, in spite of the difficulties in cultivating and maintaining many of these bacteria in the laboratory.

In addition to this group of autotrophic nitrifyers, there is also a diverse group of heterotrophic bacteria capable of heterotrophic nitrite oxidation. Thus, in one such study (Sakai et al., 1996), the nitrite transforming activities of heterotrophic bacterial strains from various culture collections as well as isolates from activated sludge were studied. Of the 48 strains tested, 17 strains consumed 1–5 mM of nitrite and accumulated corresponding amounts of nitrate. Heterotrophic microorganisms are rather flexible in determining the fate of the consumed nitrite. For instance, in *Bacillus subtilis* strain I-41 (a denitrification-positive isolate), the ratio of the amount of nitrate accumulated to that of nitrite consumed varied from 0 to 100%, depending on the culture conditions. However, the mechanism of oxidation of nitrite to nitrate in these heterotrophic bacteria is very different from that in the autotrophic bacteria (Sorokin, 1991), as heterotrophic nitrite oxidation requires catalase and hydrogen peroxide (H_2O_2) generated through oxidation of organic electron donors. Also, aeration affects the nitrite- and nitrate-transforming activities of various heterotrophic bacteria. For example, *Pseudomonas pavonaceae*, a denitrification-positive strain, metabolizes both nitrite and nitrate to more reduced compounds at low oxygen pressure, and the direction of the conversion changes from reduction to oxidation at high oxygen pressure. This switching might be caused by inhibition and repression of the nitrite-reducing activity and by stimulation of nitrite-oxidizing activity by oxygen (Sakai et al., 1997).

Historic Background

Nitrite-oxidizing bacteria carry the second stage of the nitrification process, that of oxidation of nitrite to nitrate. Because nitrate is an essential ingredient of gunpowder, nitrate and nitrification were the focus of interest both to scientists and politicians for many centuries. Nitrification protocols for the manufacture of nitrates by composting organic matter in soil have existed for many centuries: from the tenth century in China and from the twelfth century in Europe (Macdonald, 1986). However, the process was considered to be of a chemical and not biological nature until the late nineteenth century. It was only in 1862 that Pasteur suggested that nitrification was of biological origin (Pasteur, 1862), and it was only when Winogradsky in 1891 succeeded in isolating a nitrite-oxidizing bacterium that the debate about whether nitrification is a one-stage process (carried by a single bacterium) or a two-stage process (carried by two distinct classes of microorganisms) was settled (Macdonald, 1986).

Nitrite Oxidation

A detailed account of the mechanism of nitrite oxidation as an energy source has been described previously (Bock et al., 2001). The key enzyme that makes the nitrite-oxidizing bacteria so important in the global nitrogen cycle is nitrite oxidoreductase (NOR), which carries out the stoichiometric reaction:

$$NO_2^- + H_2O \Leftrightarrow NO_3^- + 2H^+ + e^- \quad (1)$$

$$2H^+ + 2e^- + 0.5O_2 \rightarrow H_2O \quad (2)$$

Reaction (1) is reversible, and many nitrite oxidizers are capable of reducing nitrate to nitrite under anaerobic conditions, but the significance of the reverse reaction to their survival is not clear, as both nitrite and nitrate are rapidly reduced in anaerobic environments.

Nitrite oxidoreductase was isolated from various *Nitrobacter* strains, such as from mixotrophically grown cells of *Nitrobacter hamburgensis*; the enzyme purified from heat-treated membranes was homogeneous by the criteria of polyacrylamide gel electrophoresis and size exclusion chromatography. The monomeric form consisted of two subunits with molecular weights of 115 kDa and 65 kDa, respectively. The dimeric form of the enzyme contained 0.70 molybdenum, 23.0 iron, 1.76 zinc and 0.89 copper gram-atoms per molecule. The catalytically active enzyme was investigated by visible and electron paramagnetic resonance spectroscopy (EPR) under oxidizing (as isolated), reducing (dithionite), and turnover (nitrite) conditions. As isolated, the enzyme exhibited a complex set of EPR signals between 5–75 K, originating from several iron-sulfur and molybdenum (V) centers. Addition of the substrate nitrite or the reducing agent dithionite resulted in a set of new resonances. The molybdenum and the iron-sulfur centers of nitrite oxidoreductase from *Nitrobacter hamburgensis* were involved in the transformation of nitrite to nitrate (Meincke et al., 1992).

Systematics

As late as 1981, all bacteria capable of chemolithotrophic growth using ammonia ornitrite as energy source and capable of assimilating CO_2 via the Calvin cycle were grouped in one family, the Nitrobacteraceae (Watson et al., 1981). This classification was based on the fact that all the bacteria grouped in this family carry out oxidation reactions of inorganic nitrogen compounds, and all are capable of chemolithotrophic growth using these as energy sources. All are Gram-negative bacteria and usually found in association; as a physiological interdependence exists between the two groups, inasmuch as the ammonia oxidizers provide the substrate for the nitrite oxidizers, whereas the nitrite oxidizers are inhibited by excess ammonia.

With the advent of molecular biology and classification of bacteria through sequencing of 16S ribosomal RNA genes and other specific DNA sequences, as well as the use of monoclonal antibodies, it became evident that the two groups are not related, and that there is no phylogenetic relationship between ammonia and nitrite oxidizers. Almost all nitrite oxidizers (with the exception of *Nitrospira moscoviensis*, which seems to occupy an intermediate position; Schramm et al., 1998) are a homogenous group belonging to the α-subdivision of the Proteobacteria and are very closely related to the nonsulfur purple photosynthetic bacterium *Rhodopseudomonas palustris*. None of the ammonia-oxidizing bacteria belong to the α-subdivision, and they are all distributed between the β- and γ-subdivisions (Woese et al., 1984; Woese et al., 1985).

Comparative 16S rDNA sequencing was used to evaluate phylogenetic relationships among selected strains of ammonia- and nitrite-oxidizing bacteria (Teske et al., 1994). All characterized strains were shown to be affiliated with the proteobacteria. The study extended recent 16S rDNA-based studies of phylogenetic diversity among nitrifiers by the comparison of eight strains of the genus *Nitrobacter* and representatives of the genera *Nitrospira* and *Nitrospina*. The latter genera were shown to be affiliated with the δ-subdivision of the proteobacteria but did not share a specific relationship to each other or to other members of the δ-subdivision. All characterized *Nitrobacter* strains constituted a closely related assemblage within the α-subdivision of the Proteobacteria. As previously observed, all ammonia-oxidizing genera except *Nitrosococcus oceanus* constitute a monophyletic assemblage within the β-subdivision of the proteobacteria. Consideration of physiology and phylogenetic distribution suggests that nitrite-oxidizing bacteria of the α- and γ-subdivisions are derived from immediate photosynthetic ancestry. Each nitrifier retains the general structural features of the specific ancestor's photosynthetic membrane complex. Thus, the nitrifiers, as a group, apparently are not derived from an ancestral nitrifying phenotype.

Watson et al. (1981) identified three genera of nitrite-oxidizing bacteria (the *Nitrospira* being discovered only in 1986; Watson, 1986) and classified them on the basis of morphology and nutritional requirements. Bartosch et al. (1999) also divided the nitrite-oxidizing bacteria into four genera, *Nitrobacter*, *Nitrospina*, *Nitrococcus* and

Nitrospira, according to their reactions with monoclonal antibodies prepared against the nitrite oxidoreductase of the genus *Nitrobacter*. Among these, *Nitrobacter* and *Nitrospira* strains are capable of heterotrophic or mixotrophic growth, the rest being strict chemolithotrophs.

Because of the difficulties in culturing nitrifying bacteria on solid media, the methods for enumerating and identifying nitrifiers in environmental samples conventionally depended on incubating serial dilutions for very long periods, and then testing these for appearance of nitrate. Thus, the dominant nitrite-oxidizing species in an environmental sample was usually identified only after incubation of 3–6 months and was acertained on the basis of morphological and physiological properties such as sensitivity to salts and nutrient concentration. These observations led over the years to a general consensus that *Nitrobacter* strain were the dominant nitrite-oxidizing organisms in practically all tested environments such as soil, marine waters, wastewater treatment plants, fresh water and reservoirs; *Nitrococcus* and *Nitrospina* were rarely identified (Watson et al., 1981).

In 1995, a Gram-negative, nonmotile, nonmarine, nitrite-oxidizing bacterium was isolated by enrichment culture of a sample from a partially corroded area of an iron pipe located in a heating system in Moscow, Russia (Ehrich et al., 1995). The cells (0.9–2.2 μm × 0.2–0.4 μm in size) were helical to vibroid-shaped and often formed spirals with up to three turns 0.8–1.0 μm in width. The organism possessed an enlarged periplasmic space and lacked intracytoplasmic membranes and carboxysomes. The cells excreted extracellular polymers and formed aggregates. The bacterium grew optimally at 39°C and pH 7.6–8.0 in a mineral medium with nitrite as sole energy source and carbon dioxide as sole carbon source. The doubling time was 12 h in a mineral medium with 7.5 mM nitrite. The cell yield was low; only 0.9 mg of protein/liter was formed during oxidation of 7.5 mM nitrite. Under anoxic conditions, hydrogen could be used as electron donor with nitrate as electron acceptor. Organic matter (yeast extract, meat extract and peptone) supported neither mixotrophic nor heterotrophic growth. At concentrations as low as 0.75 g of organic matter/liter or higher, growth of the nitrite-oxidizing cells was inhibited. The cells contained cytochromes of the *b*- and *c*-type. The G+C content of DNA was 56.9 ± 0.4 mol%. This chemolithoautotrophic nitrite-oxidizer differed from the terrestrial members of the genus *Nitrobacter* with regard to morphology and substrate range and was similar to *Nitrospira marina* in both characteristics. The isolated bacterium was designated as a new species of the genus *Nitrospira*. Recent studies suggest that this genus dominates most of the nitrite oxidation activities in natural as well as artificial habitats (see below).

With the introduction of molecular technologies to environmental microbiology, the true dominant nitrite oxidizers could be identified in situ in environmental samples, either by polymerase chain reaction (PCR, using specific rDNA primers) or fluorescence in situ hybridization (FISH, using specific probes). These studies led to the surprising conclusion that *Nitrospira* strains are the dominant nitrite oxidizers in most environmental samples tested so far, whereas *Nitrobacter* sp. are seldom identified without prior enrichment (Burrell et al., 1998; Burrell et al., 1999; Schramm et al., 1998; Juretschko et al.,1998; Okabe et al., 1999).

There is, however, no doubt that within the next years, with the advent of oligonucleotide microchip technology, oligonucleotide microchips will dominate as genosensors for determinative and environmental studies in microbiology (Guschin et al., 1997). Precise identification of bacterial communities and of the genes switched on by each member of these communities under various environmental conditions will become routine in the environmental microbiology lab, and these procedures will enable the fine resolution of the community of nitrifying bacteria and its activities in different biotopes.

DNA sequencing methodologies also were used to identify bacteria in situ, in biofilms and activated sludge particles (Schramm et al., 1998). Bacterial aggregates from a chemolithoautotrophic, nitrifying fluidized bed reactor were investigated with microsensors and rDNA-based molecular techniques. The microprofiles of O_2, NH_4^+, NO_2^- and NO_3^- demonstrated the occurrence of complete nitrification in the outer 125 μm of the aggregates. FISH analysis showed that the dominant populations were of *Nitrospira* spp. and *Nitrosospira* sp. and that they formed separate, dense clusters which were in contact with each other and occurred throughout the aggregate. Significantly, no ammonia- or nitrite-oxidizing bacteria of the genera *Nitrosomonas* or *Nitrobacter*, respectively, could be detected by FISH. To identify the nitrite oxidizers, a 16S ribosomal DNA clone library was constructed and screened by denaturing gradient gel electrophoresis (DGGE), and selected clones were sequenced. The organisms represented by these sequences formed two phylogenetically distinct clusters affiliated with the nitrite oxidizer *Nitrospira moscoviensis*. The dominant *Nitrospira* sp. formed clusters with the nitrosospira sp. and occurred throughout the aggregate while the second, smaller, morphologically and genetically different population of *Nitrospira* sp. was

restricted to the outer nitrifying zones. The phylogeny of bacteria belonging to the genus *Nitrobacter* was investigated by sequencing the whole 16S rRNA gene (Orso et al., 1994). The average level of similarity for three *Nitrobacter* strains examined was high (99.2%), and the similarity level between *Nitrobacter winogradsky* and *Nitrobacter* sp. strain LL, which represent two different genomic species, was even higher (99.6%). When all of the *Nitrobacter* strains and their phylogenetic neighbors *Bradyrhizobium* and *Rhodopseudomonas* species were considered, the average similarity level was 98.1%. When complete sequences were used, *Nitrobacter hamburgensis* clustered with the two other *Nitrobacter* strains, though this was not the case when partial sequences were used. The two *Rhodopseudomonas palustris* strains examined exhibited a low similarity level (97.6%) and were not clustered.

In another study (Okabe et al., 1999), the in situ spatial organization of ammonia-oxidizing and nitrite-oxidizing bacteria in domestic wastewater biofilms and autotrophic nitrifying biofilms was investigated by using microsensors and FISH, performed with 16S rDNA-targeted oligonucleotide probes. The combination of these techniques made it possible to relate in situ microbial activity directly to the occurrence of specific nitrifying bacterial populations. In situ hybridization revealed that bacteria belonging to the genus *Nitrobacter* were not detected; instead, *Nitrospira*-like bacteria were the main nitrite-oxidizing bacteria in both types of biofilms. *Nitrospira*-like cells formed irregularly shaped aggregates consisting of small microcolonies, which bound the clusters of ammonia oxidizers. Whereas most of the ammonia-oxidizing bacteria were present throughout the biofilms, the nitrite-oxidizing bacteria were restricted to the active nitrite-oxidizing zones, which were inside the biofilms. Microelectrode measurements showed that the active ammonia-oxidizing zone was located in the outer part of a biofilm, whereas the active nitrite-oxidizing zone was located just below the ammonia-oxidizing zone and overlapped the location of nitrite-oxidizing bacteria, as determined by FISH.

The use of fluorescent monoclonal antibodies was also very useful for the rapid quantification and in situ detection of specific nitrifiers in a mixed bacterial habitat such as a biofilm (Noda et al., 2000). In that study, 12 monoclonal antibodies against *Nitrosomonas europaea* (IFO14298) and 16 against *Nitrobacter winogradsky* (IFO 14297) enabled a direct cell count of *N. europaea* and *N. winogradsky*. Moreover, the distribution of N. europaea and N. winogradsky in a biofilm could be examined. Most of *N. winogradsky* existed near the surface and most of *N. europaea* existed within the polyethylene glycol (PEG) gel pellet.

