# Section III

## Biotechnology

# 11 Microbial Genomics as an Integrated Tool for Developing Biosensors for Toxic Trace Elements in the Environment

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## 11.1 INTRODUCTION

The process of the origin of life in the course of a long irreversible development of natural systems belongs to the great mysteries of nature. At present, we are only approaching its solution. There can be no doubt that the emergence and organization of living matter are connected with the properties of the atoms entering into its composition — primarily, with the properties of carbon. Thus, what brought about the origin of life consisted of those particular processes in space evolution of the substance of the solar system or, more precisely, in the nuclear synthesis (nucleosynthesis) that preceded the appearance of this system.

This nuclear synthesis led to the formation of the atoms of biophile elements H, C, N, and O in a relationship that proved to be favorable for the formation of complex organic compounds — the direct predecessors of life. When the primary gaseous nebula that was genetically associated with the early Sun was cooling, organic compounds appeared. They emerged mainly in the last stages of congelation, which was registered in the space rocks of the further periods: meteorites and particularly the carbonaceous chondrites. Thus, all the data on cosmochemistry of meteorites, asteroids, and comets prove that the formation of organic compounds in the solar system in the early stages of its development was a typical and mass phenomenon [1].

The biosphere represents a highly complicated organization of matter in which various inorganic forms of movement are related to living substances. Any form of life is connected with a particular environment; thus, the problem of the origin of life may well be regarded as precisely the problem of the emergence of the biosphere. It is not difficult to infer that the living organisms use, in the first place, the most accessible atoms, which are furthermore capable of forming stable and multiple chemical bonds.

It is common knowledge that carbon can give rise to the emergence of long chains, leading to the origin of innumerable polymers. Sulphur and phosphorus can also form multiple bonds. Sulphur enters into the composition of proteins, and phosphorus forms a constituent part of nucleic acids. The metabolism with the outer environment had become *a sine qua non* for the existence of every single organism. Presumably, the whole biosphere of this planet should be regarded as the part of its matter that undergoes the process of metabolism with the living matter. Any form of living organism consists of combinations of a few chemical elements (e.g., H, C, N, and O). The other

elements enter into the composition of living organisms in relatively insignificant quantities, in spite of the exclusively important role that some of them have in performing physiological functions [1]. One may well think that all the elements of Mendeleev's table enter into the composition of the living matter of this planet, though in different quantities.

The simplest and earliest organized living forms, the bacteria, possess a selective ability of making use of the chemical elements from the environment according to their physiological needs. As with nutrient organic compounds, whatever was available in the environment provided selection for transport and metabolism. Most cell types found economy in accumulating trace elements for immediate and future needs through utilizing relatively unspecific uptake systems for a wide range of metal ions. Unlike carbon compounds, however, some inorganic nutrients at low levels are toxic when present at higher levels ($Cu^{2+}$, $Co^{2+}$, and $Ni^{2+}$, for example); other inorganic ions (e.g., $Hg^{2+}$ and $AsO_2^-$) are always toxic and have no metabolic functions in bacteria [2].

With the emergence of intelligence, the highest form in the process of evolution from inanimate matter to human brain, the question that remains the most intriguing is the origin of life. Whatever complexity the process is, nature must drive to organize the system to evolve from simple molecules to multicellular organisms. Thus, the fundamental units in so many complex and diverse life systems seen today are the atoms of only little more than 100 elements. On the other hand, because every cell retains its history, all the ancestral evidences must be preserved in the living body. Thus, the present day molecules are able to guide understanding of the present biological phenomenon at the molecular level, as well as serve as the museum of all the prehistoric evolutionary processes. A biologist thus must find the answer to the fundamental question: selectivity of an essential metal atom (ion) encrypted in the life system, within the molecules.

This review hypothesizes that the toxicity potential of heavy metal ions has forced life in its early evolution to develop metal ion homoeostasis factors or metal resistance determinants. The amazing process of evolving resistance to heavy metal ions is not a result of successful accumulation of mistakes in replication of the genetic code, but is rather the outcome of designed creative processes. In the present context, it is apparent that the metabolic penalty for having uptake pumps more specific is greater than the genetic cost of having plasmid genes, which confers resistance to heavy metal ions, in the population that can spread when needed [2].

The new picture of creative cooperative evolution is based on the cybernetic capacity of the genome as suggested by Ben–Jacob [3]. This new picture of the genome as an adaptive cybernetic unit with self-awareness changed the neo-Darwinian paradigm in the life sciences because it has been shown that the genome is a dynamic entity capable of changing itself [4–7]. In this scenario, genome includes the chromosomes, all the extrachromosomal elements, and all the "chemical machinery" (like enzymes) involved in genomic activity and production of proteins. Every genome that has been sequenced to date has provided new insight into biological processes, activities, and potential of these species that was not evident before the availability of the genome sequence.

In the light of current perceptions of metabolic networks that have cropped up in the age of genomics is the one that microbial cells are not merely an assemblage of information but are constituted of atoms. The transporter determinants of genome as well as the extra cellular environment determine the atoms in a microbial cell. Therefore, if one wishes to understand how the cell is directed by the genome, one must know what atoms are present in the surroundings of the cell and how the cells react to complex mixtures of the chemical elements and compounds [8]. Genomics-based reconstruction of the cellular machinery, microbial remediation of complex environmental mixtures, and knowledge about microbial individuality have added dimension to the constellation of elements found in microbes [8]. Genome sequencing of prokaryotes of diverse origin occupying a wide range of environmental niches has resulted in shift of the primary focus of microbial genomics from the pathogen genomes to other genomes bearing potential for environmental applications.

Accessibility of bioinformatics tools enabled successful comparisons of different genomes to identify metabolic pathways and the analysis of transporter profiles across various species. For

example, the genomic sequence of *Pseudomonas putida* KT2440 was used to survey the organism's possible mechanisms of uptake and resistance to and homoeostasis regulation of several metals and metalloids [9]. In the present context, it is relevant to cite that many cell types synthesize metal-binding proteins in response to the presence of specific metals and play important roles in metal homoeostasis and detoxification mechanisms. Due to a wide spectrum of selectivity, metal-binding proteins could be used as the basis of biosensors of varying specificity, depending on the required application.

Some microorganisms have developed genes for heavy metal resistance, which tend to be specific towards a particular metal instead of a general mechanism for all heavy metals. These systems can be used as the contaminant-sensing component of the biosensor by detecting the metal for which it is designed to detoxify or excrete. The contaminant-sensing component can find a route of biotechnology in combination with reporter genes to create biosensors that can identify toxic metals at very low levels.

## 11.2 GENESIS AND CHEMISTRY OF TOXIC TRACE METALS RELEVANT TO THEIR INTERACTION WITH LIFE PROCESSES

Elements were formed in stars billions of years ago for the metals on Earth. Stars mainly burn hydrogen to helium, and later helium to carbon with oxygen and nitrogen as intermediates; higher elements are just "accidentally" formed. Thus, the probability for any element to be formed decreases hyperlogarithmically with its atomic number. The heavier any element is, the lower is the probability to find it in the Earth's crust, and the lower is the probability that evolution has used it. The three exceptions to this rule are [1]:

- The element synthesis in the stars stops at iron; thus, iron and its neighbors in the periodic system are present in high amounts on Earth despite their relatively high atomic masses.
- Li, Be, and B, which are not heavy metals, are overstepped during element synthesis; thus, they are present in low amounts on Earth, despite their relatively low atomic masses.
- Some heavy metals with extraordinarily stable nuclei were formed in higher amounts than any other heavy metals.

Metals with a density beyond 5 g/CC are heavy metals. Therefore, the term "heavy metals" is ascribed to the transition elements from V (but not Sc and Ti) to the half metal As; from Zr (but not Y) to Sb; and from La to Po, the lanthanides and the actinides. There are 21 nonmetals, 16 light metals, and 53 heavy metals (including As) constituting the total of 90 naturally occurring elements [10]. The transition elements with incompletely filled d-orbitals are heavy metals. The ability of the heavy metal cations to form complex compounds, which may or may not be redox active, is provided by the d-orbitals.

The origin of life is reduced to that of the biosphere, which from its inception was a complicated self-regulating system. A great variety of geochemical functions of living matter emerged at least as a result of the fact that any most primitive cell, being in aqueous and marine medium, had the closest possible contact with all the chemical elements of Mendeleev's table. Because life depends on water, heavy metals interesting for any living cell must form soluble ions. All the 53 heavy metals do not have a positive or adverse biological function because of the nonavailability of some heavy metals to the living cell in the usual ecosystem [11]. To enable the bioavailability, heavy metals must be present at least in nanomolar concentration in a given ecosystem because a concentration of 1 n*M* means that, in a cell suspension of $10^9$/ml, each cell may receive about 600 ions. Metal ions generally present in lower concentration may be used by a microorganism for very specific purposes; however, the lower the mean concentration of the metal ion is in an ecosystem,

the lower is the probability that any species carries around genes to use or detoxify this specific heavy metal [11].

The living conditions in the world ocean were most favorable, so it is possible that the Earth's hydrosphere was distinguished by the constancy of the biomass throughout the whole period of existence. Weast [10] has differentiated heavy metals into four classes on the basis of their concentration in seawater:

- Frequent possible trace elements with concentrations between 100 n*M* and 1 μ*M* are Fe, Zn, and Mo.
- Possible trace elements with concentration between 10 and 100 n*M* are Ni, Cu, As, N, Mn, Sn, and U.
- Rare possible trace elements would be Co, Ce, Ag, and Sb.
- Cd, Cr, W, Ga, Zr, Th, Hg, and Pb are just below the 1-n*M* level.

Other elements, e.g., Au with 55.8 p*M* in seawater, are not likely to become trace elements.

The relative solubility of heavy metals under physiological conditions dictates the difference in biological importance and the toxicity of heavy metals vis-a-vis affinity to sulfur and other interaction with macrobioelements [11]. Because of the low solubility of the tri- or tetravalent cation [10], Sn, Ce, Ga, Zr, and Th have no biological influence. Of the remaining 17 heavy metals, Fe, Mo, and Mn are important trace elements with low toxicity; Zn, Ni, Cu, V, Co, W, and Cr are toxic elements with high to moderate importance as trace elements; and As, Ag, Sb, Cd, Hg, Pb, and U have no beneficial function, but must be considered by cells as toxins [11].