Using DNA sequences from the intergenic spacer (IGS) region of the ribosomal operon, and two primers derived from 16S and 23S rDNA conserved sequences, Navarro et al. (1992a) amplified these sequences by PCR. The PCR products, cleaved by four base cutting restriction enzymes, were used to differentiate *Nitrobacter* strains. This method is convenient for the genotypic characterization of *Nitrobacter* isolates and was successfully used to characterize natural populations of *Nitrobacter* from various soils and a lake. Considerable diversity was demonstrated in various soils, and in both water and sediments of the lake.

Navarro et al. (1992b) also studied the genomic diversity of *Nitrobacter* strains by determining rDNA gene restriction patterns as well as hybridization characteristics and DNA base compositions. The DNA hybridization (S1 nuclease method) revealed five DNA genomic groups, and these groups formed three genomic *Nitrobacter* species.

Mobarry et al. (1996) prepared a hierarchical set of five 16S rRNA-targeted DNA probes for phylogenetically defined groups of autotrophic ammonia- and nitrite-oxidizing bacteria. Their environmental application was demonstrated by quantitative slot blot hybridization and whole-cell hybridization of nitrifying activated sludge and biofilm samples. In situ hybridization experiments revealed that *Nitrobacter* and *Nitrosomonas* species occurred in clusters and frequently were in contact with each other within sludge flocs.

The nitrite oxidoreductase (NOR) from the facultative nitrite-oxidizing bacterium *Nitrobacter hamburgensis* X14 was used to develop a probe for the gene *norB* (Kirstein and Bock, 1993). Sequence analysis of DNA fragments revealed three adjacent open reading frames in the order *norA*, *norX* and *norB*. The deduced amino acid sequence of protein NorB contained four cysteine clusters with striking homology to those of iron-sulfur centers of bacterial ferredoxins. Protein NorB shares significant sequence similarity to the β-subunits (NarH and NarY) of the two dissimilatory nitrate reductases (NRA and NRZ) of *Escherichia coli*. Additionally, the derived amino acid sequence of the truncated open reading frame of norA showed striking resemblance to the α-subunits (NarG and NarZ) of the *E. coli* nitrate reductases. Additionally, the derived amino acid sequence of the truncated open reading frame of norA showed resemblance to the α-subunits of the *E. coli* nitrate reductases.

Monoclonal antibodies prepared against nitrite oxidoreductases of nitrite oxidizers also

were used successfully for the identification of nitrite oxidizers (Bartosch et al., 1999). Immunoblot analyses performed with three monoclonal antibodies (MAbs) that recognized the nitrite oxidoreductase (NOR) of the genus *Nitrobacter* were used for taxonomic investigations of nitrite oxidizers. These MAbs were able to detect the nitrite-oxidizing systems (NOS) of the genera *Nitrospira*, *Nitrococcus* and *Nitrospina*. When the genus-specific reactions of the MAbs were correlated with 16S rDNA sequences, they reflected the phylogenetic relationships among the nitrite oxidizers. In ecological studies, the immunoblot analyses demonstrated that *Nitrobacter* or *Nitrospira* cells could be enriched from activated sludge by using various substrate concentrations.

The microbiology of the biomass from a nitrite-oxidizing sequencing batch reactor (NOSBR) as well as the seed sludge used were investigated by microscopy, by culture-dependent methods, and by molecular biological methods (Burrell et al., 1998). The NOSBR was fed with an inorganic salts solution and nitrite as the sole energy source. It was operated for 6 months, and 16S ribosomal DNA clone libraries were prepared both from the seed sludge and from the reactor. Analysis of the seed sludge revealed that it contained three clones (4% of biomass) that were closely related to the autotrophic nitrite-oxidizer *Nitrospira moscoviensis*, whereas the NOSBR sludge itself was overwhelmingly dominated by bacteria closely related to the *N. moscoviensis* (89%). Only two clone sequences were similar to those of the genus *Nitrobacter*. Near-complete insert sequences of eight clones of *N. moscoviensis* isolated from the NOSBR and one clone from the seed sludge were determined and phylogenetically analyzed. This report was the first to show the presence of bacteria from the *Nitrospira* genus in wastewater treatment systems.

Nitrite-oxidizing bacteria belonging exclusively to the genus *Nitrospira* also dominated the nitrite-oxidizing community of a phosphate-removing biofilm from a sequencing batch biofilm reactor (Gieseke et al., 2001).

In another study (Burrell et al., 1999), a sequencing batch reactor (SBR) was operated to selectively grow a nitrite-oxidizing microbial community, and it was found that the nitrite oxidation was due the presence of bacteria from the *Nitrospira* genus and not the *Nitrobacter* genus, which were in very low numbers. It was hypothesized that the unknown nitrite-oxidizing bacteria in wastewater treatment plants are a range of species related to *Nitrospira moscoviensis*.

Oxidation of nitrite to nitrate in aquaria is typically attributed to bacteria belonging to the genus *Nitrobacter* that are members of the α-subdivision of the class Proteobacteria. To identify bacteria responsible for nitrite oxidation in aquaria, clone libraries of rRNA genes were developed by Hovanec et al. (1998) from biofilms from several freshwater aquaria, and analysis of the rDNA libraries, along with results from denaturing gradient gel electrophoresis (DGGE) on frequently sampled biofilms, indicated the presence of putative nitrite-oxidizing bacteria closely related to other members of the genus *Nitrospira*. Hybridization experiments with rRNA from biofilms of freshwater aquaria demonstrated that *Nitrospira*-like rRNA comprised nearly 5% of the rRNA extracted from the biofilms during the establishment of nitrification. Nitrite-oxidizing bacteria belonging to the α-subdivision of the class Proteobacteria (e.g., *Nitrobacter* spp.) were not detected in these samples. Aquaria that received a commercial preparation containing *Nitrobacter* species did not show evidence of *Nitrobacter* growth and development but did develop substantial populations of *Nitrospira*-like species. Time series analysis of rDNA phylotypes on aquaria biofilms by DGGE, combined with nitrite and nitrate analysis, showed a correspondence between the appearance of *Nitrospira*-like bacterial ribosomal DNA and the initiation of nitrite oxidation. In total, the data suggest that *Nitrobacter winogradsky* and close relatives were not the dominant nitrite-oxidizing bacteria in freshwater aquaria. Instead, nitrite oxidation in freshwater aquaria appeared to be mediated by bacteria closely related to *Nitrospira* sp.

Isolation and Nutritional Requirements

The information in this section is compiled from the chapter by Bock and Koops (1992) on Nitrobacter in the second edition published in the previous edition of {The Prokaryotes}.

Obligate and facultative lithoautotrophic nitrite oxidizers can be successfully isolated only if mineral nitrite media free of any organic contaminants are used, otherwise heterotrophs will overgrow nitrite oxidizers. A sample of 1–2 g (soil, stone, mud etc.) is placed in 100 ml of enrichment medium selective for the growth of either terrestrial or marine strains (Table 1). For the enrichment of dominant nitrifiers, a combination of the most probable number (MPN) technique followed by the serial dilutions method is suitable. In the highest dilutions, nitrite oxidation can be detected only after several months because of the slow growth of the nitrite oxidizers. Plating samples from enrichment cultures on rich and poor solid agar media

Table 1. Four culture media[a] for lithoautotrophic and mixotrophic growth of nitrite oxidizers.

Ingredient	1a	2b	3b	4c
Distilled water (ml)	1,000	1,000	1,000	300
Seawater (ml)				700
$NaNO_2$ (mg)		2,000	2,000	69
KNO_2 (mg)	300			
$MgSO_4 \cdot 7H_2O$ (mg)	187.5	50	50	100
$CaCl_2 \cdot 2H_2O$ (mg)	12.5			6.0
$CaCO_3$ (mg)		3.0	3.0	
KH_2PO_4 (mg)	500	150	150	
K_2HPO_4 (mg)	500			1.74
$FeSO_4$ (mg)	10	0.15	0.15	
Chelated iron[b] (mg)				1.0
Na_2Mo_4 (μg)				30
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (μg)		50	50	
$MnCl_2 \cdot 4H_2O$ (μg)				66
$CoCl_2 \cdot 6H_2O$ (μg)				0.6
$CuSO_4 \cdot 5H_2O$ (μg)				6.0
$ZnSO_4 \cdot 7H_2O$ (μg)				30
$KHCO_3$ (mg)	1,500			
NaCl (mg)		500	500	
Sodium pyruvate (mg)			550	
Yeast extract[c] (mg)			1,500	
Peptone[c] (mg)			1,500	

[a]pH is adjusted to 7.4–7.8.
[b]13%, Geigy.
[c]Difco.

can check contamination by heterotrophs. It may take a year to obtain pure cultures with the technique described above.

Medium 1a for terrestrial strains is from Aleem and Alexander (1958), media 2b and 3b also for terrestrial strains are from Bock and Stackebrandt (1983), and medium 4c for marine strains is from Watson and Waterbury (1971).

Habitats

As described earlier, the nitrite-oxidizing bacteria are ubiquitous, and are usually found in association with the ammonia-oxidizing bacteria, which provide them with their energy source. In spite of many reports describing their sensitivity to light (Guerrero and Jones, 1996; Guerrero and Jones, 1997) and various toxicants (Deni and Penninckx, 1999; Jenicek et al., 1996), nitrite-oxidizing bacteria are abundant in practically all terrestrial, marine and fresh water habitats. Nitrifying bacteria play a significant role in erosion of rocks (Lebedeva et al., 1978) primarily because they release nitrates, which dissolve carbonates, in exchange for CO_2 they assimilate. This process was found to be a major cause of deterioration of historic monuments in Europe (Mansch and Bock, 1998). Proliferation of nitrifiers in sewage is also the basis for nitrogen removal from domestic and industrial waste in water treatment plants, including chemical industry wastewater. Nitrite oxidizers grow within a wide range of pH values up to 10 (Sorokin, 1998a; Sorokin et al., 1998b) and salinities (limits are difficult to determine as nitrifying bacteria resist very low water activities, such as occur when living within desiccated rocks).

Although nitrite oxidizers are considered obligate aerobes (even though many can utilize nitrate in absence of oxygen as an electron acceptor), growth of nitrite oxidizers can be observed in totally anaerobic environments such as deep in the mud at the bottom of deep anaerobic wastewater reservoirs, in the absence of nitrate or oxygen, in presence of significant concentrations of sulfides (Abeliovich, 1987). Also, nitrifying bacteria were isolated from a marine anaerobic sediment at depths of up to 8 cm (Blackburn, 1983). Nothing is at present known about the metabolism and survival mechanisms of these nitrite-oxidizing bacteria living under strict anaerobic, reducing conditions.

Light (particularly blue light) is frequently mentioned as an inhibiting factor for the nitrification process. However, the reports on the effects of light are sometimes contradictory. Nitrifiers are found in all habitats exposed to sunlight, either at the surface of fresh water or marine habitats, so obviously they must have efficient protection mechanisms to overcome photoinhibition and damage caused by direct sunlight.

Sorokin et al. (1998b) and Sorokin (1998a) have isolated five strains of lithotrophic, nitrite-oxidizing bacteria from sediments of three soda lakes after enrichment at pH 10 with nitrite as sole energy source; these strains were described as a new species of the genus *Nitrobacter*, *N. alkalicus*. Nitrite oxidation to nitrate occurred at pH 10. The nitrifiers had pear-shaped budding cells morphologically similar to those of *Nitrobacter* and formed tiny colonies on mineral nitrite agar at pH 10.

In a study aimed at evaluating the potential of wastewater treatment plants to contaminate receiving waterways, survival of *Nitrobacter* cells associated with particles in water treatment plant discharges was studied using immunofluoresence methods (Bonnet et al., 1997). It was found that *Nitrobacter* colonies can settle in freshwater sediments in a week (10^6 cells per gram of dry sediment) and can therefore colonize river sediments.

Chemolithotrophic nitrifying bacteria are dependent on the presence of oxygen for the oxidation of ammonium via nitrite to nitrate. The success of nitrification in oxygen-limited environments largely depends on the oxygen sequestering abilities of both ammonium- and

nitrite-oxidizing bacteria. Oxygen consumption kinetics were determined with cells grown in mixed culture in chemostats at different growth rates and oxygen tensions (Laanbroek and Gerards, 1993). Reduction of oxygen tension in the culture was found to repress the oxidation of nitrite before the oxidation of ammonium was affected, and hence nitrite accumulated.

In addition to the competition between ammonium- and nitrite-oxidizing bacteria, there is competition with organotrophic bacteria for the available oxygen as well. The outcome of the competition is determined by their specific affinities for oxygen as well as by their population sizes. The effect of mixotrophic growth of nitrite-oxidizing *Nitrobacter hamburgensis* on the competition for limiting amounts of oxygen was studied in mixed continuous culture experiments with the ammonium-oxidizing *Nitrosomonas europaea* at different oxygen concentrations (Laanbroek et al., 1994). The specific affinity for oxygen of *N. europaea* was in general higher than that of *N. hamburgensis*, and in transient state experiments, when oxic conditions were switched to anoxic, *N. hamburgensis* was washed out and nitrite accumulated. However, at low oxygen concentration, the specific affinity for oxygen of *N. hamburgensis* increased and became as great as that of *N. europaea*, and owing to its larger population size, the nitrite-oxidizing bacterium became the better competitor for oxygen and ammonium accumulated. Therefore Laanbroek et al. suggest that continuously oxygen-limited environments present a suitable ecological niche for the nitrite-oxidizing *N. hamburgensis*.

Biofilms

Biofilms present a unique biotope for using molecular DNA techniques as well as FISH and various sensors. The combination of microsensor and molecular techniques is highly useful for studies on the microbial ecology of biofilms in general, and in particular for the identification of activity sites of nitrifying bacteria.

Thus, the distribution of nitrifying bacteria of the genera *Nitrosomonas*, *Nitrosospira*, *Nitrobacter* and *Nitrospina* was studied by Schramm et al. (2000) in a membrane-bound biofilm system in which gradients of oxygen, pH, nitrite and nitrate were determined by means of microsensors, while the nitrifying populations along these gradients were identified and quantified using FISH in combination with confocal laser scanning microscopy. It was found that the oxic part of the biofilm was dominated by ammonia oxidizers and by members of the genus *Nitrobacter*, and *Nitrospira* sp. were virtually absent in this part of the biofilm, whereas they were most abundant at the oxic-anoxic interface. In the totally anoxic part of the biofilm, cell numbers of all nitrifiers were relatively low. These observations suggest the microaerophilic behavior of an as yet uncultured *Nitrospira* sp. as a factor affecting its environmental competitiveness.

De Beer and Schramm (1999), using microsensors and molecular techniques (such as in situ hybridization with 16S rDNA-targeted oligonucleotide probes), showed that there exists in biofilms grown in bioreactors a complex nitrifying community, consisting of members of the genera *Nitrosomonas*, *Nitrosospira*, *Nitrobacter* and *Nitrospira*.

In nitrifying biofilms from a trickling filter of an aquaculture water recirculation system, it was found that nitrification was restricted to a narrow zone of 50 μm of the very top of the film. Ammonia oxidizers formed a dense layer of cell clusters in the upper part of the biofilm, whereas the nitrite oxidizers showed less-dense aggregates in the proximity of the ammonia oxidizers. Both ammonia and nitrite oxidizers were not restricted to the oxic zone of the biofilm but were also detected in substantially lower numbers in the anoxic layers and even occasionally at the bottom of the biofilm (Schramm et al., 1996).

Enumeration

Conventional methodologies for counting nitrifiers are gradually being replaced by new technologies, although the MPN method still dominates, this because all methods based on specific probes or antibodies still leave open the possibility that some species do not crossreact with the probe used. On the other hand, the issue of optimal conditions for maximal yields using MPN counts has not been yet resolved.

One example is a new amperometric enzyme-linked immunoassay for specific enumeration of *Nitrobacter*, which uses an electrode made of glassy carbon on which an immunological reaction is carried out. The detection limit was approximately 3×10^6 *Nitrobacter* cells/ml (Sanden and Dalhammar, 2000). It was shown that this method could be applied for the enumeration of *Nitrobacter* in activated sludge and other environmental samples. The reason for the attempts to develop new methodologies is the lengthy incubation periods required for stable MPN counts: maximum most probable numbers of the ammonia-oxidizing group were attained in 20–55 days (median, 25) and MPN estimates of nitrite oxidizers required a much longer incubation (103–113 days; Matulewich et al., 1975). In addition, Both et al. (1990a) and (1990b) studied the effect of two concentrations of nitrite in the

incubation medium, 0.05 and 5.0 mM, and found that numbers of nitrite-oxidizing bacteria were highly dependent on the nitrite concentration as well as on the soil sampled.

Neither the influence of pH or nitrite concentration in the incubation medium could account exclusively for the MPN-enumeration result, which raises the issue of whether to use more than one incubation medium for the enumeration of nitrite-oxidizing bacteria. Enumeration of nitrite-oxidizing bacteria in soil samples by the MPN technique often showed relatively high cell number at a low nitrite concentration, when compared to the number of ammonium-oxidizing bacteria. When different *Nitrobacter* species as well as nongrowing cells differing in age were incubated 5 months at 20°C in presence of various nitrite concentrations and pH values, it appeared that the growth of cells taken from an early stationary phase culture of all these species was not affected by high nitrite concentrations or low pH. Growth of 8- and 18-month-old nongrowing cells of *Nitrobacter hamburgensis* was also insensitive to high nitrite concentration (5 mM). The growth of 8- and 18-month old resting cells of *N. vulgaris* was repressed only by a combination of 5 mM nitrite and a low pH. Growth of 8-month-old nongrowing cells of *N. winogradsky* was sensitive to 5 mM irrespective of pH, but growth of 18-month-old cells were inhibited by 5 mM nitrite only at a low pH. The growth of 8- and 18-month-old resting cells of *N. winogradsky* serotype *agilis* was repressed by low pH rather than by high nitrite concentration (Laanbroek and Schotman, 1991). These results emphasize the problems associated with using MPN counts for estimating size of nitrifier populations in natural samples.

A molecular approach based on PCR that was expected to detect and quantify nitrifying bacteria was also tested using specific primers of the genus *Nitrobacter*. In this study, coupled in parallel was a ^{14}C-radiotracing method used to measure potential nitrification; it was shown that *Nitrobacter* represented less than 0.1% of the total bacterial community (Berthe et al., 1999).

In another study, also aimed at counting *Nitrobacter* populations in situ by PCR, two primers from the 16S rDNA gene were used to generate a 397-bp fragment by amplification of *Nitrobacter* species DNA, and it was found that the PCR had a lower detection threshold (10^2 *Nitrobacter* cells per gram of soil) than did the MPN or fluorescent antibody method (Degrange and Bardin, 1995). In contrast, another study (Feray et al., 1999) found the best recovery yield was obtained with the immunofluorescence technique (21.3%), and the poorest detection level was reached with the MPN method (3.1%).

Wastewater

Tertiary wastewater treatment, aimed at removing all nitrogen wastes from the effluents, is costly in terms of the oxygen required for oxidizing ammonia to nitrate. However, as far as the nitrification—denitrification process is concerned, oxidation of nitrite to nitrate is an unnecessary step, as denitrification can just as well proceed from nitrite. Therefore, attempts are being made to devise operational parameters that will inhibit the activity of nitrite-oxidizing bacteria in wastewater treatment plants.

Thus, it was found (Rhee et al., 1997) that accumulation of nitrite occurred during the aerobic phase of a sequencing batch reactor (SBR) operating to remove nitrogen from synthetic wastewater: the activity of autotrophic nitrite oxidizers was reduced in the SBR and the free ammonia was the main inhibitor of nitrite oxidation.

It has been shown experimentally (Garrido et al., 1997) that it is possible to convert all ammonium to approximately 50% nitrate and 50% nitrite in the effluent of a biofilm air-lift suspension reactor, this with oxygen concentrations between 1 and 2 mg/liter. The ammonia- and nitrite-oxidizing bacterial populations occurring in the nitrifying activated sludge of an industrial plant treating sewage with high ammonia concentrations were also studied using in situ hybridization with a set of hierarchical 16S rDNA-targeted probes (Juretschko et al., 1998). Although a *Nitrobacter* strain was isolated, members of the genus *Nitrobacter* were not detectable in the activated sludge by in situ hybridization. A specific 16S rDNA-targeted probe for *Nitrospira* demonstrated that a *Nitrospira*-like bacterium was present in significant numbers (9% of the total bacterial counts).

According to Blackall (2000), even though it is common knowledge that *Nitrosomonas* and *Nitrobacter* are the major ammonia and nitrite oxidizers, today we know that these organisms may not play any role in the transformations for which they have achieved such acclaim. Using the above-mentioned methodologies, *Nitrospira*-like bacteria were found to be the dominant nitrite oxidizers in both enriched and full scale nitrifying systems.

Resistance, Inhibition and Biodegradation

Because inhibition of nitrite oxidation is economically advantageous in wastewater treatment plants, procedures were developed for identification of nitrification inhibitors in wastewater.

These include fractionation of wastewater samples and a nitrification inhibition assay with pure cultures of *Nitrobacter* to identify the inhibitory effect. In such a study, a series of unsaturated fatty acids and two monoterpenes were found to be inhibitory in an industrial wastewater sample (Svenson et al., 2000).

A shorter nitrification-denitrification cycle occurs in the presence of free ammonium. Although the occurrence of this cycle depends on both a high ammonia concentration and high pH, it was found that the pH of the wastewater is the decisive parameter (Surmacz-Gorska et al., 1997). However, a study aimed at identifying nitrification at high pH values in soda lakes and soda soils with pH 9.5–11 led to the isolation of a nitrifier morphologically similar to *Nitrobacter* that formed tiny colonies on mineral nitrite agar at pH 10 (Sorokin, 1998a).

Hwang et al. (2000), in a detailed study of the inverse relation between alkalinity and ammonia with respect to inhibition of nitrite oxidation, showed that when the molar ratio of carbonate alkalinity to ammonia increased from 4.1 to 9.4 (thus increasing the concentration of free ammonia), the ammonium removal rate doubled. At the same time the higher concentration of free ammonia in the medium was a selective inhibitor for *Nitrobacter*, causing an enhanced nitrite build-up in a biofilm reactor.

As for the effect of heavy metals, laboratory evaluations were conducted to study the toxic effects of copper and nickel on a culture of strictly obligatory nitrifiers (*Nitrosomonas* sp. and *Nitrobacter* sp.) in continuous flow stirred tank reactors. *Nitrosomonas* sp. was found to be equally or more sensitive than *Nitrobacter* sp. (Lee et al., 1997).

Attempts were made to develop a process for the simultaneous removal of organic halogens and nitrogen from kraft pulp mill effluents. A nitrifying biofilm reactor removed organic halogens from bleached kraft pulp mill effluents including chlorophenols from synthetic wastewater (Kostyal et al., 1997; Kostyal et al., 1998). In another study, none of several chemicals tested (nonylphenol, naphthalene, 2-methylnaphthalene, di-2-ethylhexylphthalate and toluene) inhibited nitrification when added to soil at various concentrations (Kirchmann et al., 1991). However, in many cases municipal wastewater did contain substances that inhibited nitrification to varying degrees (Jonsson et al., 2000).

Reports conflict on the effect of toluene on indigenous microbial populations. Nitrite oxidation potential (NOP) was reduced after incubation with high toluene concentrations for 45 days (Fuller and Scow, 1996). Trichloroethylene is also inhibitory to the soil indigenous nitrifying population (Fuller and Scow, 1997).

Volatile fatty acids such as formic, acetic, propionic, *n*-butyric, isobutyric, *n*-valeric, isovaleric and *n*-caproic acid as well as trimethylamine are inhibitory to nitrification, but only at high concentrations, suggesting that volatile fatty acids and trimethylamine alone cannot account for the inhibition of the nitrification activity in domestic wastewater (Eilersen et al., 1994).

Nitrifying bacteria were also found to be resistant to monochloramines, and the combination of increased concentrations of oxidized nitrogen with decreased total chlorine in treated water was used as an indicator of bacterial nitrification (Cunliffe, 1991).

The need to set limits on nitrite oxidation in wastewater treatment plants created a demand for assays to determine the degree of inhibition of nitrification. One such assay, developed by Grunditz and Dalhammar, was based on inhibition of pure cultures of *Nitrosomonas* and *Nitrobacter* isolated from sewage sludge (Grunditz and Dalhammar, 2001).

As for the effects of light, nitrifying bacteria (both ammonia and nitrite oxidizers) are capable of recovery from photoinhibition in the dark (see above). Recovery of oxidizing activities is both dose and wavelength dependent (Guerrero and Jones, 1996). The light-absorbing pigment was identified as a porphyrin-like pigment with an absorption maximum at 408 nm, accumulating at the late exponential phase of growth. This photoreceptor was found at higher concentrations in ammonia oxidizers than in nitrite oxidizers (Guerrero and Jones, 1997).

Lithotrophy

An evaluation of field data from historical buildings in Germany, carried on by Mansch and Bock (1998), showed that ammonia and nitrite oxidizers were found in 55 and 62% of the samples, respectively, and that nitrifying bacteria will colonize natural stone within several years of exposure. The highest cell numbers were in some cases found underneath the surface. Nitrifying bacteria showed a preference for calcareous material with a pore radius of 1–10 μm. Cell numbers of nitrifying bacteria did not correlate with the nitrate content of the stone material. Their data strongly suggest that microbial colonization of historical buildings is enhanced by anthropogenic air pollution. A comparison of samples taken between 1990 and 1995 from buildings throughout Germany showed that in eastern Germany significantly more colonization with facultatively methylotrophic bacteria and nitrifying bacteria existed. The same was true for natural stone from an urban exposure site when compared to material from a rural exposure site.

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Prokaryotes (2006) 5:873–891
DOI: 10.1007/0-387-30745-1_42

CHAPTER 3.2.18

The Genera *Azoarcus*, *Azovibrio*, *Azospira* and *Azonexus*

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Introduction

The genera *Azoarcus*, *Azospira*, *Azovibrio* and *Azonexus* harbor mostly nitrogen-fixing Betaproteobacteria. In their first taxonomic description, several strains which were later assigned to the genera *Azovibrio*, *Azospira* (Reinhold-Hurek et al., 1993) and *Azonexus* (Hurek et al., 1997b) were included in the genus *Azoarcus* sensu lato. Therefore, they are discussed in one chapter. Except for one strain, all these diazotrophic bacteria had been isolated from a similar source, roots of a pioneer grass in Pakistan or fungal resting stages from the same field; they had many physiological features in common, and they were phylogenetically related. Therefore, three groups of bacteria were included into *Azoarcus* although they were located on the *Azoarcus* rRNA branch at low $T_{m(e)}$ values (Reinhold-Hurek et al., 1993) or showed a low 16S rDNA similarity (Hurek et al., 1997b). Isolation of additional strains, which made possible a better phenotypic description, and sequencing of almost complete 16S rDNA sequences allowed the reassessment of the taxonomic structure of *Azoarcus* sensu lato. The unnamed groups C and D (Reinhold-Hurek et al., 1993) obtained the rank of different genera *Azovibrio* and *Azospira*, respectively (Reinhold-Hurek and Hurek, 2000); *Azoarcus* sensu lato group E (Hurek et al., 1997b) was proposed as *Azonexus* (Hurek et al., 1997b). Strains of these genera deserve special recognition as endophytes of grass roots or as degraders of aromatic compounds.

The Genus *Azoarcus*

Introduction

The genus *Azoarcus* harbors two ecologically different groups of bacteria: 1) soil-borne strains which can degrade aromatic hydrocarbons under denitrifying conditions, and 2) bacteria associated with grass roots epi- or endophytically, which apparently do not survive well in root-free soil. They will be referred to as "soil-borne" or "plant-associated" species, respectively, in this chapter. *Azoarcus* was originally described with two valid species of grass-associated diazotrophs (Reinhold-Hurek et al., 1993). However, a growing number of species of soil-borne strains has been added in recent years.