In addition to playing an important catalytic role by complexing with biochemicals, heavy metal cations in sophisticated biochemical reactions, such as nitrogen fixation; water cleavage during oxygenic photosynthesis; respiration with oxygen or nitrate; one-electron catalysis, rearrangement of C–C bonds; hydrogen assimilation; cleavage of urea; transcription of genes into mRNA, etc., can also form nonspecific complex compounds at higher concentrations in the cells that lead to the toxic effects. The heavy metal cations like $Hg^{2+}$, $Cd^{2+}$, and $Ag^{+}$ are immensely toxic complex formers and are often perilous for any biological function. Even most important trace elements like $Zn^{2+}$ or $Ni^{2+}$ — and especially $Cu^{2+}$ — are toxic at higher concentrations. Therefore, it was compelling to every life form to evolve some homoeostasis system for maintaining a tight control over intracellular concentration of heavy metal ions [11].

## 11.3 PHYSICAL PROPERTIES OF HEAVY METAL CATIONS AND OXYANIONS

The divalent cations $Mn^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ have ionic diameters in a range of 138 to 160 pm [10], a difference of 14%; all are, of course, double positively charged. Oxyanions like chromate, with four tetrahedrally arranged oxygen atoms, double negatively charged, differ mostly in the size of the central ion, so the structure of chromate resembles sulfate. The same is true for arsenate and phosphate. Thus, uptake systems for heavy metal ions must bind those ions tightly if they want to differentiate between a couple of similar ions. However, tight binding costs time and energy.

## 11.4 DUAL STRATEGY ADOPTION BY THE LIVING CELL FOR UPTAKE OF HEAVY METAL IONS AND THEIR COMPARISON

The uptake mechanisms that exist within a cell allow the entry of metal ions, including heavy metal ions that affect toxicity. There are two general uptake systems: the first one is driven by a

**TABLE 11.1**
**Comparison between Two Basic Mechanisms of Uptake of Heavy Metal Ions**

| Nonspecific strategy | Specific strategy |
|---|---|
| Rapid | Slow |
| Constitutively expressed | Inducible; produced by the cell in times of need, starvation, or specific metabolic situation |
| Used by a variety of substrates | High substrate specificity |
| Usually driven only by the chemiosmoticgradient across the cytoplasmic | Uses ATP hydrolysis in many membrane cases as energy sources |
| Not expensive | Expensive |
| Uptake system: | Uptake system: |
| Nn-specific CorA ($Ni^{2+}$, $Co^{2+}$, $Zn^{2+}$, and $Mn^{2+}$ uptake) [bacteria, archaea, and yeast] | P-type ATPases ($Mg^{2+}$) |
| Pit-phosphate system (arsenate) | ABC transporters ($Mn^{2+}$, $Zn^{2+}$, and $Ni^{2+}$) |
| Sulfate uptake system (chromate) | Slow and specific HoxN ($Ni^{2+}$and $Co^{2+}$) |

*Sources*: The table is constructed on the basis of the information assembled from MacDiarmid, C.W. and Gardner, R.C., *J. Biol. Chem*., 273, 1727, 1998; and Tao, T. et al., *J. Bacteriol*., 177, 2654, 1995.

chemiosmotic gradient across the membrane results in an influx of a wider variety of heavy metals, and the second is driven by energy from ATP hydrolysis (Table 11.1).

## 11.5 INCORPORATION OF METALS IN BIOACTIVE MOLECULES IN THE PROCESS OF EVOLUTION

In the modern version of Mendeleev's table, transition metals are in the center and clearly share many attributes. They exist as ($MnO_4^{2-}$, $WO_4^{2-}$ exceptions) charged cations in solution under biological conditions. Many of these ions can have similar ionic radii, so they tend to bind to similar classes of ligands. Indeed, ligating atoms that bind metal in a particular protein are found in four types of amino acid side chains: histidines, cysteine, glutamic, and aspartic acids. Less frequently, tyrosine and methionine coordination is found. Nature generally uses an array of two or five of these coordinating side chains in the majority of metalloenzymes.

Once it gathers these different metals, how does the cell know which metal to put into which enzyme-active site? A traditional view in the field of bioinorganic chemistry has been that metal selectivity is due to very sophisticated chelating properties of the individual apo-proteins. In this scenario, apo-proteins are thought to poise the exact orientation of these side chains to match the precise ionic radius and electronic preference of the functional metal ions, for example, $Zn^{2+}$, and to discriminate against all others, such as $Cu^{2+}$ or $Fe^{2+}$ ions. In some of these cases, very little difference is present in ionic radii. The proteins are thus viewed as highly specific chelating agents with finely tuned kinetic and thermodynamic properties that have been selected through evolution to bind only one type of transition metal ion. In this model, each apo-protein as it is produced in the cell simply selects a metal ion from the cytoplasm. In general, however, the field has moved to the point of thinking of membrane-bound proteins as transporters.

A breakthrough in the process of evolution of bioactive molecules from simple atoms must be the spontaneous association of small molecules to generate big molecules (i.e., polymers such as polypeptides and polynucleotides) that organized and resulted in the emergence of a giant molecule with structure and characteristics distinct from the inanimate matter in terms of its stability, spontaneous development, and awareness of environment. In that simplest form of the primitive life, it is possible that the minimum cellular processes were governed through inorganic catalysts.

In such reactions, the simplest redox reactions of the biological molecules were likely to be mediated and catalyzed by metal ions.

The primitive life processes evolved in Archean water were definitely anoxic and the environment was reducing the metal ions remained in the sparingly soluble state, limiting their concentration. When the reducing environment no longer existed upon exhaustion of readily available hydrogen, oxygen started appearing in the available form in the environment by splitting water by the photosynthetic apparatus. It is argued that metals available at that period at relatively higher concentration were selected by and encrypted in the bioactive molecules to perform the specific catalytic or other functions and thus became essential metals in the life processes. During transition from anoxic to oxic environment, some elements, like Cu, Fe, Ni, Zn, Mo, and Co, began to participate in biological functions.

The cells had to incorporate some of those metals in their life processes to scavenge the increasing oxygen availability. For instance, in catalase, cytochrome oxidase, or dismutase, metals such as copper, iron, or zinc are the cofactors and part of the active site of the enzyme is used in the production of oxygen or in the oxygen defense mechanisms. Among several thousand proteins expressed in a typical bacterium, about 30% are metalloproteins. The general order of prevalence of major transition metals in enzymes in *Escherichia coli* is Zn, Fe, Cu, Mo, Mn, Co, and Ni [8]. Although many of the metals thus became essential in the vital processes, the cell must face a difficult situation in uptake of the required metals only from the environment. The uptake system must be specific and regulated so that the system not only recognizes the specific metal ion but also senses any changes in the concentration of the metal in the surrounding environment. The microbial genome is the information bank and can be credited when needed in response to the signal from the environment.

### 11.5.1 Concepts of Heavy Metal Toxicity, Tolerance, and Resistance

Because nonspecific transporters are expressed constitutively, the cell hyperaccumulates heavy metal ions in the face of high concentration of any heavy metal in the environment. Once inside the cell, heavy metal cations like $Hg^{2+}$, $Cd^{2+}$, and $Ag^{+}$ with high atomic numbers tend to bind to –SH groups. The minimal inhibitory concentration of these metal ions is a function of the complex dissociation constants of the respective sulfides. By binding to SH groups, the metals may inhibit the activity of sensitive enzymes. Other heavy metal cations may interact with physiological ions ($Cd^{2+}$ with $Zn^{2+}$ or $Ca^{2+}$; $Ni^{2+}$ and $Co^{2+}$ with $Fe^{2+}$; $Zn^{2+}$ with $Mg^{2+}$), thereby inhibiting the function of the respective physiological cation.

Heavy metal cations may bind to glutathione; the resulting bis-glutathione complexes tend to react with molecular oxygen to oxidized bis-glutathione GS-SG [17], the metal cation and $H_2O_2$. Because the oxidized bis-glutathione must be reduced again in an NADPH-dependent reaction and the metal cations immediately catch another two glutathione molecules, heavy metal cations cause considerable oxidative stress. Finally, heavy metal oxyanions interfere with the metabolism of the structurally related nonmetal (chromate with sulfate; arsenate with phosphate) and reduction of heavy metal oxyanion leads to the production of radicals, e.g., in the case of chromate. Therefore, the "gate" that was always "open" for $Mg^{2+}$ or phosphate uptake turns out to be responsible for the heavy metal toxicity.

### 11.5.2 Emergence of Heavy Metal Tolerant Mutants: Misfit in Evolutionary Selection

Metal-tolerant mutants may arise out of mutations that affect the expression of the gene for the rapid and nonspecific transporters. In fact, *corA* and *pit* mutants with a tolerant phenotype towards cobalt and arsenate, respectively, have been isolated [18–20]. However, tolerant mutants are less

fit than the wild type in the medium without toxic heavy metal ion and are thus rapidly overgrown by the revertant strain.

### 11.5.3 Evolution of Resistance Mechanism

The "open gate" paradox leads to a new evolutionary picture, where progress is not a result of successful accumulation of mistakes in replication of the genetic code, but is rather the outcome of designed creative processes. Progress happens when organisms are exposed to paradoxical environmental conditions — conflicting external constraints that force the organism to respond in contradicting manners [3].

In an attempt to ensure protection of sensitive cellular components, a cell may develop a metal resistance system. Several factors determine the extent of resistance in a microorganism: the type and number of mechanisms for metal uptake; the role that each metal plays in normal metabolism; and the presence of genes located on plasmids, chromosomes, or transposons that control metal resistance. Summing up all the information on metal resistance in microorganisms, the involvement of six mechanisms has been outlined [21]:

- Metal exclusion by permeability barrier
- Active transport of the metal away from the cell
- Intracellular sequestration of the metal by protein binding
- Extracellular sequestration
- Enzymatic detoxification of the metal to a less toxic form
- Reduction in metal sensitivity of cellular targets

The potential of toxicity of heavy metal has thus forced life in its early evolution to develop metal ion homoeostasis factors or metal resistance determinants [11]. Resistance determinants encode proteins with actions targeted directly against a heavy metal cation. Thus, resistance is different from tolerance: here, a protein with a main function not connected to the toxic heavy metal is changed to circumvent accumulation or action of the toxic metal.