Phylogeny

Azoarcus spp. are members of the Betaproteobacteria in the Rhodocyclales according to phylogenetic analysis of almost complete 16S rRNA gene sequences. They are phylogenetically most closely related to *Thauera* with which they form the *Azoarcus/Thauera* branch (Fig. 1). *Azoarcus* species are located on two different clades, which in part reflects their physiology and ecology: the soil-borne species *A. tolulyticus*, *A. toluclasticus*, *A. toluvorans*, *A. evansii*, *A. buckelii* and *A. anaerobius* and the plant-associated species *A. indigens*, *A. communis* and *Azoarcus* sp. strain BH72. The analysis of phylogenetic relationships within the *Azoarcus*/*Thauera* 16S rDNA cluster is rendered difficult because the branching pattern between *Thauera*, the soil-borne and the plant-associated *Azoarcus* species is unstable when tested with different tree-building methods. This was also observed previously (Hurek et al., 1997b; Reinhold-Hurek and Hurek, 2000). Also in other taxa closely related to each other, like *Rhizobium* and related genera, the resolution of phylogenetic analysis is sometimes limited. Nevertheless, all named species in the genus *Thauera* cluster in one clade with highly significant support levels (Fig. 1), allowing a reliable assignment to this genus. Also most soil-borne species or strains of *Azoarcus* fall into one clade with significant support levels, and none of the species containing plant-associated strains are located on this branch. The 16S rDNA phylogenetic distances within these three clades are similar. Within the genus *Azoarcus*, they are up to 6%, while *Thauera* is 6–7% distant. Thus *Azoarcus* spp. represents a rather heterogeneous group. Moreover, since the phylogenetic distances within the three clades in the *Azoarcus*/*Thauera* group are similar, both subgroups of

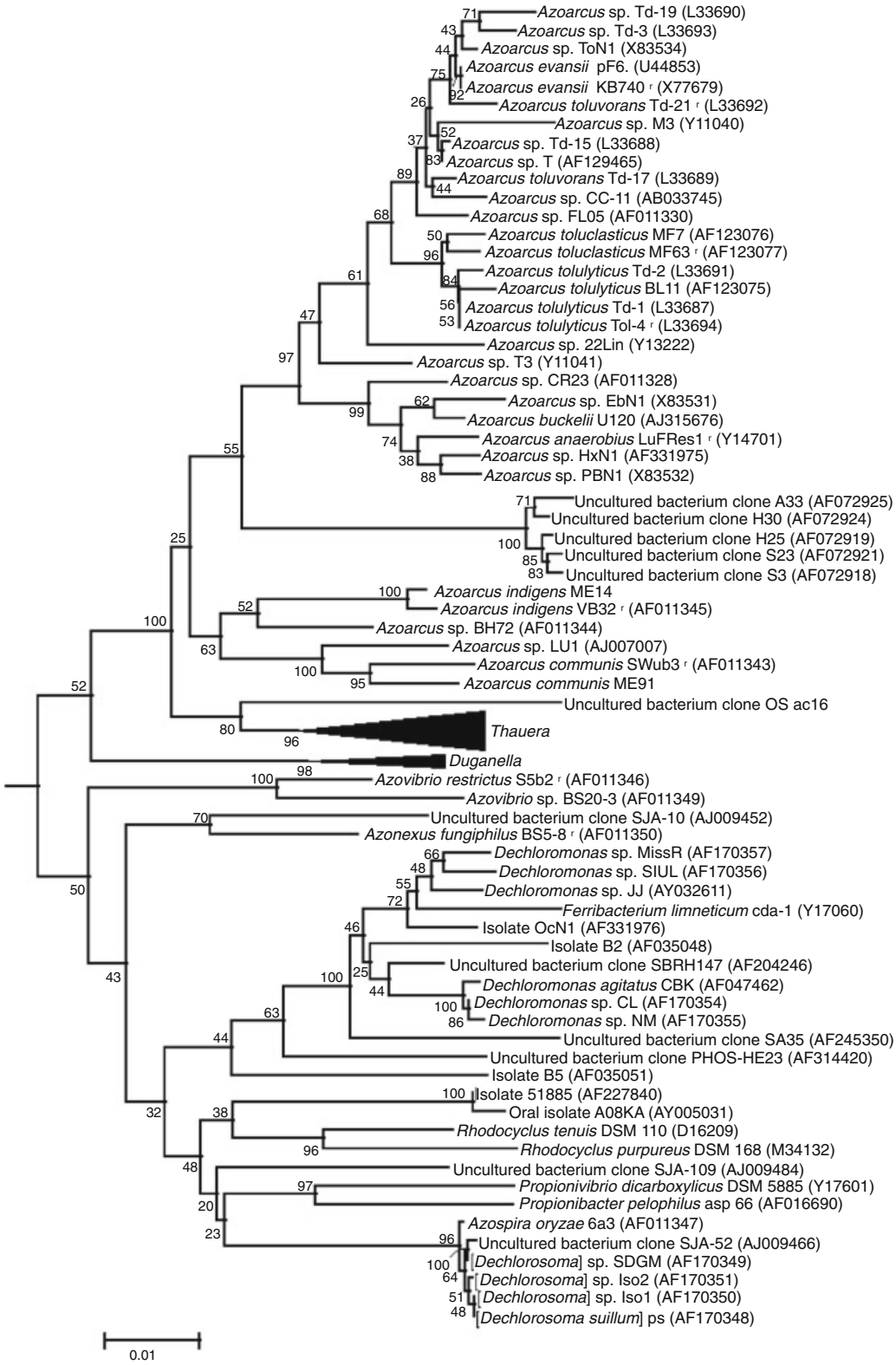

Fig. 1. Phylogenetic analysis of 16S rDNA sequences (1358 positions) of a subgroup of the Betaproteobacteria. Subtree of the *Rhodocyclus*/*Thauera*/*Azoarcus* group derived from an analysis of 159 sequences. Tree inference was carried out using the neighbor-joining algorithm and a Jukes-Cantor correction with 125 bootstrap repetitions. Sequence accession numbers are given in parentheses.

Azoarcus might also deserve the rank of different genera in the future. However, this will require a rigid polyphasic taxonomic analysis.

On the basis of 16S rDNA sequence analyses, all of the validly described species of *Azoarcus* are well resolved except for the two strains of *A. toluvorans*, which are interdispersed with several strains of uncertain affiliation (Fig. 1). Former members of *A. tolulyticus*, Td-3 and Td-19, have recently been removed from this species owing to low DNA-DNA similarity (below 40%), which is also reflected in our phylogenetic analysis (Fig. 1).

Interestingly, also a deeply-branching clade of as yet uncultured bacteria detected in nitrifying-denitrifying activated sludge of an industrial wastewater treatment plant (Juretschko et al., 2002) is localized within the *Azoarcus* cluster (Fig. 1).

Taxonomy

The definition of the genus *Azoarcus* is quite complex. It includes strains with a variety of physiological and ecological properties, which place them into at least two groups. In addition to their ecology, the species containing plant-associated strains differ from the soil-borne species in some nutritional features, e.g., in their inability to use carbohydrates as sole carbon source or, under conditions of denitrification, a wide range of aromatic compounds. The genus *Azoarcus* consists of the following eight validly described species, mostly based on polyphasic taxonomic approaches including DNA-DNA hybridization, protein profiles, fatty acid analysis, and nutritional profiles: *A. indigens* (type species) and *A. communis* (Reinhold-Hurek et al., 1993), *A. tolulyticus* (Zhou et al., 1995), *A. toluclasticus* and *A. toluvorans* (Song et al., 1999), *A. evansii* (Anders et al., 1995), *A. anaerobius* (Springer et al., 1998) and *A. buckelii* (Mechichi et al., 2002). One isolate (BH72) is different from these at the species level according to DNA-DNA similarity studies (≤25% DNA binding); however, a species name was not given because it is only a singular strain (Reinhold-Hurek et al., 1993). Numerous additional soil-borne strains are localized in the *Azoarcus* clade according to 16S rDNA sequence analysis (examples given in Fig. 1). Most have been isolated under conditions of denitrification with aromatic hydrocarbons as a C-source, but the lack of nutritional/physiological data and DNA-DNA similarity values does not yet allow a species assignment.

The phylogenetic distances within the three clades in the *Azoarcus*/*Thauera* group are similar (Reinhold-Hurek and Hurek, 2000), and the soil-borne and plant-associated species show considerable phenotypic differences (Reinhold-Hurek and Hurek, 2000; see also the section on Identification in this Chapter). Therefore, both subgroups of *Azoarcus* might deserve the rank of different genera in future. However, this will require a rigid polyphasic taxonomic analysis. As can be seen from the descriptive table (Table 3), many features have not been tested for all species or strains. There appears to be a bias in testing, e.g., nitrogen fixation and aerobic nutritional profiles are not excessively tested in soil-borne strains, whereas nutritional profiles under conditions of denitrification are missing for plant-associated species. These studies should also include the numerous soil-borne isolates not yet assigned to any species. As some strains are deeply branching in the phylogenetic analysis (e.g., 22Lin, T3 and M3; Fig. 1), the description of new species might be expected.

Habitat

Azoarcus spp. occur in terrestrial ecosystems; however, both groups of *Azoarcus* spp. strongly differ in their habitats and ecology. Members of *A. indigens*, *A. communis* and *Azoarcus* sp. BH72 occur inside roots or on the root surface of Gramineae and have never been isolated from root-free soil so far (Reinhold-Hurek and Hurek, 1998b), except for members of *A. communis* originating not from plants but from French refinery oily sludge (S2; Laguerre et al., 1987; Reinhold-Hurek et al., 1993) or from a compost biofilter in Canada (Lu1; Juteau et al., 1999). In contrast, all isolates localized in the clade of *A. tolulyticus*, *A. toluvorans*, *A. toluclasticus*, *A. evansii*, *A. buckelii* or *A. anaerobius* do not originate from living plants but mostly from soil and sediments. Therefore, their habitats will be treated separately.

The plant-associated strains were originally isolated from Kallar grass (*Leptochloa fusca* [L.] Kunth), a flood-tolerant salt marsh grass grown as a pioneer plant on salt-affected, flooded, low-fertility soils in the Punjab of Pakistan since the 1970s (Reinhold et al., 1986; Reinhold-Hurek et al., 1993). These nitrogen-fixing bacteria were found in high numbers in surface-sterilized roots or culms and rarely on the root surface (*A. communis*). Only much later, the host range was found to be extended to rice (*Oryza sativa*) from Nepal (Engelhard et al., 2000) or to resting stages (sclerotia) of a plant-associated basidiomycete found in rice field soil from Pakistan (Hurek et al., 1997b).

Soil-borne species of *Azoarcus* are very widespread but not plant-associated. Strains belonging to the validly described species have been isolated from noncontaminated soils (Song et al., 1999) or contaminated soils, either with unknown contaminations in industrial areas

(Song et al., 1999) or with petroleum contaminations (Fries et al., 1994; Zhou et al., 1995). Many strains were also cultured from sediments of creeks (uncontaminated or contaminated; Anders et al., 1995), aquifers (Fries et al., 1994; Fries et al., 1997; Zhou et al., 1995), or even from activated sewage sludge (Springer et al., 1998). Soil-borne *Azoarcus* spp. are also widespread with respect to their geographical distribution: they have been found for example in North America (United States, Canada and Puerto Rico), South America (Brazil), or Europe (Germany and Switzerland).

Isolation

Plant-associated and soil-borne members of *Azoarcus* spp. will be treated separately. Plant-associated strains are best enriched on media free of combined nitrogen. Washed or surface-sterilized roots are macerated aseptically in wash medium (enrichment medium free of a carbon source).

To avoid overgrowth by faster growing diazotrophs, inoculate enrichment cultures with serial dilutions of this material. N-free semisolid synthetic malate medium (SM-N) is used for enrichment, composed of (per liter of distilled water): $MgSO_4 \cdot 2H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.02 g; $MnSO_4 \cdot H_2O$, 0.01 g; $Na_2MoO_4 \cdot 2H_2O$, 0.002 g; Fe(III)-EDTA, 0.066 g; KH_2PO_4, 0.6 g, and K_2HPO_4, 0.4 g, adjusted to pH 6.8; DL-malic acid, 5 g, and KOH, 4.5 g adjusted to pH 6.8; agar, 2 g; and vitamin solution, 1 ml. The vitamin solution contains (mg per liter of distilled water) myo-inositol, 10,000; niacinamide, 100; pyridoxine HCl, 100; thiamine HCl, 100; calcium pantothenate, 50; folic acid, 20; choline chloride, 50; riboflavin, 10; ascorbic acid, 100; *p*-aminobenzoic acid, 1; vitamin A, 0.5; vitamin D_3, 0.5; vitamin B_{12}, 0.5; and D-biotin, 0.5. Add phosphate buffer and vitamin solution after autoclaving. Inoculate medium solidified in screw-cap tubes with 10 µl of macerate or root pieces below the medium surface. Incubate tubes without shaking at 30°C or 37°C, and check for development of a subsurface pellicle. Take samples for streaking before the pellicle has moved to the medium surface and become very dense. Isolate single colonies on SM-medium agar supplemented with 20 mg of yeast extract per liter. Use acetylene reduction method to analyze enrichment cultures or isolates for nitrogenase activity in semisolid medium. Check purity and colony color on complex medium, VM ethanol (which is based on SM medium but supplemented [per liter] with "Lab Lemco" powder [Oxoid] or Bacto Peptone [Difco], 3 g; yeast extract, 1 g; NaCl, 1 g; NH_4Cl, 0.5 g; and agar, 15 g). Alternatively, for salt-affected habitats, use SSM medium which is adapted to high salt concentrations of saline-sodic soil (Reinhold et al., 1986) instead of SM medium for enrichment and isolation.

For soil-borne strains, enrichment is routinely done under strictly anaerobic conditions, with nitrate as terminal electron acceptor. A variety of aromatic carbon sources have been used, depending on the species or strain under study. Preparation of media and cultivation of bacteria have to be carried out under strictly anoxic conditions.

Use a typical medium (for *A. evansii*), which consists of (per liter of distilled water): KH_2PO_4, 0.816 g; K_2HPO_4, 5.920 g; NH_4Cl, 0.530 g; $MgSO_4 \cdot 7H_2O$, 0.200 g; KNO_3, 0.5 g; and $CaCl_2 \cdot 2H_2O$, 0.025 g; dissolve phosphate and other ingredients separately from each other, adjust both to pH 7.8 for *A. evansii* or neutral pH for other strains, and combine both autoclaved solutions after cooling. Add 10 ml of sterile trace elements SL-10 and 5 ml of vitamin solution. SL-1 contains (per liter of distilled water): HCl (25%; 7.7 M), 10 ml; $FeCl_2 \cdot 4H_2O$, 1.5 g; $ZnCl_2$, 70 mg; $MnCl_2 \cdot 4H_2O$, 100 mg; H_3BO_3, 6 mg; $CoCl_2 \cdot 6H_2O$, 190 mg; $CuCl_2 \cdot 2H_2O$, 2 mg; $NiCl_2 \cdot 6H_2O$, 24 mg; and $Na_2MoO_4 \cdot 2H_2O$, 36 mg. First dissolve $FeCl_2$ in HCl, then dilute in water, add and dissolve the other salts. The vitamin solution contains (mg per liter of distilled water): vitamin B_{12}, 50 mg; pantothenic acid, 50 mg; riboflavin, 50 mg; pyridoxamine-HCl, 10 mg; biotin, 20 mg; folic acid, 20 mg; nicotinic acid, 25 mg; nicotinamide, 25 mg; α-lipoic acid, 50 mg; *p*-aminobenzoic acid, 50 mg; and thiamine-HCl $\cdot 2H_2O$, 50 mg. Add potentially toxic carbon sources at low concentrations (toluene, 5 ppm; phenol, 1 mM; and Na-benzoate, 5 mM) and spike cultures again with the carbon source after its degradation. Use ascorbic acid (4 mM) to reduce the medium. Carry out enrichment in anoxically sealed serum bottles. When using rich sediments as an inoculum, deplete when necessary from readily oxidizable carbon sources by repeated incubation in medium free of carbon sources (Fries et al., 1994). Plate the enriched samples on agar media such as M-R2A medium (Fries et al., 1994) or on agar used for dilution series (Widdel and Bak, 1992). M-R2A medium contains (per liter of distilled water): yeast extract, 0.5 g; peptone, 0.5 g; casamino acids, 0.5 g; glucose, 0.5 g; soluble starch, 0.5 g; sodium pyruvate, 0.3 g; K_2HPO_4, 0.4 g; KH_2PO_4, 0.25 g; KNO_3, 0.505 g; $CaCl_2 \cdot 2H_2O$, 0.015 g; $MgCl_2 \cdot 2H_2O$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.007 g; Na_2SO_4, 0.005 g; NH_4Cl, 0.8 g; $MnCl_2 \cdot 4H_2O$, 5 mg; H_3BO_3, 0.5 mg; $ZnCl_2$, 0.5 mg; $CoCl_2 \cdot 6H_2O$, 0.5 mg; $NiSO_4 \cdot 6H_2O$, 0.5 mg; $CuCl_2 \cdot 2H_2O$, 0.3 mg; $NaMoO_4 \cdot 2H_2O$, 0.01 mg; and agar, 15 g. Adjust the pH to 7.0 before autoclaving.

For cultivation on poorly water-soluble compounds such as alkylbenzenes, special procedures are required (Rabus and Widdel, 1995).

Identification

Since members of the genus *Azoarcus* are quite heterogeneous with respect to ecology and physiology, the description of the genus is complex. The phenotypic characteristics of the genus *Azoarcus* are as follows: Cells are straight to slightly curved rods. Cell pairs often appear slightly S-shaped, with the exception of *A. buckelii* cells, which occur as cocci. Cell width is 0.4–1.5 μm and length 1.1–4.0 μm. In many species, some elongated cells (8–12 μm) occur in late-log or stationary-phase cultures on medium containing malic acid and molecular nitrogen (N_2) or ammonia as nitrogen source. Cells accumulate polyhydroxybutyrate granules and are Gram negative. Cells are highly motile by means of one, rarely two polar flagella. Many species fix nitrogen and then require microaerobic conditions for growth on N_2. On semisolid nitrogen-free media, microaerophilic growth can be observed as veil-like pellicles developing several mm below the surface and moving to the medium surface during growth. Colony morphology differs in plant-associated and soil-borne species. *Azoarcus communis*, *A. indigens* and *Azoarcus* sp. BH72 grow well on complex media such as trypticase soy agar (TSA) or VM medium containing beef extract or peptone, yeast extract, and ethanol (colony diameter 2–4 mm after 4 d); on the latter medium, they produce a yellowish, nondiffusible pigment. In contrast, the soil-borne species *A. tolulyticus*, *A. evansii*, *A. buckelii*, *A. toluvorans* and *A. toluclasticus* grow poorly or not at all on complex media, and some may show different pigmentation. *Azoarcus anaerobius* is an exception since it is strictly anaerobic and therefore does not develop colonies on aerobically incubated agar plates. Optimum temperature for growth is 28–40°C, and no growth occurs at 45°C (Fig. 2). The soil-borne species are chemoorganoheterotrophic. Bacteria have a strictly respiratory metabolism with O_2 as the terminal electron acceptor, except one species (*A. anaerobius* which is strictly anaerobic). Alternatively, under anaerobic conditions, nitrate can be used for dissimilatory nitrate reduction. Nitrate can be also assimilated. Bacteria are oxidase positive. They grow well on salts of organic acids (such as L-malate, succinate, fumarate and DL-lactate), ethanol and L-glutamate, but not on mono- or disaccharides except for species that are not plant-associated. These soil-borne species use few carbohydrates and a variety of aromatic substrates as sole carbon sources under denitrifying conditions. Growth factor requirement varies: some strains depend on *p*-aminobenzoic acid or on cobalamin. All investigated species have 16:1, and all except one *cis*-9 16:1 and 18:1 as major cellular fatty acids. The mol% G+C of the DNA is 62–68 (Tm). The type species is *Azoarcus indigens*.

Since the genera *Azovibrio*, *Azospira* and *Azonexus* were formerly included in *Azoarcus* sensu lato (Reinhold-Hurek and Hurek, 2000) and are physiologically very similar, some characteristics distinguishing these genera from each other are given in Table 1. Genera harboring plant-associated diazotrophs and *Thauera* were also included in Table 1. The genus *Thauera* (Macy et al., 1993; Anders et al., 1995), which is phylogenetically closely related, is physiologically similar to soil-borne species and thus difficult to distinguish by classical tests.

The validly described species of *Azoarcus* are *A. indigens* (type species) and *A. communis* (Reinhold-Hurek et al., 1993), *A. tolulyticus* (Zhou et al., 1995), *A. toluclasticus* and *A. toluvorans* (Song et al., 1999), *A. evansii* (Anders et al., 1995), *A. anaerobius* (Springer et al., 1998) and *A. buckelii* (Mechichi et al., 2002). These species can be distinguished from each other

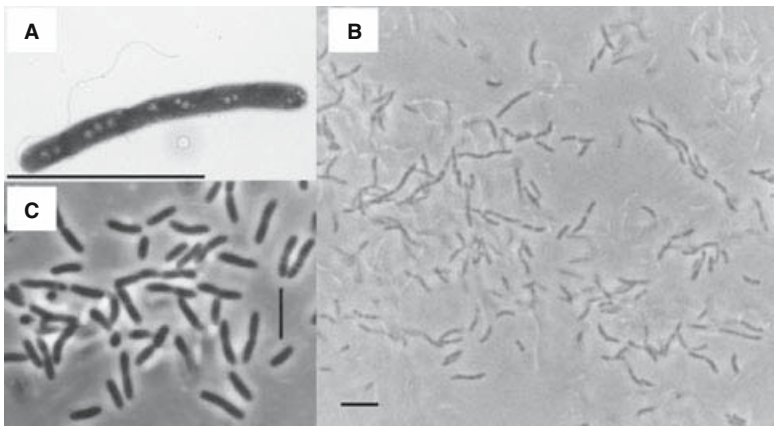

Fig. 2. Microcopic images of *Azoarcus* spp. Transmission electron (A) and phase contrast (B, C) images. A) *Azoarcus* sp. strain BH72 cultured in liquid VM medium for 24 h. B) *Azoarcus indigens* $VB32^T$ cultured on N_2 in semisolid SM medium for 24 h. C) *Azoarcus tolulyticus* BL2 grown on nutrient plates for 48 h. Bars, 5 μm. A) and B) are from Reinhold-Hurek et al. (1993) and C) is from Song et al. (1999).

Table 1. Differential characteristics of the genera *Azoarcus*, *Azovibrio*, *Azospira*, *Azonexus* and physiologically similar diazotrophs or related bacteria of the Proteobacteria.

Character	*Azoarcus*	*Azovibrio*	*Azospira*	*Azonexus*	*Herbaspi-rillum*	*Burkholderia vietnamiensis*	*Azospirillum*	*Gluconacetobacter diazotrophicus*	*Thauera*
Subclass	β	β	β	β	β	β	α	α	β
Cells curved	d[a]	+	+	+	+	–	+	–	–
Cell width (μm)	0.4–1.0[b]	0.6–0.8	0.4–0.6	0.6–0.8	0.6–0.7	0.3–0.8	0.8–1.4	0.7–0.9	0.7–1.0
Colony color	Yellowish to beige[c]	Beige	Pinkish translucent	Ocher	Cream	Cream	Pinkish opaque	Brown	White-yellowish[c]
Fermentative	–	–	–	–	–	–	d	+	–[d]
Growth on sugars	d[a]	–	–	–	+	+	+	+	–[e]
Requirement for cobalamine	–	–	–	+	–	–	–	–	–
Growth in presence of O_2 on									
n-Butylamine	+[f]	–	–	–	d	–	ND	ND	ND
4-Hydroxybenzoate	+[g]	–	–	–	+	+	ND	ND	+
Phenylacetate	+[h]	–	–	–	–	+	–	ND	+
Glutarate	+[i]	–	–	–	–	+	ND	ND	+[j]
2-Oxoglutarate	+[k]	–	+	+	+	d	d	ND	ND
n-Caproate	d	–	+	–	–	+	ND	ND	d
Proprionate	d	+	+	–	+	+	+	ND	+[d]
D-Mannose	–[l]	–	–	–	+	+	d	ND	ND
L-Proline	d	–	–	+	+	+	+	+	+[d]

Symbols and abbreviations: +, positive for all strains; –, negative for all strains; d, 11–89% of strains are positive; and ND, not determined.
[a]Positive for soil-borne species, negative for plant-associated species.
[b]Except for *A. anaerobius* and *A. buckelii* (1.5 μm).
[c]Several strains do not grow on nutrient agar.
[d]Tested for *T. aromatica.*
[e]Except for *T. selenatis*.
[f]ND for *A. toluvorans*, *A. toluclasticus*, *A. anaerobius*, *A. buckelii*.
[g]Negative for *A. evansii*.
[h]Negative for *A. toluclasticus*, d for *A. tolulyticus*.
[i]Negative for *A. indigens*, ND for *A. toluvorans*, *A. toluclasticus*, *A. anaerobius*.
[j]ND for *T. mechernichensis*.
[k]Negative for *A. tolulyticus*, ND for *A. toluvorans*, *A. toluclasticus*, *A. anaerobius*, *A. buckelii*.
[l]ND for *A. buckelii*.

by phenotypic characteristics given in Table 2. Strain BH72 is included in Table 2 and may represent a species of its own according to DNA-DNA-similarity studies (Reinhold-Hurek et al., 1993). It has to be noted, however, that in some cases characteristics have been tested for one strain only (see also Table 3). A more complete list of the characteristics of these species is given in Table 3. Further comments on the species are given in the text below.

Azoarcus indigens is the type species and can be differentiated from the others by its requirement for *p*-aminobenzoic acid, growth on itaconate, and a combination of characteristics given in Table 2. Cells are thin (0.5–0.7 μm wide) and curved, and cell pairs look slightly S-shaped. Colonies are very compact and difficult to disperse; growth in liquid media is clumpy; aggregation is very strong on peptone media. Optimum temperature for growth is 40°C. The major fatty acids are 3-OH-10:0, 12:0, *cis*-9 16:1, *cyclo*-17:0, and 18:1. *Azoarcus indigens* was isolated from roots and stem bases of *Leptochloa fusca* (L.) Kunth from the Punjab of Pakistan (Reinhold-Hurek et al., 1993), and black sclerotia of an *Ustilago*-related basidiomycete from rice soil in the Punjab of Pakistan (Hurek et al., 1997b) and from rice roots (*Oryza sativa*) from Nepal (Engelhard et al., 2000). The type strain is strain VB32 (=LMG 9092), which has a G+C content of 62.4 mol% (T_m). Accession number of the 16S rDNA sequence is AF011345 (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=AF011345).

Azoarcus anaerobius can be distinguished from others by its strictly anaerobic life style with nitrate as the only electron acceptor. Nitrate is quantitatively reduced to N_2 gas, nitrite accumulates intermediately, and nitrous oxide (N_2O) is not detected. Additional differences are given in Table 2. Optimum growth temperature is 28°C. Enhanced salt concentration impairs growth. Cells are superoxide-dismutase-positive. Sulfate, thiosulfate, sulfite, sulfur, trimethyl-amine *N*-oxide, dimethyl sulfoxide (DMSO), $Fe(OH)_3$, $K_3[Fe(CN)_6]$, or fumarate are not reduced, and oxygen is not reduced, not even at low pressures. Characteristics in addition to those given in Table 3 are: Sole carbon sources for growth are propanol, valerate, pyruvate, cyclohexane carboxylate, phenol, resorcinol, *p*-cresol; no growth with L-malate, formate, 5-oxocaproate, pimelate, catechol, hydroquinone, 2-hydroxybenzoate, *o*-cresol, and *m*-cresol. No autotrophic growth with hydrogen or thiosulfate. The pH range is 6.5–8.2 and optimum pH 7.2. Cells were isolated from sewage sludge. The species consists of only one strain, which is the type strain LuFRes1 (= DSM 12081). It has a G+C content of 66.5 ± 0.5 mol% (T_m). Accession number of the 16S rDNA sequence is Y14701 (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=Y14701).

Azoarcus buckelii can be distinguished from others by its cell shape and a combination of characteristics given in Table 2. Cells are coccoid of irregular size (1.5 ± 0.5 μm). Optimum temperature for growth is 28°C with a range from 4 to 40°C. It requires vitamin B_{12} for growth. Also nitrite can be used as terminal electron acceptor. Under oxic conditions, methanol and pyruvate are utilized in addition to carbon sources listed in Table 3. No growth occurs on formate, 2-aminobenzoate, catechol, pimelate, hexadecane, *o*-cresol, *p*-cresol, cyclohexane carboxylate, and phenol. Under denitrifying conditions, they utilize benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 2-aminobenzoate, *o*-phtalate, protocatechuate, phenol, *o*-cresol, *p*-cresol, cyclohexane carboxylate, 4-hydroxyphenylacetate, heptanoate, 2-hydroxybenzaldehyde, 3-hydroxybenzaldehyde, 4-hydroxybenzaldehyde, 2-hydroxybenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 2,4-dimethylphenol, 4-hydroxy-3-methylbenzoate, or for slow growth, 3-methylbenzoate, 2-fluorobenzoate, gentisate, phenylacetate, phenylpropionate, L-tyrosine, L-phenylalanine, and indole 3-acetate; no growth occurs on *m*-cresol, 3-aminobenzoate, 4-aminobenzoate, 2-methylbenzoate, 4-methylbenzoate, catechol, resorcinol, hydrochinone, cinnamate, *p*-anisate, L-tryptophan, indole, ethylbenzene, benzene, 3-chlorobenzoate, adipate, pimelate, D-glucose, D-fructose, D-ribose, lactose, cyclohexanol, cyclohexanone, 1,3-cyclohexanedione, D,L-mandelate, D,L-4-hydroxymandelate, and 2-hydroxybenzyl alcohol. The species consists of only one strain, which is the type strain U120 (= DSMZ 14744). It has a G+C content of 66 mol%. Accession number of the 16S rDNA sequence is AJ315676 (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=AJ315676).

Azoarcus communis can be distinguished from others by its cell width (0.8–1.0 μm) in combination with growth on D-mandelate, by growth on citrate, and a combination of characteristics given in Table 2. Cells are only slightly curved and plump. Optimum temperature for growth is 37°C. Some strains still grow well in 2% NaCl. The major fatty acids are 3-OH-10:0, 14:0, *cis*-9 16:1, 16:0, and 18:1. This species was isolated from roots of *L. fusca* (L.) Kunth from the Punjab of Pakistan ($SWub3^T$; Reinhold-Hurek et al., 1993; Engelhard et al., 2000) and from refinery oily sludge in France (Laguerre et al., 1987). The species affiliation of strain LU1 isolated from a compost biofilter in Canada (Juteau et al., 1999)

Table 2. Characteristics differentiating species of the genus *Azoarcus* from each other.