The paradoxes are the gears of creativity, serving as the new principle on which the new paradigm is established. Heavy metal ions cannot be degraded or modified like toxic organic compounds and, paradoxically, cells obligately are required to maintain certain cytoplasmic concentrations of some trace metals in physiology; thus, microorganisms employ a number of mechanisms to establish proper equilibrium, including the uptake, chelation, and extrusion of metals [2]. In addition to the existing genetic mechanisms for heavy metal resistance affecting efflux [13,22], chelating, or conversion to a less toxic oxidation state by reduction, indications or possibilities of the involvement of diverse genes in metal (loid) homeostasis, tolerance, and resistance could be explored through data mining of genome databases [9].

### 11.5.4 Physicochemical Restriction in Detoxification Process vis-à-vis Choice for Getting Rid of Excess Heavy Metal Ions

The redox potential is an important criterion for detoxification by reduction. The redox potential of a given heavy metal should be between the hydrogen–proton couple (0 V) and the oxygen–hydrogen couple (1.229 V) [10], which is the physiological redox range for most aerobic cells. Thus, $Hg^{2+}$ (0.851 V); chromate (1.350 V); arsenate (0.560 V); and $Cu^{2+}$ (0.153 V) may be reduced by the cell, but $Zn^{2+}$ (–0.762 V); $Cd^{2+}$ (–0.4030 V); $Co^{2+}$ (–0.28 V); and $Ni^{2+}$ (–0.257 V) may not [10].

The second constraint arises in the context of scavenging the reduced product. The reduced product should be able to diffuse out of the cell or it might reoxidize itself. In fact, most reduction products are quite insoluble ($Cr^{3+}$) or even more toxic ($AsO_2$) than the educts. Logically, even if the cell decides to detoxify a compound like that by reduction, an efflux system should be present

to push off the reduced products to the environment. Only in case of mercury do reducibility and a low vapor pressure of the metallic reduction product fit together; Hg is thus detoxified by reduction of $Hg^{2+}$ to $Hg^0$ plus diffusional loss of the $Hg^0$. Thus, if the desirability of the reduction fails, the only alternatives left are complexation and efflux.

However, if a fast growing cell adopts complexation, the cost–benefit ratio rules out this strategy. This can be exemplified by assuming an aerobic cell that detoxifies $Cd^{2+}$ by forming CdS. For this, sulfate must be taken up, which costs one ATP; PAPS must be formed (three ATPs) and reduced to sulfite (2 $e^-$ lost, which may yield three ATPs during respiration); and, finally, sulfide (6 $e^-$ = nine ATPs). This amounts up to 16 ATPs for the formation of one sulfide, which complexes one $Cd^{2+}$. If it is not only sulfide but also a thiole-group attached to cysteine or glutathione or derivatives thereof, or even a ribosomally synthesized protein like metallothionin, these costs are immense.

Because reduction is not possible or may not be sensible as a sole mechanism of detoxification, heavy metal ions must be detoxified by efflux, alone or in combination, in any organism growing rapidly in an environment contaminated with high concentrations of heavy metals. Heavy metal metabolism is therefore transport metabolism [11] and the protein families involved in heavy metal transport will be examined.

## 11.6 TOXIC METAL IONS AND MECHANISMS OF RESISTANCE

### 11.6.1 Mercury

With the strongest toxicity, mercury does not have any beneficial function. The major form of mercury in the atmosphere is elemental mercury ($Hg^0$), which is volatile and is oxidized to mercuric ion ($Hg^{2+}$) photochemically. $Hg^{2+}$, the predominant form entering aquatic environments, readily adsorbs to particulate matter. The major microbial reaction observed is the methylation of mercury, yielding methylmercury, $CH_3Hg^+$. Because bacteria confront toxic $Hg^{2+}$ and $CH_3Hg^+$, several methods of detoxifying mercury species exist. An NADPH-linked enzyme called mercuric reductase, which is related to glutathione reductase and other proteins [23], transfers two electrons to $Hg^{2+}$, reducing it to $Hg^0$, which is essentially nontoxic and leaves the cell through passive diffusion [2,24,25].

In Gram-negative bacteria, mercury resistance genes, called *mer* genes, are arranged in an operon and are under control of the regulatory protein MerR, which can function as a repressor and an activator [26–41]. In the absence of $Hg^{2+}$, MerR binds to the operator region of the *mer* operon and prevents transcription of *mer* genes. However, when $Hg^{2+}$ is present, it forms a complex with MerR, which then functions as an activator of transcription of the *mer* operon [42,43]. MerD, the product of *merD*, also plays a regulatory role by preventing an overshot during induction [44].

The mercuric reductase is the product of the *merA* gene, whereas *merP* encodes a periplasmic $Hg^{2+}$-binding protein. This MerP binds $Hg^{2+}$ and transfers it to a membrane protein, MerT, the product of merT, which transports $Hg^{2+}$ into the cell for reduction by mercuric reductase [45,46]. Alternatively or in addition to MerTP, an alternative uptake route exists that involves the MerC protein [47,48]. The product of the reduction, $Hg^0$, which is volatile, escapes from the cell to the environment.

Methylmercury, as stated earlier, is soluble and can be concentrated in the aquatic food chain, primarily in fish, or further methylated by microorganisms to yield the volatile compound called dimethylmercury. Metabolically, methylation of mercury occurs by donation of methyl groups from $CH_3– B_{12}$. Methylation has been observed for arsenic, mercury, tin, lead, selenium, and tellurium [49]. Methylmercury and dimethylmercury bond to proteins and tend to accumulate in animal tissues, especially muscle. Organomercurials, which are always much more toxic than the $Hg^{2+}$ is (e.g., methylmercury is about 100 times more toxic than $Hg^0$ or $Hg^{2+}$), may also be detoxified if the *mer* resistance determinant encodes a MerB organomercurial lyase in addition to the other proteins [35,38–40,50–54]. After cleavage by MerB, MerA reduces the resulting $Hg^{2+}$. The high

toxicity of organomercurials and other methylated and alkylated heavy metal compounds makes it very unlikely that these kinds of chemical modifications of heavy metals are metal resistance mechanisms [11].

### 11.6.2 Lead

Use of *lead* and its toxicity has been well known for a long time [55,56]. It is no transition element, but belongs to the element group IVa, C–Si–Ge–Sn–Pb. In seawater, it is even more rare than mercury [10]. Due to its low solubility, especially, lead phosphate is insoluble with a solubility product of $10^{-54}$; its biologically available concentration is low. Molecular information on lead uptake is not available but Pb-tolerant bacteria have been isolated [57], and a process involving precipitation in intracellular lead phosphate granules in *Staphylococcus* has been reported [58,59].

### 11.6.3 Antimonite

Antimonite enters the *E. coli* cell by the glycerol facilitator, GlpF [60]. It is detoxified by all systems giving resistance to arsenite by efflux [60,61].

### 11.6.4 Cadmium

Uptake of cadmium is understood very little at the molecular level. In *Ralstonia* sp. CH34, cadmium is accumulated by the magnesium system(s) [22]. In contrast to cadmium transport by $Mg^{2+}$ uptake systems in Gram-negative bacteria, the $Mn^{2+}$ uptake system is primarily responsible for cadmium entry into the Gram-positive cells [62–64] and maybe also in *S. cerevisiae* [65].

Resistance to cadmium in bacteria is based on chemiosmotic antiporter efflux systems. These antiporter devices are three-component systems involved in drug or metal efflux and include proteins belonging to the RND (resistance, nodulation, cell division) family of integral membrane proteins. The best known archetype is the *czcCBA* system of *Ralstonia metallidurans*, which confers resistance to Cd, Zn, and Co. The cyanobacterium *Synechococcus* sp. contains metallothionein SmtA [66]. Amplification of the smt metallothionein determinant increases cadmium resistance [67] and deletion of it decreases resistance [68–70]. The SmtB regulator controls the actual metallothionein gene, smtA, which is also a metal-fist repressor [69,71,72]. This repressor also regulates a zinc-transporting P-type ATPase [73]; thus, in cyanobacteria, which are jammed with RND- and P-type transport systems, metal transport may play a more important role in cadmium resistance than the results concerning the metallothionein suggest.

In Gram-negative bacteria, cadmium seems to be detoxified by RND-driven system like Czc, which is mainly a zinc exporter [74,75], and Ncc, which is mainly a nickel exporter [76]. The CzcA protein of *R. metallidurans* is associated with the inner membrane and appears to have 12 transmembrane domains [77]. CzcC belongs to the outer membrane efflux protein (OEP) family that function as an auxiliary element for the export of metals. CzcB has the HylD (RND-associated membrane fusion proteins) domain in which the N-terminus is anchored to the inner membrane. Neither CzcB nor CzcC appears to be necessary for resistance to metals, but they may increase the efficiency and specificity of the main component, CzcA, of the system [77].

In Gram-positive bacteria, the first example for a cadmium-exporting P-type ATPase was the CadA pump from *Staphylococus aureus* [78,79]. This protein was the first member of a subfamily of heavy metal P-type ATPases, and all the copper (including Menke's and Wilson's), lead, and zinc transporters found later are related to this protein. Other cadmium resistances in Gram-positive bacteria were found to be mediated by CadA [65]. Regulator of cadA in most cases is the CadC metal-fist repressor [80–82]. Genomics of *P. putida* KT2440 revealed the presence of two CadA homologues, where putative *cadA2* gene is transcribed divergently under the control of the putative regulator CadR and putative cadA1 is located in the middle of the *czc* region [9]. The translated sequence of cadA2 gene (the putative N-terminal) bears resemblance to the N-terminal fragment

of the P-type ATPase responsible for resistance to Cd and partial resistance to Zn (reported earlier in *P. putida* 06909 [83]) [9].

The mechanism by which an ABC transporter, YCF1p, transports the glutathione-bound cadmium (cadmium–bis-glutathionate complex) to the vacuole in yeast [84,85] may have a commonality in all eukaryotes because the multidrug resistance-associated protein from man can complement a YCF1 mutation with respect to cadmium resistance [86]. Another ABC transporter, HMT1p, is reported to transport cadmium–phytochelatin complexes and also bears resemblance to a transporter detected in *A. thaliana* to the vacuoles [86–90]. Despite reports on involvement of a cation diffusion facilitator, like ZRC1 transporter in cadmium extrusion, and binding of cadmium by metallothioneins, the most predominant cadmium-detoxification method in eukaryotes appears to be the vacuolar transport of glutathione/phytochelatin by ABC transporters [11].