Characteristic	*A. indigens*	*A. communis*	*A. sp.* BH72	*A. tolulyticus*	*A. toluvorans*	*A. toluclasticus*	*A. evansii*	*A. anaerobius*	*A. buckelii*
Cell width (μm)	0.5–0.7	0.8–1.0	0.6–0.8	0.8–1	0.8–1.0	0.6–0.8	0.4–0.8	1.5	1.5
O_2 terminal electron acceptor	+[a]	+	+	+	+	+	+	−	+
Catalase	+	+	+	d	+	−	+	−	+
Nitrogen fixation	+	+	+	+	ND	ND	−	−	−
Requirement for *p*-aminobenzoic acid	+	−	−	−	−	−	−	−	−
Sole carbon sources for growth									
3-Hydroxybenzoate	+	+	+	d	−	−	+	+	+
Phenylacetate	+	+	+	d	+	−	d	+	±
L-Phenylalanine	+	+	−	d	±	d	+	+	+
D-Tartrate	+	d	+	−	−	−	+	ND	ND
n-Caproate	−	+	+	d	−	−	−	ND	−
Isovalerate	−	+	+	+	±	−	+	+	ND
L-Proline, D(+)-galactose, sucrose	−	−	−	+	+	+	+	ND	ND
Maltose	−	−	−	+	+	+	d	ND	ND
D(+)-Glucose	−	−	−	+	−	d	d	−	−
D(+)-Fructose	−	−	−	+	−	d	+	−	−
D(−)-Ribose, adipate	−	−	−	+	+	+	−	ND	ND
Itaconate	+	−	−	−	ND	ND	−	ND	ND
p-Aminobenzoate	+	−	−	−	ND	ND	+	ND	ND
D-Mandelate	+	+	−	−	ND	ND	−	ND	ND
Citrate	−	+	−	−	ND	ND	−	ND	−
L(+)-Arabinose, D(+)-xylose	−	−	−	+	+	+	−	−	ND

Symbols and abbreviations: +, positive for all strains; −, negative for all strains; ±, weak reaction for all strains; d, 11–89% of strains are positive; and ND, not determined.

Table 3. Characteristics of species of the genera *Azoarcus*, *Azovibrio*, *Azospira* and *Azonexus*.[a]

Characteristic	*Azoarcus indigens* (n = 5)	*Azoarcus communis* (n = 3)	*Azoarcus sp.* BH72 (n = 1)	*Azoarcus tolulyticus*[b] (n = 9)	*Azoarcus toluvorans* (n = 2)	*Azoarcus toluclasticus* (n = 5)	*Azoarcus evansii*[b] (n = 2)	*Azoarcus buckelii* (n = 1)	*Azoarcus anaerobius* (n = 1)	*Azovibrio restrictus* (n = 5)	*Azovibrio sp.* BS20-3 (n = 1)	*Azospira oryzae* (n = 12)	*Azonexus fungiphilus* (n = 5)
Cell width (μm)	0.5–0.7	0.8–1.0	0.6–0.8	0.8–1[b]	0.8–1.0	0.6–0.8	0.4–0.8	1.5 ± 0.5	1.5	0.6–0.8	0.6–0.8	0.4–0.6	0.6–0.8
Cell length (μm)	2.0–4.0	1.5–3.0	1.5–4.0	1.4–2.8	1.4–2.8	1.7–4.0	1.5–3.0	1.5 ± 0.5	2.7–3.3	1.5–3.5	1.5–3.5	1.1–2.5	1.5–4.0
Elongated cells in stationary cultures	r	r	r	–	ND	ND	–	–	ND	r	ND	r	+
Colony diameter (mm, VM/CR)[c]	2–3	2–4	2–3/1	1–1.5[b]	ND	ND	0.2–0.7[b]	ND	0	1.5–2/1	Negligible	1–2	1–2/0.7
Colony color (VM)[c]	Opaque yellowish	Translucent yellowish	Translucent yellowish	Opaque yellowish[b]	ND	ND	Translucent beige[b]	ND	–	Opaque beige	Negligible	Translucent pink-salmon	Opaque ocher
Colony color (CR)[c]	Whiteish pink white margin	Whiteish pink pink center	Orange red	Orange red[b]	ND	ND	Orange red[b]	ND	–	Orange red	Negligible	Translucent orange	Dark red
Colony surface	Rough	Smooth	Shining	Shining	ND	ND	Shining	ND	–	Shining	ND	Shining	Shining
Growth at 40°C	+	+	+	–[b]	–	–	–[b]	+	–	d	ND	+	+
Requirement for *p*-aminobenzoic acid	+	–	–	–	–	–	–	–	–	–	–	–	–
Requirement for cobalamin	–	d	–	–	–	–	–	+	–	–	+	d	+
Nitrogen fixation	+	+	+	+	ND	ND	+	–	–	+	+	+	+
Oxidation/fermentation of glucose	–	–	–	ND	ND	ND	+[d]	ND	–	–	–	–	–
Catalase	+	+	+	D	+	–	+	+	–	+	ND	+	+
Sole carbon sources for growth[g]													
n-Butylamine	+	+	+	+[b]	ND	ND	+[b]	ND	ND	–	–	–	–
3-Hydroxybenzoate	+	+	+	d	–	–	+[b]	+	+	–	–	–	–
Benzylamine	d	v	+	+[b]	ND	ND	+[b]	ND	ND	–	–	–	–
Phenylacetate	+	+	+	d	+	–	d	±	+	–	–	–	–
Benzoate	d	d	+	+	+	+	+	+	+	–	–	–	–
L-Phenylalanine	+	+	–	d	±	d	+	+	+	–	–	–	–

Table 3. *Continued*

Characteristic	*Azoarcus indigens* (n = 5)	*Azoarcus communis* (n = 3)	*Azoarcus sp.* BH72 (n = 1)	*Azoarcus tolulyticus*[b] (n = 9)	*Azoarcus toluvorans* (n = 2)	*Azoarcus toluclasticus* (n = 5)	*Azoarcus evansii*[b] (n = 2)	*Azoarcus buckelii* (n = 1)	*Azoarcus anaerobius* (n = 1)	*Azovibrio restrictus* (n = 5)	*Azovibrio sp.* BS20-3 (n = 1)	*Azospira oryzae* (n = 12)	*Azonexus fungiphilus* (n = 5)
D(+) Malate	+	+	+	+[b]	ND	ND	–[b]	ND	–	D	–	–	–
L-Alanine	d	+	+	+[b]	ND	ND	+[b]	ND	ND	D	+	–	–
2-Oxoglutarate	+	+	+	–[b]	ND	ND	+[b]	ND	ND	–	–	+	+
D-Tartrate	+	d	+	–	–	–	+	ND	ND	–	–	+	–
n-Caproate	–	+	+	d	–	–	–[b]	+[f]	ND	–	–	+	–
Propionate	+	+	+	d	–	–	+	ND	+	+	+	+	–
Isovalerate	–	+	+	+	±	–	+[b]	ND	+	–	+	+	–
L-Proline	–	–	–	+[b]	+	+	+	ND	ND	–	–	–	+
Malonate	–	–	–	–	ND	ND	–	ND	ND	–	–	–	d
Maltose	–	–	–	+	+	+	d	ND	ND	–	–	–	–
D(+)-Glucose	–	–	–	+	–	d	d	–	–	–	–	–	–
D(+)-Fructose	–	–	–	+	–	d	+[b]	–	–	–	–	–	–
D(+)-Galactose, sucrose	–	–	–	+	+	+	+[b]	ND	ND	–	–	–	–
D(–)-Ribose	–	–	–	+	+	+	–[b]	ND	ND	–	–	–	–
Maltotriose, palatinose, D(+)-melezitose	–	–	–	+	ND	ND	+[b]	ND	ND	–	–	–	–
DL-Glycerate	d	–	–	+[b]	ND	ND	+[b]	ND	ND	–	–	–	–
Toluene (denitrifying)	–	–	–	+	+	d	ND	ND	ND	–	–	–	–
Protocatechuate, L-mandelate	+	+	+	–[b]	ND	ND	–[b]	ND	ND	–	–	–	–
4-Hydroxybenzoate	+	+	+	+[f]	+[f]	+[f]	–	±	+[f]	–[b]	–	–	–
m-Coumarate	d	+	+	–[b]	ND	ND	–[b]	ND	ND	–	–	–	–
Tryptamine	+	v	+	–[b]	ND	ND	–[b]	ND	ND	–	–	–	–
Gentisate	+	+	+	–[b]	ND	ND	+	–	ND	–	–	–	–
L(+)-Tartrate	–	–	–	–	–	–	+[b]	ND	ND	–	–	–	–
meso-Tartrate, betaine[b]	–	–	–	–	ND	ND	+[b]	ND	ND	–	–	–	–
p-Aminobenzoate	+	–	–	–[b]	ND	ND	+	ND	ND	–	–	–	–
Itaconate	+	–	–	–[b]	ND	ND	–[b]	ND	ND	–	–	–	–
Glutarate	–	+	+	+[b]	ND	ND	+[b]	+	ND	–	–	–	–
D-Mandelate	+	+	–	–[b]	ND	ND	–[b]	ND	ND	–	–	–	–

Citrate	–	+	–	–	ND	ND	–	–	ND	–	–	–	–
Glycerol, D-alanine	–	d	–	–	ND	ND	–	+[h]	ND	–	–	–	–
(–)-Quinate	–	d	–	+[b]	ND	ND	–	ND	ND	–	–	–	–
Isobutyrate	–	+	+	+[b]	ND	ND	+[b]	ND	ND	d	+	v	–
L-Aspartate	+	v	+	+[b]	+	+	+	±	ND	+	–	+	+
3-Hydroxybutyrate	+	+	+	+[b]	ND	ND	+[b]	ND	ND	–	+	+	+
L-Malate	+	+	+	+	ND	ND	+	+	–	+	+	+	+
Fumarate	+	+	+	+	+	d	+	ND	+	+	+	+	+
Adipate	–	–	–	+	+	+	–	ND	ND	–	–	–	–
L-Arginine	–	–	–	–	+	+	–	ND	ND	–	–	–	–
L-Tyrosine	–	–	–	–	–	–	–	ND	+	–	–	–	–
L(+) Arabinose, D(+)-xylose	–	–	–	+	+	+	–	ND	–	–	–	–	–
∀-Lactose	–	–	–	+	d	+	–	ND	ND	–	–	–	–

Symbols and abbreviations: +, positive for all strains; –, negative for all strains; ±, weak reaction for all strains; d, 11–89% of strains are positive; r, rarely; v, variable result; and ND, not determined.

[a]All strains have the following features: cells are straight to curved rods except *A. buckelii*; oxidase positive; no growth in the presence of 5% NaCl and no growth-rate increase when NaCl is added to medium (ND for *A. toluvorans*, *A. toluclasticus*, *A. anaerobius* and *A. buckelii*); denitrification (ND for *Azonexus* sp.); no spore formation; no starch hydrolysis (ND for *A. evansii*, *A. buckelii* and *A. anaerobius*).

[b]Marked characters were determined for *A. tolulyticus* strain Td-1 or *A. evansii* strain KB740^T (Reinhold-Hurek and Hurek, 2000).

[c]Growth on VM ethanol agar (VM) or Congo red agar (CR) at 37°C for 4 d.

[d]Not tested for strain pF6.

[e]All strains grow on DL-lactate, succinate, acetate, L-glutamate, butyrate (ND for *A. buckelii*), ethanol (ND for *A. toluvorans* and *A. toluclasticus*; no substrate but L-malate tested for *A. evansii* pF6); no growth on (ND for *A. buckelii*) D(+)-mannose, maltitol, *N*-acetyl-D-glucosamine, D-gluconate, and caproate; no growth on[b] (ND for *A. toluclasticus*, *A. toluvorans*, *A. anaerobius* and *A. buckelii*) D(+)-trehalose, L(+)-sorbose, ∀-D(+)-melibiose, D(+)-raffinose, lactulose, 1-0-methyl-β-galactopyranoside, 1-0-methyl-∀-galactopyranoside, D(+) cellobiose, β-gentiobiose, 1-0-methyl-β-D-glucopyranoside, ∀-L-rhamnose, ∀-L-fucose, D(+)-arabitol, L(–)-arabitol, xylitol, dulcitol, D-tagatose, *myo*-inositol, D-sorbitol, adonitol, hydroxyquinoline-β-glucuronide, D-lyxose, erythritol, 1-0-methyl-∀-D-glucopyranoside, 3-0-methyl-glucopyranose, D-saccharate, mucate, D-glucuronate, D-galacturonate, 2-keto-D-gluconate, 5-keto-D-gluconate, 3-phenylpropionate, caprylate, 4-aminobutyrate, DL-∀-amino-*n*-valerate, trigonelline, putrescine, ethanolamine, and D-glucosamine.

[f]Under denitrifying conditions.

[g]Tested with O_2 as terminal electron acceptor.

[h]Only glycerol tested.

From Reinhold-Hurek et al. (1993), Anders et al. (1995), Hurek and Reinhold-Hurek (1995a), Zhou et al. (1995), Hurek et al. (1997), Rhee et al. (1997), Springer et al. (1998), Song et al. (1999), Engelhard et al. (2000), Reinhold-Hurek and Hurek (2000), and Mechichi et al. (2002).

is currently not clear. The type strain is strain SWub3 (= LMG 9095), which has a G+C content of 62.4 mol% (T_m). Accession number of the 16S rDNA sequence is AF011343 (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=AF011343).

Azoarcus evansii can be distinguished from other species by a combination of characters given in Table 2. Cells are rods with rounded ends and motile by means of a subpolarly inserted flagellum. Yeast extract (0.1% [w/v]) inhibits growth. Optimum growth temperature is 35–37°C, and optimum pH is 7.8. During denitrifying growth with aromatic compounds, nitrite is an intermediate and is reduced mainly to N_2O (strain KB740^T). Characteristics in addition to those listed in Table 3 are: Under anaerobic conditions, strain KB740^T uses benzoate, phenylacetate, phenylglyoxylate, 3- and 4-hydroxybenzoate, 2-aminobenzoate, 4-hydroxyphenylacetate, L-phenylalanine, *p*-cresol, 2-fluorobenzoate, benzaldehyde, benzyl alcohol, indolylacetate, *o*-phtalate, adipate, pimelate, cyclohexane carboxylate, succinate, fumarate, L-malate, acetate, acetone, D-fructose, D-maltose, and slow growth occurs on glutarate, D-glucose, but not on toluene, phenol, 2-hydroxybenzoate, protocatechuate, *o*- and *m*-cresol, indole, ethanol, D-ribose, or D-lactose. Pyridine is used by strain pF6 under aerobic and anaerobic conditions. The major cellular fatty acids are *cis*-9 16:1, 16:0, 12:0, 3-OH 10:0, and *cis*-9, 11 18:1. The major quinone is ubiquinone 8. The species description is based on one strain isolated from creek sediment in the United States (Braun and Gibson, 1984); however, a second strain, pF6, sharing 100% 16S rDNA sequence identity was isolated on pyridine from industrial wastewater in Korea (Rhee et al., 1997). The type strain is strain KB740 (= DSM6898), which has a G+C content of 67.5 mol%. Accession number of the 16S rDNA sequence is X77679 (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=X77679).