### 11.6.5 Silver

Since time immemorial, silver has been used in medicine as an antimicrobial agent [91]. Even today, silver is used in hospitals as an antiseptic on burned skin and in implanted catheters. It is not considered an essential trace metal because of its toxicity. In nature, silver-resistant bacteria have been evolved [92–101]. Although reduced accumulation of silver by silver-resistant bacteria was observed, binding of silver to other intracellular compounds like $H_2S$ and phosphate also always occurred [11].

The copper-effluxing ATPase CopB from *E. hirae* was found to transport Ag as well as Cu [102]; the $K_m$ for both substrates was identical. Genome analysis of *Pseudomonas putida* revealed that SilP and PacS are the prospective proteins involved in the transport of monovalent cations (Cu and/or Ag). The corresponding genomic segment is located in a large gene cluster probably involved in Ag resistance and/or Cu homoeostasis [9]. The Ag/Cu-related genomic segment could encode a P-type ATPase (putative SilP), a three polypeptide cation/proton antiporter (putative CusCBA), as well as metal-binding proteins (CopAB1); this could all be regulated by a two-component regulatory system (putative CopSR1) [9] resembling the *sil* gene of *Salmonella* plasmid pMG101 isolated from a hospital burn ward [103]. Summarily, silver resistance may be based on RND-driven transenvelope efflux in Gram-negative bacteria; efflux by P-type ATPases in Gram-positive organisms; and additional complexation by intracellular compounds.

### 11.6.6 Molybdenum

In the form of molybdate capable of performing oxyanion catalysis without being as toxic as chromate, molybdenum is an important trace metal. The principal mechanism of molybdate uptake is mediated by an inducible ABC transporter [104–111]. Molybdate in bound to a specific molybdate cofactor [112,113], a pterin-mono- or dinucleotide in most enzymes; the exception is the nitrogenase enzyme where the specific iron-molybdenum cofactor does not involve a pterin, and Mo is bound to homocitrate, sulfur, and a histidine residue [114,115]. Genome analysis of *P. putida* KT2440 revealed another ABC metal transporter system for Mo uptake, modABC, where ModA, ModB, and ModC are putative proteins for periplasmic-binding protein, inner membrane permease, and the ATP-binding protein, respectively [9].

### 11.6.7 Arsenic

Like silver, arsenic is predominantly toxic and devoid of any function as a trace element. Different forms of arsenic appear in any environmental sample, of which a fraction may be available to the biological system and are likely to be variable depending on the physicochemical status of that environmental niche. However, toxicity, solubility, and mobility can vary depending upon which species of arsenic is present, thus affecting the bioavailability of the arsenic contamination. Arsenate enters the bacterial cell via the rapid, nonspecific, and constitutive uptake systems for phosphate.

Mutation of this system leads to tolerance to arsenate. Both anions, phosphate and arsenate, may still be accumulated by the inducible, specific transport system Pst, but this system discriminates 100-fold better between both anions than Pit does [13,20,116,117]. However, tolerant mutant strains are always impaired in their phosphate metabolism and tend to revert readily.

In Gram-negative bacteria, the arsenic-resistance gene remains inactive with the absence of As (III) in the cell due to the binding of the ars operon repressor protein to the promoter region of the gene. The system is activated by As (III) binding to the repressor protein and freeing the promoter region for transcription. The freed promoter region is transcribed to produce various components of the mechanism such as ArsB, an arsenite-translocating protein that serves as a transmembrane efflux channel. This protein functions chemiosomotically, without any energy source, or by ATP hydrolysis when coupled with ArsA, an arsenite-specific ATPase [61,118–121].

The best studied example is the plasmid-encoded arsenic resistance from *E. coli* [122]. The ArsB protein in these systems is able to function alone [123]; therefore, arsenite efflux by the ArsAB complex is energized chemiosomotically and by ATP [124]. ArsA acts as a dimer with four ATP-binding sites, and homologs to this protein have been found in eubacteria, archebacteria, fungi, plants, and animals [121,125,126]. ArsC, the enzyme arsenate reductase, is also transcribed to reduce As(V) to As(III) because As(V) cannot pass through the ArsB/ArsA pump [127,128]. For the resistance determinant in *E. coli*, arsenate reduction by the ArsC protein is coupled to glutathione [129] via glutaredoxin [130,131]. For ArsC from *Staphylococus aureus*, the electron donor is thioredoxin [127].

ArsD is a regulatory protein for additional control over the expression of the system and ArsR is a transcriptional repressor. The mechanism varies slightly in Gram-positive bacteria, which lack ArsA and ArsD. Arsenical resistance is regulated mainly by the ArsR repressor, the first example of a "metal-fist" repressor in which an inducing metal — in this case As (III) — binds to the dimeric protein, thereby preventing binding to the operator region [117,132–137]. Related regulatory proteins regulate a Cd-P-type ATPase in *Staphyloccoccus aureus* and metallothionein synthesis in cyanobacteria.

In other arsenic resistance determinants, which are more expensive to run, an additional regulator exists, ArsD [138]. Although ArsR controls the basal level of expression of the ars operon, ArsD controls maximal expression, and both regulators compete for the same DNA-binding site [139]. Two copies of an *arsRBCH* operon are found in the chromosome of *P. putida* [9]. The *arsH*, originally identified in the *ars* cluster of a Tn*2502* transposon, appears to be necessary for arsenic resistance in *Yersinia enterocolitica* [140]. It has been suggested that ArsH might be a transcriptional activator because a plasmid containing the genes arsRBC without arsH in *Y. enterocolitica* did not cause an increase in arsenic resistance [140].

The protozoon *Leishmania*, in addition to having a P-glycoprotein-related transporter, an ABC-transporter responsible for arsenite resistance [141], also gets rid of the toxicity by conjugating As (III) with glutathione or trypathione let out by the glutathione conjugate transporter [142–146]. Homologous arsenite transporters related to ArsB were found in *S. cerevisiae*, the ARS3p protein [61,147], and also in man [148,149]. Thus, metabolism of arsenic resistance seems to follow the same pattern in all organisms again: uptake by the phosphate system; reduction; efflux by ArsB-related proteins or ABC-transporters; and maybe even additional energizing by ArsA-related ATPases.

### 11.6.8 Zinc

Zinc is an important trace element that is toxic at elevated concentrations. The other reality is that it must be transported into cells against concentration gradients. In the usual housekeeping cellular environment, some constitutively expressed broad ion range uptake systems are capable of satisfying the demand of macronutrients and trace elements of the bacterial cells [150]. Zinc is, however, important in forming complexes (such as Zn-fingers in DNA) and as a component in cellular

enzymes [151–154]. Zinc is rapidly uptaken by $Mg^{2+}$ transport systems, as shown in *Ralstonia* sp. CH34 [22]. Zn-uptake systems/transporters can be categorized into three groups.

The first group comprises CorA transporters found to be present in many bacteria, archaea, and yeast [12,14,15]. The second, narrowly distributed group comprising potential chemiosomotically driven transporters representing MgtE family is present in *Providencia stuartii* and few other Gram-negative and Gram-positive bacteria [155,156]. The third magnesium/zinc transporter is MgtA from *S. typhimurium*, a P-type ATPase that may transport zinc more efficiently than magnesium [156–159]. MgtA is regulated by magnesium starvation [16,160], and zinc may interfere with this process, which is at least partially dependent on the PhoPQ two-component regulatory system.

However, the MgtA P-type ATPase is not the inducible high-specificity uptake system for zinc. A periplasmic zinc-binding protein was found in *Haemophilus influenzae* to be important for zinc uptake [161], and ABC-transporters or the evidence for ABC-transporter involved in zinc uptake were found in *Streptococcus pneumoniae, Streptococcus gordonii,* and *E. coli* [162–164]. Proteins belonging to the Fur family were found to regulate iron/zinc uptake [164]. The first protein, Zur, is probably involved in the regulation of Zn uptake. Zur protein of *E. coli* binds two atoms of Zn per monomer of polypeptide. In *E. coli*, *zur* is located away from the *znuABC* genes, and acts as a repressor when it binds Zn [164]; in *P. putida*, zur is located within the znuACB1 genes [9]. The issue of iron regulons in pseudomonads involving not only the Fur proteins but also dedicated sigma factors, extra activators, and perhaps some small regulatory RNAs, is also related to metal homoeostasis [9].

Uptake of zinc in *S. cerevisiae* is also mediated by ZRT1p high-affinity and ZRT2p low-affinity transporters of the ZIP family having homologous counterpart in *Arabidopsis thaliana* (plants), protozoa, fungi, invertebrates, and vertebrates [165–169]. Two general efflux mechanisms are responsible for bacterial resistance to zinc. The first one is a P-type ATPase efflux system that transports zinc ions across the cytoplasmic membrane by energy from ATP hydrolysis. A chromosomal gene, *zntA*, was isolated from *E. coli* K12 and was found to be responsible for the ATPase that transports zinc and other cations across cell membranes' systems [170,171]. The related zinc transporter in the cyanobacterium *Synechocystis* is ZiaA [73]. In most cases, P-type ATPases mediating cadmium resistance can also efflux zinc.

The other mechanism involved in zinc efflux is an RND-driven transporter system that transports zinc across the cell wall of Gram-negative bacteria and is powered by a proton gradient and not ATP [22,74]. Czc from *Ralstonia* sp. is the first of these systems that has been cloned and sequenced [172,173]. The Czc determinant contains three structural genes coding for the three subunits of the membrane-bound efflux complex CzcCB2A [174,175]. The concept of "transenvelope transport" [176–180] has been recently developed as a model to describe common themes in certain efflux systems. The organization of the *czc*-like systems is generally conserved in all organisms studied, although regulatory proteins may be located in a variety of orientations with respect to the core *czcCBA* genes. CzcA is responsible for the translocation of metals across the plasma membrane; CzcCB would avoid their release into the periplasmic space and export them out of the cell.