Azoarcus toluclasticus can be differentiated from the other species by the lack of catalase activity despite aerobic growth and by a combination of characters given in Table 2. Cells are short motile rods. Optimum temperature for growth is 30°C. Under denitrifying conditions, they can grow on acetate, benzoate, pyruvate, succinate, D-xylose, L-arabinose, D-ribose, D-galactose, sucrose, lactose, maltose, adipate, lactate, mannitol, L-aspartate, L-proline or L-arginine. All strains except strain MF23 can use toluene as a growth substrate and strains MF58 and MF63^T can also grow on phenol under denitrifying conditions. The strains grow on brain-heart infusion, nutrient and trypticase soy agar (TSA), except for strain MF63^T, which does not grow on nutrient agar. The predominant cellular fatty acids are 16:0 and 16:1ω7*c*. DNA-DNA hybridizations show intermediate similarities (47–55%) among genomovar I (strains MF7 and MF23) and genomovar II (strains MF58, MF63^T and MF441). They were isolated from aquifer sediments in the United States. The type strain is strain MF63 (= ATCC 700605), which has a G+C content of 67.3 mol% (HPLC). Accession number of the 16S rDNA sequence is AF123077 (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=AF123077).

Azoarcus tolulyticus can be differentiated from the other species by a combination of characters given in Table 2. Cells are short motile rods, which are slightly elongated (to 2.8 μm) when grown on M-R2A agar. Optimum temperature for growth is 30°C, and growth still occurs at 37°C. Under denitrifying conditions they can utilize acetate, adipate, L-arginine, L-arabinose, L-aspartate, benzoate, fumarate, D-galactose, D-glucose, lactate, lactose, maltose, mannitol, L-proline, pyruvate, D-ribose, succinate, sucrose, toluene, D-xylose, or 4-hydroxybenzoate. The strains grow on brain-heart infusion agar, but not or only poorly on nutrient and TSA. The predominant cellular fatty acids are 16:0 and 16:1ω7*c*. Strains Td-19 and Td-3 (Zhou et al., 1995) have been removed from the species owing to low DNA-DNA similarity values (Song et al., 1999). The source of strains is aquifer sediments and petroleum-contaminated soils in the United States (Fries et al., 1994; Fries et al., 1997; Chee-Sanford et al., 1996). The type strain is strain Tol-4 (= ATCC 51758), which has a G+C content of 66.9 mol% (HPLC). Accession number of the 16S rDNA sequence is L33694 (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=L33694).

Azoarcus toluvorans can be differentiated from the other species by a combination of characters given in Table 2. Optimum temperature for growth is 30°C. Grow on brain-heart infusion, nutrient and TSA. Under denitrifying conditions, they can grow on acetate, benzoate, butyrate, fumarate, phenylacetate, pyruvate, succinate, toluene, D-xylose, L-arabinose, D-ribose, D-galactose, sucrose, maltose, adipate, lactate, mannitol, L-aspartate, L-proline, L-phenylalanine, L-arginine, 4-hydroxybenzoate or phenol, and under aerobic conditions, on benzene or ethylbenzene. They were isolated from soil from an industrial area in Brazil (Td17) or noncontaminated organic soil in the United States (Td21^T; Fries et al., 1994). The type strain is strain Td21 (= ATCC 700604), which has a G+C content of 67.8 mol% (HPLC). Accession number of the 16S rDNA sequence is L33692 (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=L33692).

Azoarcus sp. strain BH72 can be differentiated from the other species by its lack of growth on L-phenylalanine and a combination of characteristics given in Table 2. Cells are long, thin (0.6–0.8 μm wide) and slightly curved, elongated (8–12 μm) cells occurring in late-log or stationary phase culture on N-free or ammonium-supplemented SM-medium. Optimum temperature for growth is 40°C. The major fatty acids are16:0, *cis*-9 16:1, 18:1, 18:1, and 14:0. It was isolated from the root interior of *Leptochloa fusca* (L.) Kunth from the Punjab of Pakistan (Reinhold et al., 1986). The G+C content is 67.6 mol% (T_m). Accession number of the 16S rDNA sequence is AF011344 (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=AF011344).

Preservation

Although organic acids are typical carbon sources for many strains, medium containing organic acids becomes alkaline in the stationary phase. Thus, subculturing on organic acids should be avoided and VM ethanol medium should be used instead. Soil-borne strains may also be subcultured on M-R2A medium. Preservation by lyophilization is possible. Long-term preservation is achieved by storage in liquid nitrogen with 5% DMSO as a cryoprotectant.

Physiology

The optimum growth temperature is 30°C or 37–40°C, which is lower for *A. anaerobius* (28°C). Optimum pH is neutral, and the bacteria are not halophilic. All *Azoarcus* species have a strictly respiratory type of metabolism, and no fermentative abilities have been observed. All species except one, *A. anaerobius*, use oxygen as the terminal electron acceptor. In the original description of *Azoarcus* spp., standard procedures had failed to reveal the capacity for denitrification (Reinhold-Hurek et al., 1993). In a closer examination, the capacity to use nitrate as the terminal electron acceptor was demonstrated for these plant-associated strains (Hurek and Reinhold-Hurek, 1995a), a feature which they share with all other species (Anders et al., 1995; Zhou et al., 1995; Springer et al., 1998; Song et al., 1999). Most strains belonging to *A. tolulyticus*, *A. evansii*, *A. toluclasticus*, *A. toluvorans* and *A. anaerobius* were enriched or isolated anaerobically on nitrate, whereas the plant-associated species were enriched under conditions of nitrogen fixation.

In the original description of the genus *Azoarcus*, the capacity to fix nitrogen is listed as a genus character (Reinhold-Hurek et al., 1993). Nitrogen fixation (Fries et al., 1994; Zhou et al., 1995) or the occurrence of a nitrogenase gene *nifH* (Hurek et al., 1997a) has also been demonstrated for *A. tolulyticus*. However, owing to the addition of new species, this cannot be generalized for *Azoarcus* anymore. Nitrogen fixation was not detected in *A. anaerobius* (Springer et al., 1998) or *A. buckelii* (Mechichi et al., 2002). In the absence of physiological data (Anders et al., 1995), *A. evansii* was thought unlikely to be diazotrophic since no polymerase chain reaction (PCR)-amplifiable *nifH* fragment (T. Hurek and B. Reinhold-Hurek, unpublished observations) or hybridization with a *nifH* probe (Hurek et al., 1997a) occurs. However, recently this species was reported to fix nitrogen (Mechichi et al., 2002). For most other isolates enriched under denitrifying conditions, this character was not tested at all. In all diazotrophic strains (*A. indigens*, *A. communis*, *A. tolulyticus* and *Azoarcus* sp. strain BH72), nitrogenase activity occurs only under microaerobic conditions, probably due to the lack of efficient oxygen protection mechanisms. It was shown that nitrogen fixation is more tolerant to oxygen in strain BH72 than in *Azospirillum* spp., reaching steady states in an oxygen-controlled chemostat until dissolved O_2 exceeds 25 μM (Hurek et al., 1987). Similarly, the expression of nitrogenase genes is transcriptionally regulated in response to oxygen (fully repressed at 4% oxygen in the headspace) or combined nitrogen (repressed by 0.5 mM ammonium or nitrate; Egener et al., 1999). This strain shows augmented rates and efficiency of nitrogen fixation, called "hyperinduction," in empirically optimized batch cultures when shifting down to extremely low oxygen concentrations (30 nM; Hurek et al., 1994a). In the course of hyperinduction, novel intracytoplasmic membrane stacks are formed which might participate in efficient nitrogenase activity, since the iron protein of nitrogenase is mainly associated with these membranes (Hurek and Reinhold-Hurek, 1995a). The formation of these so-called "diazosomes" is most abundant and reproducible in coculture of strain BH72 and endophytic *Acremonium alternatum*, a mitosporic deuteromycete related to the Hypocreales within the euascomycetes and an isolate from Kallar grass (Hurek et al., 1995b; Hurek and Reinhold-Hurek, 1998). As in many Proteobacteria, nitrogenase genes are organized in an *nifHDK* operon (Egener et al., 2001). Phylogenetically, the nitrogenase in the genus *Azoarcus* follows either the organismic phylogenetic tree or appears to have been obtained by lateral gene transfer, depending on the species. In *A. indigens*, *A. communis* and *Azoarcus* sp. strain BH72, a fragment of the iron protein of nitrogenase encoded by the *nifH* gene is most closely related to nitrogenases occurring in diazotrophs of the γ-subclass of Proteobacte-

ria, while in *A. tolulyticus*, it is located within a clade of Alphaproteobacterial nitrogenases (*Bradyrhizobium* and *Azospirillum*; Hurek et al., 1997a).

In general, carbohydrates are not the preferred carbon sources of *Azoarcus* spp. None of the plant-associated species is able to utilize any out of 50 mono- and disaccharides or sugar alcohols tested (Reinhold-Hurek et al., 1993). *Azozrcus anaerobius* shows no growth on common carbohydrates such as D(+)-glucose or D(–)-fructose (Springer et al., 1998). In contrast, among the soil-borne species, all strains tested so far are able to utilize at least some carbohydrates (Table 1). All strains grow well on organic acids and a few amino acids (Table 1). The carbon sources listed here (except for *A. anaerobius*) were tested with O_2 as the terminal electron acceptor. For some strains, tests were also carried out under anaerobic conditions with nitrate as electron acceptor. For the majority of carbon sources, results were identical; however, for several carbon sources, strain-dependent differences were found (Song et al., 1999). In contrast to plant-associated species (Reinhold-Hurek et al., 1993; Hurek and Reinhold-Hurek, 1995a), most soil-borne species grow on aromatic compounds (such as toluene or phenol) under denitrifying conditions (Zhou et al., 1995; Song et al., 1999), benzoate (Anders et al., 1995), or resorcinol (Springer et al., 1998). Owing to the anaerobic degradation of aromatic compounds, this bacterial group receives particular attention for their biodegradation and biotransformation abilities. While aerobic metabolism is characterized by the extensive use of molecular oxygen (which is essential for the hydroxylation and cleavage of the ring structures), different strategies are necessary for the anaerobic degradation and are currently under study. Toluene, which can be decomposed anaerobically by three species, is activated by the addition to fumarate to form benzylsuccinate (Beller and Spormann, 1997) catalyzed by benzylsuccinate synthase via a glycyl radical (Krieger et al., 2001).

The aerobic metabolism of benzoate is unusual in *A. evansii* KB740^T. None of the known pathways, including the conversion of benzoate to catechol (1,2-dihydroxybenzoene) or protocatechuate (3,4-dihydroxybenzoate), appears to operate in this species (Mohamed et al., 2001). The first step is the activation of benzoate to benzoyl-CoA by a benzoate-CoA ligase, and the second step involves the hydroxylation of benzoyl-CoA by a novel benzoyl-CoA oxygenase (Mohamed et al., 2001). The first step of phenylacetate degradation is catalyzed by two different phenylacetate-CoA ligases under aerobic and anaerobic conditions, respectively (Mohamed, 2000). Strain PF6 degrades pyridine aerobically and anaerobically with nitrate as electron acceptor (Rhee et al., 1997). Benzoyl-CoA is also a central intermediate in the anaerobic degradation of aromatic compounds (Harwood et al., 1998). In *A. evansii* KB740, nucleotide sequence analysis of the gene cluster including a gene for benzoate-CoA ligase indicates that the degradation of benzoate is probably similar to the benzoate-CoA pathway in *Thauera aromatica* (Harwood et al., 1998). For further reading on anaerobic oxidation of aromatic compounds, the review of Boll et al. (2002) is recommended.

Resistance of *Azoarus* spp. to antibiotics has not been extensively tested. *Azoarcus indigens*, *A. communis* and strain BH72 are not resistant to ampicillin, kanamycin, streptomycin, spectinomycin or tetracycline (B. Reinhold-Hurek and T. Hurek, unpublished observation).

Genetics

For the three plant-associated species of *Azoarcus*, DNA reassociation experiments led to the estimation of a genome size from 4.5 to 5 Mb. The genome of *Azoarcus* sp. strain BH72 is currently sequenced (Hurek and Reinhold-Hurek, 2003), with an estimated genome size of 4.4 Mb.

Nothing is known about bacteriophages of the genus *Azoarcus*. Also the plasmid content is not well studied. In strain BH72, plasmids could not be detected in Eckhardt gels or by pulse field gel electrophoresis (B. Reinhold-Hurek and T. Hurek, unpublished observations). Several strains of *Azoarcus* spp. have been shown to be transformable. Broad host range plasmids based on RK2 such as pRK290 or pAFR3 can be transferred by triparental mating and are stably replicated in strain BH72 (Egener et al., 2001). Transformation can also be achieved by electroporation (Hurek et al., 1995b), and mutagenesis by allelic exchange (Hurek et al., 1995b) or transposon insertion (Dörr et al., 1998) is established.

Ecology

As already stated in the section on Habitat, the two phylogenetic clusters of *Azoarcus* strains appear to differ substantially in their ecology: the plant-associated and the soil-borne species. Among the plant-associated species (*A. indigens*, *A. communis* and strain BH72), most strains (except strain S2 from sludge [Laguerre et al., 1987] or strain LU1 from a compost biofilter [Juteau et al., 1999], both related to *A. communis* [Fig. 1]) appear to be strictly plant-associated and have never been isolated from root-free soil (Reinhold-Hurek and Hurek, 1998b). In contrast, no members of soil-borne species have been isolated from plant material as yet.