Regulation of Czc is highly complicated and requires about six additional proteins [74,150,181]. Two or three genes of regulators, *czcN*, and Orf69 and *czcI*, are located upstream of the *czcCBA* structural genes. Transcription is initiated from three different promoters: *czcNp, czcIp*, and *czcCp*; possible RNA processing together with transcription initiation delivers an unstable *czcN*-, a *czcI*-, and a large *czcCBA*-message with a variety of rare large mRNAs such as *czcICBA* and *czcNICBA*. Downstream of *czcCBA*, three more regulator genes exist and are transcribed together as a *czcDRS* message. CzcRS forms a two-component regulatory system, which is not essential for Czc regulation. This system acts specifically on *czcNp* and seems to increase the sensitivity of the main Czc-regulator, probably an unknown sigma factor of the ECF (extracellular functions) sigma-70 factor family. The complexity of Czc regulation indicates the importance of this efflux system for metal homosostasis in *A. eutrophus*.

CzcD, a model cation diffusion facilitator (CDF) zinc transporter protein [13,182], finds its relative in *S. cerevisiae*, the CDF transporter, ZRC1 and COT1p, mediating resistance to zinc and cobalt [183,184]. ZRC1p is also involved in regulation of the glutathione level [185] and COT1p, which is basically a cobalt transporter, may substitute from ZRC1p [186,187]. Because COT1p transports its substrate across a mitochondrial membrane, both proteins could be involved in heavy metal metabolism of the yeast mitochondria. Thus, ZRC1p and COT1p might function in the efflux of surplus cations from the mitochondria [11]. Similar enough is the existence of four closely related mammalian CDF proteins: ZnT1, ZnT2, ZnT3, and ZnT4; these are responsible for Zn-efflux across the cytoplasmic membrane; Zn-transport into lysosomes; Zn-transport into synaptic vesicles; and Zn-secretion into milk, respectively [188–191].

### 11.6.9 Copper

Because copper is used by all living entities in cellular enzymes like cytochrome c oxidase, it is an important trace element. However, due to its radical character, copper is also very toxic and, because it is so widely used in mining, industry, and agriculture, high levels of copper may exist in some environments. Copper toxicity is based on the production of hydroperoxide radicals [192] and on interaction with the cell membrane [193]. As such, bacteria have evolved several types of mechanisms to resist toxicity due to high copper concentrations. Copper metabolism has been studied in *E. coli*; some species related to *Pseudomonas*; the Gram-positive bacterium *Enterococus hirae*; and in *S. cerevisiae*, which sheds some light on the copper metabolism of higher organisms [194–199].

The mechanism of a plasmid-encoded copper resistance [200] in *E. coli* is based on an efflux mechanism. The efflux proteins are expressed by plasmid-bound *pco* genes (structural genes, *pcoABCDE*; and the regulatory genes *pcoR* and *pcoS*), which in turn depend on the expression of chromosomal *cut* genes. Two cut genes, *cutC* and *cutF*, were shown to encode a copper-binding protein and an outer membrane lipoprotein [201–206]. In *Pseudomonas syringae*, resistance to copper via accumulation and compartmentalization in the periplasm and outer membrane is due to four proteins encoded on the plasmid-borne *cop* operon.

The two periplasmic blue copper proteins are CopA and CopC, and the inner and the outer membrane proteins are CopD and CopB, respectively. However, a mutant *cop* operon containing *copD* but lacking one or more of the other genes conferred hypersensitivity and hyperaccumulation of cellular copper, indicating a role for CopD in copper uptake by the cell [196,197,207–209]. As in *E. coli*, copper resistance in *Pseudomonas* is regulated by a two-component regulatory system composed of a membrane-bound histidine kinase and a soluble response regulator, which is phosphorylated by the kinase and switches on transcription of the *cop* genes [210,211].

The copper transport and resistance system in the Gram-positive bacterium *Enterococcus hirae* is the best understood system. The two genes, copA and copB, determining uptake and efflux P-type ATPases, respectively, are found in a single operon. Although CopA is probably responsible for copper uptake and copper nutrition, the 35% identical CopB is responsible for copper efflux and detoxification [167,212]. Copper and silver induce the system and they seem to transport silver besides copper [213]; obviously, the monovalent cations are being transported [102]. The first two genes in the cop operon, *copY* and *copZ*, determining a repressor and activator, respectively, constitute the regulatory protein pair [214,215].

P-type ATPases also seem to control copper flow in two pathogens: *Helicobacter pylori* [216] and *Listeria monocytogenes* [217]. Copper-transporting P-type ATPases have been found in cyanobacteria [218,219] and in yeast. The copper P-type ATPase does not transport copper across the cytoplasmic membrane. In yeast cells, the iron/copper-specific reductases FRE1p and FRE2p reduce Cu (II) to Cu (I) [220,221], which is transported into the cell by the CTR1p transporter [221–223]. A functional homologue of a novel protein in yeast with two related possible copper transporters (CTR2p, CTR3p) [167] is found in man [224]. Additionally, Cu (II) is accumulated by the CorA-related transporters ALR1p and ALR2p [221,223].

The metallothioneins of yeast, CUP1p and CRS5p, are probably copper-storage devices in yeast [225]. COX1p delivers copper into the mitochondria for the synthesis of cytochrome c oxidase [226–228]. A copper P-type ATPase, CCC2p, and a "copper chaperone," ATX1p, are also capable of transporting copper [229,230]. The protein factors, ACE1p, ACE2p, and MAC1p factors regulate the copper homoeostasis [220,231–234]. The progress in understanding of the copper homoeostasis in yeast has contributed significantly to copper homoeostasis in general [235–239]. In man, defects in function or expression of copper transporting P-type ATPases are responsible for two heredity diseases: Menke's and Wilson's diseases [240]; their functional homologues have been identified in mouse, rat, and *Caenorhabditis elegans* [241–243]. Thus, the copper-dependent cycling of the transporter may be true for all animals or maybe even for all eukaryotes.

### 11.6.10 Nickel

Although nickel is a required nutrient, at high levels it can be toxic to microorganisms. It enters the bacterial and yeast cell mainly by the CorA system [12,158,244]. An additional nickel transporter identified in *Alcaligenes eutrophus* [245] as part of the hydrogenase gene cluster [246] was cloned [247] and expressed. The purified product HoxN [168] was found to be an archetype of a new class of transport proteins. Nickel is probably bound to hisitidine residues in the *Ralstonia eutropha* HoxN [248] and NixA from *Helicobacter pylori* [249]. Uptake of nickel (and cobalt in a related protein) [250,251] is probably driven by the chemiosmotic gradient. Surprisingly, nickel for hydrogenase formation in *E. coli* is supplied by an ABC-transporter [252–254], which involves a periplasmic nickel-binding protein. In Gram-negative bacteria, the *nikRABCDE* system is responsible for the uptake of nickel. In *E. coli*, NikA is the periplasmic-binding protein; NikB and NikC form a heterodimer inner membrane pore for the translocation of the metal; and NikD and NikE are the heterodimeric ATP-binding proteins.

The organization of these genes in *Pseudomonas putida* is different from that in *E. coli* but the same as in *Brucella suis*, where *nikR* is upstream of and transcribed divergently from *nikABCDE* [9]. Nickel detoxification may be done by sequestration and/or transport, as with most heavy metal cations. Metal resistance based on extracellular sequestration has been hypothesized only in bacteria, but has been found in several species of yeast and fungi [255–257]. Nickel uptake in *Saccharomyces cerevisiae* may be reduced by excreting large amounts of glutathione, which binds with great affinity to heavy metals [258]. Nickel is bound to polyphosphate in *Staphyloccus aureus* [259] and to free histidine in nickel hyperaccumulating plants [260]. In *S. cerevisiae*, nickel is disposed off into the vacuole and probably bound to histidine in there [256]. The transport into the vacuoles requires a proton-pumping ATPase [261]; thus, this kind of nickel transport may be also driven by a chemiosomotic gradient. Other yeasts and fungi probably detoxify nickel using similar mechanisms and by a mutation of the CorA uptake system [257,262].

The best known nickel resistance in bacteria is the nickel efflux driven by an RND transporter in *Ralstonia* sp. CH34 and related bacteria. Two systems, a nickel–cobalt resistance [*cnr]* [263] and a nickel–cobalt–cadmium resistance [*ncc*] [76] have been described that are closely related to the cobalt–zinc–cadmium resistance system *czc* from strain CH34. Regulation of *cnr/ncc* on one hand and czc on the other, however, is completely different. The nickel resistance systems are regulated by a sigma factor, a membrane-bound antisigma factor, and a periplasmic anti-antisigma factor, which probably senses the nickel. A P-type ATPase described to bind $Ni^{2+}$, $Cu^{2+}$, and $Co^{2+}$, ATPase 439, may be regarded as the first example of a nickel P-type ATPase in bacteria [11].

### 11.6.11 Cobalt

Cobalt is required as a trace element in nitrile hydratases and cofactor $B_{12}$; however, in the ionized form, at higher concentration, it can directly inhibit microorganisms. It is accumulated in most bacteria by the CorA system [14,15,157,258]. Members of the CDF protein family have also

been found to transport cobalt, like a protein in the Gram-positive bacterium *Staphylococcus aureus* [184] and COT1p protein from *S. cerevisiae* that transports cobalt across a mitochondrial membrane [186,187]. Excess $Co^{2+}$ is generally pumped from Gram-negative bacteria in conjunction with nickel or zinc by a three-polypeptide membrane complex that is not an ATPase but functions as a divalent cation/2 $H^+$ antiporter [76,173,263]. A bacterium containing a nitrile hydratase, *Rhodococcus rhodochrous*, was reported to possess a system related to the nickel transporter HoxN from *R. eutropha* [250,251], thus implying that HoxN homolog might play a role to supply $Co^{2+}$ for the production of this non-$B_{12}$-cobalt protein [11]. Thus, $Co^{2+}$ is detoxified by RND-driven systems in Gram-negative bacteria and by CDF transporters in eukaryotes and Gram-positive bacteria.

### 11.6.12 Chromium

Chromium does not have any essential function in microorganisms. Chromate enters the cell of *Alcaligenes eutrophus* and many other bacteria by the sulfate uptake system [13,22]. Changing the valence state by reducing Cr (VI) under aerobic and anaerobic conditions through electron-transport systems containing cytochromes may (though this is inconclusive) confer resistance to chromate. Chromate-resistant *Pseudomonas fluorescens* strain LB300 and other bacteria have been reported to reduce chromate [264–278]. The DNA sequences determining chromate resistance of the *Pseudomonas aeruginosa* and *Alcaligenes eutrophus* share homologous *chrA* genes encoding membrane. An additional upstream gene, *chrB*, that has been postulated to be responsible for the inducibility of the resistance in *A. eutrophus* is absent in *P. aeruginosa* [22,174,266,267].

### 11.6.13 Vanadium

The heavy metal with the lowest atomic number is *Vanadium*. Vanadate, bearing structural similarity to phosphate, may be taken up by phosphate uptake systems and is known as an inhibitor of ATPases [279,280]. In addition to toxicity, there are few examples of the physiological role of vandate like vanadate-dependent nitrogenase in *Azotobacter chroococcum* for nitrogen fixation in absence of molybdate in the environment [281–293]. More functions as a trace element are obscure [294]; it also can be used as an electron acceptor for anaerobic respiration [295,296]. Vanadate resistance has been studied in an extremophile *Sulfolobus* and a eukaryote *S. cerevisiae* [297,298]; however, the detailed mechanism of vanadate resistance remains elusive.

## 11.7 Evaluation of the Uptake and Efflux Capabilities of Organisms Based on Whole Genome Sequence Analysis

By taking advantage of the availability of multiple complete genomes, some approaches offer new opportunities for predicting gene functions in each of these genomes. All these approaches rely on the same basic premise: the organization of the genetic information in each particular genome reflects a long history of mutations; gene duplications; gene rearrangements; gene function divergence; and gene acquisition and loss that has produced organisms uniquely adapted to their environment and capable of regulating their metabolism in accordance with the environmental conditions. This means that cross-genome similarities can be viewed as meaningful in the "evolutionary" sense and thus are potentially useful for functional analysis.

The most promising comparative methods specifically employ information derived from multiple genomes to achieve robustness and sensitivity that is not easily attainable with standard tools. It seems that they are indeed the tools for the "new genomics," whose impact will grow with the

increase in the amount and diversity of genome information available. A disproportionate number of these examples are from the COG (clusters of orthologous groups) system.

- *Phylogenetic patterns (profiles)*. The COG-type analysis applied to multiple genomes provides for the derivation of phylogenetic patterns, which are potentially useful in many aspects of genome analysis and annotation [299]. Similar concepts have been introduced by others in the form of phylogenetic profiles [300,301]. The phylogenetic pattern for each protein family (COG) is defined as the set of genomes in which the family is represented. A pattern search tool that allows the user to select COGs with a particular pattern accompanies the COG database. Predictably, genes that are functionally related tend to have the same phylogenetic pattern.
- *Profile scan*. Based on the classic Gribskov method of profile analysis [302,303], ProfileScan uses a method called pfscan to find similarities between a protein or nucleic acid query sequence and a profile library [304]. In this case, three profile libraries are available for searching. First is PROSITE, an ExPASy database that catalogs biologically significant sites through the use of motif and sequence profiles and patterns [305]. Second is pfam, which is a collection of protein domain families that differ from most such collections in one important aspect: the initial alignment of the protein domains is done by hand, rather than by depending on automated methods. As such, pfam contains slightly over 500 entries, but the entries are potentially of higher quality. The third profile set is referred to as the Gribskov collection.

All organisms for which complete genome sequences are available were analyzed for their content of cytoplasmic membrane transport proteins (work done in the Institute of Genomic Research). The transport systems present in each organism were classified according to (1) putative membrane topology; (2) protein family; (3) bioenergetics; and (4) substrate specificities. The overall transport capabilities of each organism were estimated and differences in their reliance on primary vs. secondary transport and the range of transporter substrate specificities in each organism were found to correlate generally with the respective ecological niches and metabolic capabilities of each organism. More than 80 distinct families of transport proteins were identified. The efficacy of a phylogenetic approach for predicting function was demonstrated.

Five major families of multidrug efflux transporters have been analyzed: the ATP-dependent ABC superfamily and four families of secondary transporters: MFS, RND, SMR, and MATE families. The ABC and MFS are large superfamilies including uptake and efflux proteins. *These two superfamilies are the two largest families of transporters known; they are extremely diverse and appear to be (almost) ubiquitous, suggesting a very ancient origin* [306]. The RND, SMR, and MATE families are smaller families, which to this date only include efflux systems; they are found in prokaryotes as well as eukaryotes. The major findings of these analyses and the complete complement of transports in each completely sequenced organism are available at http://www-biology.ucsd.edu/~ipaulsen/transport/.

## 11.8 TRANSPORTERS INVOLVED IN HEAVY METAL UPTAKE AND EFFLUX

Transporters of trace elements can be classified into four distinct groups [2,13,167,180,182,307–323]:

- *Electrochemical potential-driven transporters* utilize a carrier-mediated process to catalyze: transport of a single species by mediated diffusion or in a membrane potential-dependent manner (uniport); two or more species in opposite directions in a tightly coupled process without utilizing chemical free energy (antiport); or two or more species

in the same direction in a coupled process (symport), again without using any form of energy other than the electrochemical potential gradient.

- *Primary active (P–P bond hydrolysis driven) transporters* utilize the free energy of the P–P bond hydrolysis to drive the movement of ions against their chemical or electrochemical potential gradient. The transport protein may be transiently phosphorylated during the transport cycle but the substrate is not phosphorylated. These transporters occur universally in all domains of life.
- *Transporters whose mode of transport or energy coupling is unknown* are awaiting their final placement after their transport mode and energy coupling have been resolved. These families include at least one member for whom a transport function has been described, but the mode of transport or the energy coupling is not known.
- *Auxiliary transport proteins* in some way facilitate transport across one or more biological membranes, but they do not participate directly in the transmembrane translocation of a substrate. They may provide a function connected with energy coupling to transport; play a structural role in complex formation; serve a biogenic or stability function; or function in regulation.

Transporter families of the four groups involved in trace metal metabolism are summarized in Table 11.2.

## 11.9 INFLUX AND EFFLUX ARE COORDINATELY REGULATED

In the process of evolution of the ion-transport system, it was necessary to evolve specificity of the transporters. The occurrence of influx and efflux transporters for the same metal ion necessitated evolution of not only a specialized but also a highly regulated system. For a cell exposed to toxic metal ions, like $Hg^{2+}$, $Cu^{2+}$, $Cd^{2+}$, or oxyanions of arsenic and antimony, it is obligatory to maintain the cytosolic ion concentration below the toxic level. In general, the cells survive the toxic ion effect with the aid of a specific ion-efflux system. Thus, for a nonessential but toxic ion, the necessity and advantage of simultaneously operating an uptake- and an efflux-transport system associated in several metal ion resistance operons remains speculative. In many instances, however, cells had evolved a transport system for a particular essential ion that can be used by a structurally similar toxic ion to enter the bacterial cell.

One can also advocate that metabolic penalty for having uptake pumps more specific is greater than the genetic cost of having plasmid genes in the population that can spread when needed [2]. Thus, most of the toxic metal (or metalloid) resistance is encoded by mobilizable extrachromosomal replicons or transposons. Among the known resistance systems, mercury, cadmium (along with zinc, cobalt, and nickel), and arsenic (along with antimony) resistance genetic determinants have been well characterized. All these toxic metal ion resistance operon systems, except the ars operon, are common in having coordinated regulation with the specific gene(s) for the ion-uptake system associated with the plasmid encoded resistance operon or encoded by the genome [324].

Mutation in the resistance gene of the operon causes hypersensitivity if the associated uptake gene(s) remains unaltered. In the mercury resistance (*mer*) operon, at least three genes *merTPC* are cotranscribed with *merA* and specify $Hg^{2+}$ uptake system. Thus, the mer operon, lacking merA gene, which encodes the mercuric reductase to convert mercuric ion to volatile mercury, resulted in more hypersensitivity to $Hg^{2+}$ than the isogenic strains lacking mer operon [325]. This observation, in fact, had helped to identify the mercuric ion uptake genes merTPC of the *mer* operon [47].

Similarly, a *copCD* clone of copper resistance *copABCD* [2] operon was phenotypically hypersensitive to copper [326]. Unlike mercuric ion, copper (or zinc) is an essential element but toxic at a higher level; thus, a homeostasis condition is maintained to ensure that the cells do not become copper (or zinc) depleted or copper (or zinc) surfeited [194]. Thus, complex and distinct operon systems having certain coordination between metabolism of these metals including uptake and resistance including efflux (and or binding) were evolved.

## TABLE 11.2
## Transporters Involved in Trace Metabolism

| Family | Substrates | Size range[a] | Number of transmembrane segments[b] | Distribution | Examples |
|---|---|---|---|---|---|
| **Electrochemical potential-driven transporters** | | | | | |
| CDF | Cd2+, Co2+, Ni2+ | 300–750 | 6 | Archaea, bacteria, eucarya | Heavy metal uptake and efflux transporters of bacteria, eukaryoyic plasma membranes and mitochondria (CzcD of *Ralstonia eutropha*) |
| ZIP | Zn2+, Fe2+ | 376 | 8 | Eucarya | Zn uptake transporter Zrt1 of *Saccharomyces cerevisiae*. |
| RND | Heavy metal ions, multiple drugs, organic solvents, etc. | 800–1200 | 6, 12 | Archaea, bacteria, eucarya | Drug efflux pump AcrA of *Escherichia coli* |
| TDT | Tellurite | 300–350 | 10 | Archaea, bacteria, eucarya | Arsenical resistance efflux pump of *Staphylococcus aureus* |
| ArsB | Arsenite, antimonite | 400–900 | 12[c] | Archaea, bacteria, eucarya | Arsenical resistance efflux pump of *Staphylococcus aureus* |
| CHR | Chromate; sulfate (uptake or efflux) | c. 400 | 6,[c] 10 | Archaea, bacteria | The chromate transporter ChrA of *Alcaligenes eutrophus* |
| NiCoT | Ni2+, Co2+, Ni2+ | 300–400 | 8[c] | Bacteria | Ni2+ uptake permease HoxN of *Ralstonia eutropha* |
| Nramp | Divalent metal ions (uptake) | 500–600 | 8–12[c] | Archaea, bacteria, eucarya | Divalent metal ion, H+ symporter Nram2 of *Homo sapiens* |
| ACR3 | Arsenite | c.400 | 10 | Archaea, bacteria, eucarya | Arsenical resistance-3 protein Acr3 of *S.cerevisiae* |
| CadD | Cd, cations | 150–200 | 5 | Bacteria | Cadmium resistance protein CadD of *S. aureus* |
| **Primary active transporters** | | | | | |
| ABC | All kinds of inorganic and organic molecules from simple ions to macromolecules | 1000–2000 (multidomain; usually mutisubunit) | (5[c]); (6[c]); 12[c]_ variable | Archaea, bacteria, eucarya | MDR of *Homo sapiens* |
| P-ATPase | Cations (uptake and/or efflux) | 600–1200 (sometimes mutisubunit) | (6–12); 8,[c] 10[c] | Archaea, bacteria, eucarya | KdpABC (K+ uptake) of *E. coli* |
| ArsAB | Arsenite, antimonite, (tellurite?) | c. 1100 (multidomain; two subunit) | 12[c] | Archaea, bacteria, eucarya | Arsenite efflux pump ArsAB of *E. coli* |
| **Transporters of unknown classification** | | | | | |
| MerTP | Hg2+ (uptake) | c. 200 | 3[c] | Bacteria | Mercuric ion transporter MerTP encoded on the incJ plasmid pMERPH of *Shewanella putrifaciens* |