Plant-associated *Azoarcus* spp. have been up to now isolated from a limited number of samples, such as Kallar grass (*Leptochloa fusca* L. Kunth) from the Punjab of Pakistan (Reinhold et al., 1986; Reinhold-Hurek et al., 1993), rice roots (*Oryza* spp.) from Nepal (Engelhard et al., 2000), or resting stages of plant-associated fungi (Hurek et al., 1997b). However they may be more widely distributed than assumed; in molecular-ecological studies on root material or fungal spores, *Azoarcus* 16S rDNA genes (Hurek and Reinhold-Hurek, 1995a) or *nifH* genes (Ueda et al., 1995; Engelhard et al., 2000; Hurek et al., 2002) have been retrieved which did not correspond to genes of cultivated strains or species. Interestingly, the sequences retrieved up to now from plant material did not cluster with genes from soil-borne species, confirming that the latter do not appear to be plant associated. Since bacteria corresponding to the detected sequences could not be isolated from the same plant samples, they may occur in a state in which they are difficult to cultivate and thus overlooked by classical microbiological techniques. This is in agreement with the observation that in an inoculation experiment of Kallar grass with strain BH72, already two months after inoculation, attempts to reisolate the bacteria failed; however, nitrogenase gene (*nifH*) mRNA of this species was present at high levels in plant roots, suggesting high metabolic activity (Hurek et al., 2002). Using *nif*$^-$ and wild type BH72, it was demonstrated by N-balance and $^{15}N_2/^{14}N_2$ isotope studies that *Azoarcus* sp. can supply fixed nitrogen to its host plant Kallar grass (Hurek et al., 2002), an important observation with respect to the ecological role and possible applications of these bacteria.

Since plant-associated *Azoarcus* spp. cannot be isolated from root-free soil, the question arises how do they newly infect plants in nature. Survival in the absence of plants may be achieved in association with fungal resting stages in the soil (Hurek). Black sclerotia of a basidiomycete (a relative of *Ustilago hordei*) collected from a Kallar grass site in Pakistan harbored culturable strains of *Azoarcus indigens* and of the related genera *Azovibrio* spp., *Azospira oryzae* and *Azonexus fungiphilus* (Hurek). This suggests that plant-associated fungi might serve as shuttle vectors for plant-associated bacterial strains. Indeed, when these sclerotia were used to inoculate surface-sterilized rice seedlings in gnotobiotic culture, diazotrophs could be isolated from roots after 2–3 weeks of incubation. The abundant diazotrophs were identified as *Azospira oryzae* (B. Reinhold-Hurek, unpublished results).

Mechanisms of plant-microbe-interactions are best studied for *Azoarcus* sp. strain BH72, originating from the pioneer plant Kallar grass from Pakistan (Reinhold et al., 1986). In gnotobiotic cultures in the laboratory, these bacteria have a wider host range. They can invade rice roots and to a lesser extent stems (Hurek et al., 1994b), where they mainly colonize the root cortex tissue intercellularly and rarely the stele including xylem cells (Hurek et al., 1994b). Despite a high density of colonization of the root interior, the bacteria do not cause symptoms of plant disease (Hurek et al., 1994b) and thus have an endophytic and not a pathogenic lifestyle. Unlike rhizobia, they do not form an endosymbiosis in living plant cells (Reinhold-Hurek and Hurek, 1998a). Nevertheless they show endophytic nitrogen fixation, expressing nitrogenase genes in the apoplast of aerenchymatic air spaces of flooded rice seedlings (Egener et al., 1999) or field-grown Kallar grass plants (Hurek et al., 1997a).

Soil-borne species occur in a wide range of environments as stated in the section on Habitat. In most cases they were readily isolated under denitrifying conditions from nonmarine habitats such as contaminated or noncontaminated soils or sediments. Since many strains can degrade a wide range of aromatic compounds—which are the second most abundant class of organic compounds in nature—they can be expected to play an important role in carbon degradation in the biosphere. Their occurrence in anoxic sediments or sewage sludge indicates that their lifestyle in situ might be anaerobic (on nitrate as terminal electron acceptor), in contrast to the plant-associated strains that might be fixing nitrogen microaerobically. Besides the numerous isolates, there are also strains which have as yet escaped cultivation. One example is a deeply-branching clade of *Azoarcus* sp. found in high numbers in nitrifying-denitrifying activated sludge of an industrial wastewater treatment plant (Juretschko et al., 2002).

The Genera *Azovibrio*, *Azospira* and *Azonexus*

Introduction

Bacteria of these genera were first described to belong to the genus *Azoarcus* sensu lato (Reinhold-Hurek et al., 1993; Hurek et al., 1997b), to group C (*Azovibrio*), group D (*Azospira*) and group E (*Azonexus*). The availability of new isolates recently allowed a reassessment of the genus by a polyphasic taxonomic approach, leading to the proposal of three new genera. Since they are relatively similar in physiology and ecology, they are treated together.

Recently, perchlorate-reducing bacteria were described as a new genus (*Dechlorosoma suillum*; Achenbach et al., 2001); however, a polyphasic taxonomic analysis by us (Tan and Reinhold-Hurek, 2003) demonstrated that they are members of *Azospira oryzae*. Therefore, these bacteria are also included in this chapter.

Phylogeny

Analysis of almost complete 16S rDNA sequences placed all three genera into the Betaproteobacteria. Phylogenetic analyses using different algorithms showed that *Azoarcus* sensu lato was not monophyletic, but *Azoarcus* sensu stricto was divided from the other groups by the *Thauera* clade (Reinhold-Hurek and Hurek, 2000; see Fig. 1). The three new genera clearly represent different lineages, despite their exact phylogenetic relationships within the *Rhodocyclus* subgroup not being well resolved. Their phylogenetic distances from each other are similar to that of the *Thauera*/*Azoarcus* group or *Duganella* (formerly Zoogloea; Fig. 1). Other related genera are *Dechloromonas*, *Propionivibrio* and *Rhodocyclus*, and none are typically plant-associated bacteria.

Taxonomy

The three groups are divided from each other and to *Azoarcus* by a polyphasic taxonomic analysis including 16S rDNA sequence analysis, DNA-DNA hybridization, protein profiles, fatty acid analysis, and nutritional profiles. Each genus consists of only one validly described species: *Azovibrio restrictus*, *Azospira oryzae* and *Azonexus fungiphilus* (Reinhold-Hurek and Hurek, 2000). The members of each species are quite homogeneous in protein patterns and substrate utilization, except for *Azovibrio* sp. strain BS20-3. It shares only 97% 16S rDNA sequence identity with *Azovibrio restrictus* S5b2^T, which is strictly microaerobic and might thus deserve the rank of a separate species in future.

Azovibrio now harbors new members recently described as a misnamed genus "*Dechlorosoma suillum*" (Achenbach et al., 2001). The type strain shares almost 100% of 16S rDNA sequence identity (1433 out of 1435 bp) with *Azospira oryzae* 6a3, is very similar in sodium dodecylsulfate (SDS)-soluble protein patterns and carbon source profiles, and shows approx. 90% binding in DNA-DNA-hybridization studies; thus the type strain of *Dechlorosoma suillum* and *Azospira oryzae* have to be regarded as the same species (Tan and Reinhold-Hurek, 2003).

Habitat and Ecology

Bacteria of all three genera occur in terrestrial habitats. *Azovibrio* and *Azospira* strains have typically been isolated from surface-sterilized plant roots and are thus considered to be diazotrophic endophytes of grasses.

Members of both genera were found in roots of Kallar grass (*Leptochloa fusca* (L.) Kunth) in the Punjab of Pakistan (Reinhold-Hurek et al., 1993) and in roots of wild rice and cultured rice *Oryza sativa* in Nepal (Engelhard et al., 2000). *Azospira oryzae* also was found in roots of cultured rice in Italy (Engelhard et al., 2000) and wild rice in the Philippines (Hurek et al., 2000; Reinhold-Hurek and Hurek, 2000). Some strains of both genera and *Azonexus fungiphilus* were additionally found in another microniche, the resting stages (sclerotia) of a basidiomycete related to *Ustilago hordei* (Hurek et al., 1997b); they were collected in rice field soil at the site in the Punjab of Pakistan, where formerly Kallar grass had been grown. The finding that culturable bacteria appear to persist in fungal resting stages but not in soil suggests that plant-associated fungi may play a role as shuttle vectors for reinfection of plants by these diazotrophic endophytes. However, *Azonexus fungiphilus* was thought to be exclusively fungus-associated (Reinhold-Hurek and Hurek, 2000) but was recently cultured from surface-sterilized roots of wild rice, as well (B. Reinhold-Hurek and Z. Y. Tan, unpublished observations).

A new group of *Azospira oryzae* (Tan and Reinhold-Hurek, 2003), which was misnamed "*Dechlorosoma suillum*" has an entirely different habitat. It was isolated from a primary treatment lagoon of swine waste in the United States (Achenbach et al., 2001).

Isolation

Since all known strains are diazotrophic, enrichment and isolation are done on nitrogen-free media. The procedures are the same as described for plant-associated *Azoarcus* spp.

Identification

Azovibrio, *Azonexus* and *Azospira* are quite similar in physiological features but differ especially in cell morphology. Characters differentiating them from *Azoarcus* and other related genera are given in Table 1. More detailed phenotypic features are listed in Table 3. All three genera are diazotrophic. They do not use carbohydrates as sole carbon sources, but they do use a few organic and amino acids. Additional features are given below.

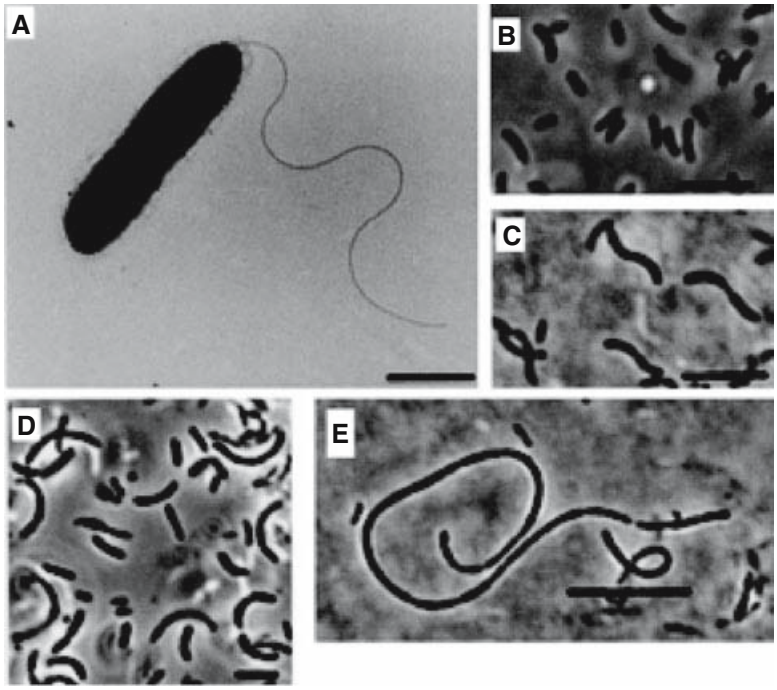

Fig. 3. Microscopic images of cells of the genera *Azovibrio*, *Azospira* and *Azonexus*. Transmission electron (A) and phase contrast (B-E) images of *Azovibrio* restrictus S5b2^T (A, B), *Azospira oryzae* 6a3^T (C), and *Azonexus fungiphilus* BS5-8^T (D, E). Cells were grown on N_2 in semisolid medium and photos taken during exponential (B), stationary (E) and late stationary (C, D) growth phase. Bars, 1 µm (A), 5 µm (B-D) or 10 µm (E). From Reinhold-Hurek and Hurek (2000).

Azovibrio cells are motile, slightly curved rods of 0.6–0.8 × 1.5–3.6 µm (Fig. 3), and elongated cells occur very rarely in stationary phase cultures. Cells grow well at 37°C. The major cellular fatty acids are *cis*-9-16:1, 16:0, 18:1, 14:0 and 3-OH-10:1. They have no dissimilatory nitrate reductase. The only validly described species is *A. restrictus*; however, strain BS20-3 (isolated from black sclerotia) might represent another species. In contrast to *A. restrictus*, BS20-3 requires cobalamin; it is also unusual because it is strictly microaerobic with negligible colony growth (Reinhold-Hurek and Hurek, 2000). The type strain is *A. restrictus* S5b2 (= LMG9099) which has a G+C content of 64.8 mol% and was isolated from surface-sterilized roots of Kallar grass. Accession number of the 16S rDNA sequence is AF011349 (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=AF011349).

Azospira cells are thin curved rods of 0.4–0.6 × 1.1–2.5 µm (Fig. 3), and elongated cells with one to several spiral windings of up to 8 µm occur rarely in late-stationary phase nitrogen-fixing cultures. Cells are highly motile with corkscrew-like motion. Growth is optimum at 37°C or 40°C. The major cellular fatty acids are *cis*-9-16:1, 16:0, 18:1 and 3-OH-10:1. They have no dissimilatory nitrate reductase. Plant-associated strains can use oxygen and nitrate as terminal electron acceptors, while strains originating from wastewater (formerly [*Dechlorosoma suillum*]) can also use perchlorate. The only validly described species is *Azospira oryzae*. The type strain is strain 6a3 (= LMG9096) which has a G+C content of 65.2 mol% and was isolated from surface-sterilized roots of Kallar grass. Accession number of the 16S rDNA sequence is AF011347 (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=AF011347).

Azonexus cells are highly motile, slightly curved rods of 0.6–0.8 × 1.5–4.0 µm; elongated straight to coiled cells of up to 50 µm length occur in the stationary phase of nitrogen-fixing cultures (Fig. 3). Cells grow well at 37°C and require cobalamin for growth. The only validly described species is *Azonexus fungiphilus*. The type strain is strain BS5-8 (= LMG 19178), which was isolated from resting stages (black sclerotia) of a basidiomycete related to *Ustilago* found in a rice field in Pakistan. Accession number of the 16S rDNA sequence is AF011350 (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide=search=AF011350).

Preservation

Preservation and storage are carried out as for plant-associated species of *Azoarcus*.

Physiology

All three genera (even the perchlorate-reducing isolates from swine wastewater) are diazotrophic, showing microaerophilic nitrogen fixation (Tan and Reinhold-Hurek, 2003). As the plant-associated *Azoarcus* strains, cells require microaerobic conditions for nitrogen fixation

and form subsurface pellicles due to microaerophilic growth in semisolid media. The optimum growth temperature is 37–40°C. Optimum pH is neutral, and the bacteria are not halophilic. All genera have a strictly respiratory type of metabolism, and no fermentative abilities have been observed. All genera use oxygen or nitrate as the terminal electron acceptor, and some strains of *Azospira oryzae* also use perchlorate. All are oxidase and catalase positive. Similarly to plant-associated *Azoarcus* species, organic acids, amino acids, and ethanol but no carbohydrates are utilized.

Genetics

Extrachromosomal genetic elements have not been intensively studied in these genera. Transposon mutagenesis has been established for *Azonexus fungiphilus* (Gemmer and B. Reinhold-Hurek, unpublished), while *Azospira oryza* appears to be difficult to transform.

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