**TABLE 11.2**
**Transporters Involved in Trace Metabolism** (continued)

| Family | Substrates | Size range[a] | Number of transmembrane segments[b] | Distribution | Examples |
|---|---|---|---|---|---|
| MerC | Hg2+ (uptake) | c. 140 | 4 | Bacteria | Hg uptake transporter MerC encoded on the incJ plasmid pMERPH of *Shewanella putrifaciens* |
| MerF | Hg2+ | c. 80 | 2 | Bacteria | MerF importer of *Pseudomonas aeruginosa* plasmid |
| FeT | Fe2+ (Co2+, Cd2+) (uptake) | c. 550 | 6 | Eucarya (yeast) | Fe2+ transporter FeT4p of *S. cerevisiae* |
| Ctr1 | Cu2+ (uptake) | 150–200 | 3 | Eucarya | Copper transporter Ctr2p of *S. cerevisiae* |
| PbrT | Pb2+ | 400–650 | 7 | Bacteria | PbrT of *Ralstonia metallidurans* |
| MgtE | Mg2+, Co2+ (uptake) | 300–500 | 4–5 | Archaea, bacteria | Mg2+ transpoter MgtE of *Bacillus firmus* |
| **Auxiliary transport proteins** | | | | | |
| MFP | Proteins; polypeptides, signalling molecules, heavy metal ions, etc. | 350–500 | 1 | Bacteria | EmrA of *E. coli* |

[a] Size range (in number of amino acid residue).

[b] Number of (putative) transmembrane alpha-helical segments.

[c] Number is established by substantial experimental data/x-ray crystallographic data.

Despite tremendous effort and progress made in the understanding of the molecular mechanism of arsenic resistance operon, including the characterization of structural genes and their products, nothing is known about the specific uptake system of this toxic metalloid [60,327,328]. In the early studies of phosphate transport system, it became apparent that the arsenate ion, being analogue of inorganic phosphate, acts as a substrate of the inorganic phosphate transport system, Pit and Pst (the low-affinity Pit system in particular) to enter the bacterial cell. As a result, mutation in phosphate transport may be associated with arsenate resistance phenotype. No other transport mechanism or arsenate-specific transporter is known in the microbes.

Arsenite or antimonite cannot use phosphate transport system; no investigation has been made to understand how these metalloids enter the cell. Moreover, because the solution chemistry of the metalloids is significantly complex, it adds further complications to investigate in the interactions of these toxic elements with microbes. However, elucidation in the molecular mechanism of arsenic or antimony transport (uptake and efflux) is essential [143]:

- Arsenite is a ubiquitous pollutant in the environment and a proven carcinogen.
- Antimony compounds are the only effective drug for treatment of leshmaniasis.
- Arsenic compounds are still used as an antitrypanosomal drug.
- Studies on the molecular biology of the arsenic–antimony resistance in microbes have proven highly beneficial in investigations of arsenic metabolism in higher organisms, including humans. Particularly, the ATP-dependent, efflux-mediated, multidrug resistance-associated protein is functionally similar to the ATP-coupled ArsAB pump.

A major difference in the *ars* and *mer* or *copABCD* (or *pcoABCD*) encoded resistance for arsenic and the mercury or copper, respectively, is the absence of any known arsenic (and antimony) uptake determinant in the *arsRDABC* or *arsRBC* operon. No coordinated regulation between arsenic uptake and efflux is known yet. Thus, the attempt to search the cellular transporters in the uptake mechanism of these metalloids in the bacterial genome should be rewarding.

Only a single antimonite-resistant mutant was identified from a pool of random transposon insertional mutants [60]. The insertion of the transposon was mapped in the glpF gene. Though the authors had selected for antimonite- and arsenite-resistant phenotype, only a single antimonite-resistant mutant (1 m*M* in LA) that showed wild type arsenite-sensitive phenotype was described. The results suggested that glpF gene coding for glycerol facilitator could be the major route of Sb (III) uptake.

The authors succeeded in selecting spontaneous resistant mutants of three kinds: arsenite-, arsenate-, and antimonite-resistant. The associated metal and antibiotic resistance phenotype of the two arsenite resistant mutants is similar to multidrug-resistant (MDR) bacterial and tumor cells [329]. Emergence of multidrug-resistant mutants in a bacterial population under drug selection pressure constitutes a serious concern in modern medicine; thus, a relevant field of study in adaptive mutation or evolutionary biology [7] has become pertinent. To understand the emergence of drug resistance in the protozoan-parasite *Leshmania*, arsenite-resistant *Leshmania* cells have been described since 1990 [330].

Arsenic drugs have been used since ancient times and most of those applications had been abandoned in the development of modern medicine. Nevertheless, several arsenical drugs of melarsen derivatives are still used to treat African sleeping sickness caused by a protozoan parasite *Trypanosoma brucei* [331]. The trypanolytic effect of melarsen oxide was abrogated by adenine or adenosine. This observation led to the conclusion that adenine transporters can be used by arsenic and thus parasites lacking P2 adenosine transport are resistant to melarsen due to loss of uptake. The other mechanism of arsenite-resistant development in a population of *Leshmania* exposed to drugs was due to amplification of drug resistance genes, such as *ltpgpA* encoding for P-glycoprotein [332–334].

## 11.10 EVOLUTION OF SPECIFIC GENETIC ELEMENTS TO SENSE AND RESPOND TO METALS IN THE ENVIRONMENT: A DOMAIN FOR DIVERSE METALS

### 11.10.1 Transcription Repressor Protein ArsR Binding with the Ars Operator–Promoter and ArsR Binding with Inducer Molecule

The *arsR* gene of R773 encodes the repressor protein of 117 amino acids. Transcriptional fusion with β-lactamase (*blaM*) demonstrated that the first 83 amino acid residues of ArsR protein were sufficient for repression [135]. Subsequently, experimental evidence also suggested that a core sequence of about 80 amino acids (9 to 89 residues) contained all the information for dimerization (the active form of ArsR), repression, and induced recognition [136]. ArsR binds to the operator sequence spanned from nt. –64 to –40 of the transcriptional start site identified by DNaseI protection assay [135]. More precisely, two short stretches, nt. –61 to –58 and –50 to –47, within the DNaseI protection region were suggested as the contact sites between the ArsR of R773 and the specific DNA sequence of the operon.

However, ArsR of pSX267 was shown to bind at –35 and –10 regions of the promoter [61]. This *in vitro* ArsR binding was substantiated by the constitutive expression with the clones having operator mutations or mutation in *arsR* of R773 [135]; deletion of *arsR* in pSX267 [61]; or mutation in *arsR* of pKW301. In order to perform the ArsR on the *ars* operon, it should have at least three domains specific for (1) metal binding; (2) DNA binding; and (3) dimerization, the active form of ArsR [136]. The ArsR of R773 is the first member of the ArsR metaloregulatory protein family [136,335]. The members of this family respond to a single or diverse metal-ion species, namely, arsenite, antimonite, bismuth (III), cadmium (II), and zinc (II). All these proteins possess a sequence [134,335] characteristic of DNA-binding helix–turn–helix (H–T–H) motif shown in Figure 11.1.

It was proposed that the N-terminal cysteine and at least one (generally two) histidine residues at C-terminal are the feature of this motif. These cysteines and histidines participate in metal binding, which thus disrupts DNA–ArsR binding [134]. Furthermore, ArsR of pSX267 was suggested to possess motifs of typical zinc-finger and leucine-zipper, identified in several DNA-binding proteins [336]. Arsenite, antimonite, or bismuth induces the transcription by releasing the repressor protein from the promoter, allowing the RNA polymerase to initiate the transcription. It is puzzling how these diverse oxyanions, arsenite, antimonite, and the cation bismuth ($Bi^{3+}$) could recognize the same metal-binding site, even though the efficiency of binding of these diverse metalloid species to the specific operator site is different [135,138].

## 11.11 PROBLEM OF TRANSPORTING TOXIC LEVELS OF THREE DIFFERENT DIVALENT CATIONS WITH A COMMON SYSTEM; THE NATURE OF THE MEANS REQUIRED TO COPE WITH THE DIFFICULTIES; AND THE TYPE OF GENETIC CHANGES: AN EXAMPLE OF SELF-AWARENESS — OUTCOME OF DESIGNED CREATIVE PROCESSES IN *RALSTONIA*

$Ni^{2+}$ is accumulated by the rapid and nonspecific CorA (MIT) $Mg^{2+}$ transport system. Highly specific nickel transporters are HoxN chemiosmotic transporters or ABC uptake transporters, which use a periplasmic nickel-binding protein, depending on the bacterial species. Characterized nickel-resistance systems are based on inducible, RND-driven transenvelope transporters. Moreover, a nickel efflux P-type ATPase may exist in *Helicobacter pylori*.

$Zn^{2+}$ is accumulated by the rapid and nonspecific CorA (MIT) $Mg^{2+}$ transport system in some bacterial species, and MgtE system in others. Inducible, high-affinity ABC transporters supply zinc in times of need. P-type ATPases may transport zinc in both directions: zinc uptake as a by-product

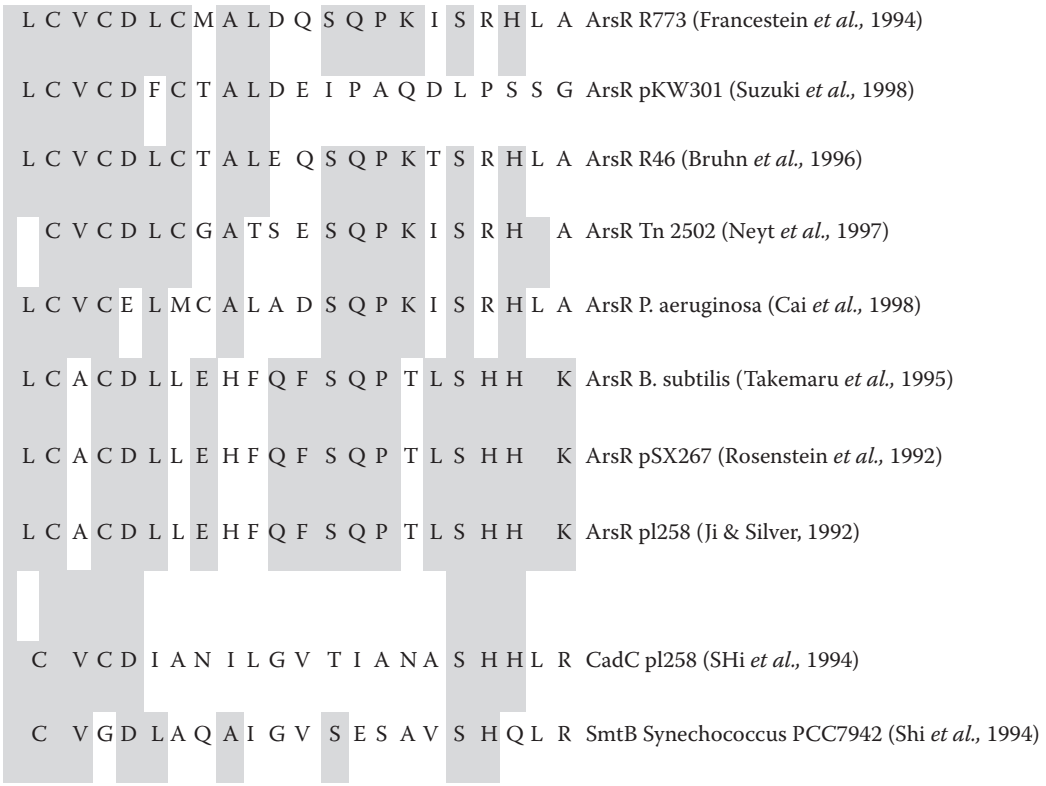


**FIGURE 11.1** Helix–turn–helix-DNA-binding motif of ArsR family of metalloregulatory proteins.

of $Mg^{2+}$-uptake again and efflux as detoxification. CDF transporters catalyze slow efflux, high-efficiency transenvelope efflux by inducible RND-driven transporters like Czc.

Magnesium and/or manganese uptake systems are responsible for the uptake of $Cd^{2+}$. Only in cyanobacteria, metallothionein-like proteins were characterized (Smt). Efflux is done in Gram-positive bacteria by P-type ATPases, in Gram-negative by RND-driven transenvelope transport, and maybe additionally to these systems by CDF transporters.

This part of the review will present the results of the conserved domain search with query sequences of resistance proteins derived from gene bank databases to support the findings of the earlier authors who have attempted to solve the evolutionary puzzles. Consider how a *Ralstonia* sp. strain CH34 (earlier known as *Alcaligenes eutrophus* CH34) solved the problem of transporting toxic levels of the three different divalent cations with a common system without causing starvation for the two, $Co^{2+}$ and $Zn^{2+}$, that are essential trace nutrients.

It can well be assumed that regulation must occur not only at the level of transcription but also at the level of cation pump function. The kinetic properties of CzcABC complex assure that the toxic cation cadmium is always pumped out of the cell. Zinc, in contrast, is exported slowly at low concentration and rapidly at high concentration. Cobalt provides a problem with the *czc* system because it is a poor inducer of *czc* and is bound and transported with low affinity. One solution for problems with cobalt efflux for *Ralstonia* was to evolve related cobalt efflux systems that are not induced by zinc, such as the *cnr* resistance determinant.

The three subunits of CzcABC protein complex differ in structure and function, as first demonstrated by the analysis of *czc* deletion mutants [337]. CzcA and CzcB alone are able to catalyze a highly efficient Zn efflux. On the other hand, CzcC is needed to modify the substrate specificity of the complex to include cadmium and, for practical purposes, cobalt.

The CzcC was shown to be located in the membrane fraction, independent of the presence of CzcA and CzcB. The CzcC subunit does not contain apparent motifs for a metal-binding site; is membrane bound by itself; and does not need CzcA or CzcB as an anchor. Therefore, CzcC may function by altering the conformation of the CzcAB complex. Conserved domain search with CzcC query sequence produced significant alignments with COG1538 and pfam02321. COG1538 is TolC, an outer membrane protein involved in cell envelope biogenesis, outer membrane/intracellular trafficking, and secretion. The pfam02321 constitutes the outer membrane efflux protein (OEP). The OEP family form trimeric channels that allow export of a variety of substrates in Gram-negative bacteria. Each member of this family is composed of two repeats. The trimeric channel is composed of a 12-stranded, all-beta sheet barrel that spans the outer membrane, and a long all-helical barrel that spans the periplasm.

It has been demonstrated that after expression of *czcC* or *czcB* under the control of the phage T7 promoter, the cells containing the $^{35}$S-labeled Czc proteins present in the crude extract were mainly found in the membrane fraction, and a part remained in the cytoplasmic fraction. Because it has been suggested that CzcC belongs to outer membrane-associated proteins, CzcC might be located at the periplasmic face of the outer membrane. Due to its high hydrophobicity [337], however, CzcC does not contain sufficient hydrophobic beta sheets or alpha helices to be an integral outer membrane protein [175]. Deletion of the *czcC* gene resulted in a loss of cadmium resistance and most of the cobalt resistance; however, most of the zinc resistance was retained [337]. On the other hand, deletion of the histidine-rich motifs of CzcB led to a decrease in zinc resistance, but resistance to cadmium and cobalt was only slightly affected. Therefore, cation transport through the Czc complex might use two different pathways: a CzcC-dependent pathway is used by cadmium, cobalt, and half of the zinc ions, and a CzcC-independent pathway that involves the histidine-rich motifs of CzcB is used by the other half of the zinc cations.

The predicted amino acid sequence of CzcB starts with a highly hydrophobic amino terminus followed by two small histidine-rich segments, which are homologous to each other. These two motifs are very good candidates for the required zinc-binding sites because they are absent in the otherwise related CnrB protein that does not recognize $Zn^{2+}$. Therefore, CzcB is a membrane-bound protein located on the cytoplasmic face of the membrane. CzcB shows some homology to membrane fusion proteins; it might space the periplasm and draw the outer membrane close to the CzcA antiporter, thereby connecting both membranes in a flexible fashion as has been proposed by HlyD. To the authors' query, position-specific scoring matrices (PSSMs) producing significant alignments were COG0845, AcrA, membrane fusion protein (cell envelope biogenesis, outer membrane); pfam00529, HlyD, HlyD family secretion protein; and COG1566, EmrA, multidrug resistance efflux pump (defense mechanisms).

CzcA alone may function as a cation–proton antiporter, although with low efficiency. From hydropathy analysis of the predicted sequence, four domains were predicted [150]: two hydrophobic regions composing the transport "tunnel" and two cytoplasmic hydrophilic domains. These regions are arranged in the following order: hydrophobic amino terminus; hydrophilic domain I; tunnel region I; hydrophilic domain II; tunnel region II; and hydrophilic carboxy terminus. This structure appears to be the result of an ancient gene duplication event shared by a family of proteins for which CzcA is the prototype [180].

A conserved domain search with the CzcA protein sequence produced significant alignments with gnl|CDD|13017 COG3696, putative silver efflux pump, inorganic ion transport, and metabolism; gln|CDD|15262, pfam00873, ACR_tran, AcrB/AcrD/AcrF family; and gln|CDD|10708, COG0841, AcrB, cation/multidrug efflux pump. The members of the protein family pfam00873 are integral membrane proteins. Some are involved in drug resistance. AcrB cooperates with a membrane fusion protein, AcrA, and an outer membrane channel, TolC. It is known that eight genes apparently are involved in bacterial silver resistance. Two of these, *silS* and *silR* encode a pair of cation-sensing sensor kinase (SilS) and transphosphorylated responder (SilR), homologous to PcoS/PcoR for copper resistance and CzcS/CzcR for cadmium, zinc, and cobalt resistances. As

with *pco* system, silRS is followed by silE, which codes for a periplasmic $Ag^+$-binding protein, homologous to PcoE. However, the remainder of the silver resistance genes is transcribed in the opposite direction (unlike the situation in *pco*) and encodes a three-component CBA system, weakly homologous to CzcCBA, and a P-type ATPase [338].

CzcD protein is involved in the regulation of the Czc system. It is a membrane-bound protein with at least four transmembrane alpha helices and is a member of a subfamily of the cation-diffusion facilitator (CDF) protein family, which occurs in all three domains of life. CzcD protein sequence produced significant alignments with COG1230, Co/Zn/Cd efflux system component of inorganic ion transport and metabolism; pfm01545, Cation efflux family; and COG0053, MMT1, predicted Co/Zn/Cd cation transporters. Members of pfam01545 are integral membrane proteins that are found to increase tolerance to divalent metal cations such as cadmium, zinc, and cobalt. These proteins are thought to be efflux pumps that remove these ions from cells. The deletion of *czcD* in a *Ralstonia* sp. led to partially constitutive expression of the Czc system due to an increased transcription of the structural *czc CBA* genes in absence and presence of inducer. The *czcD* deletion could be fully complemented in trans by CzcD and two other CDF proteins from *Saccharomyces cerevisiae*, ZRC1p and COT1p. Thus CzcD appeared to repress the Czc system by an export of the inducing cations [338].

The ZRC1 protein is required in yeast for a zinc–cadmium resistance based on an unknown mechanism. Although multiple copies of the ZRC1 gene enable yeast cells to grow in the presence of high concentrations of zinc, disruption of the chromosomal ZRC1 locus renders the respective mutant strain more sensitive to $Zn^{2+}$ [183]. The COT1 protein has a similar function as ZRC1 with respect to cobalt cation. Overexpression of COT1 protein increases cobalt tolerance and mutation of the COT1 gene makes the cells more sensitive to $Co^{2+}$. The COT1 protein is located in the mitochondrial membrane fraction and is involved in the uptake of $Co^{2+}$ by mitochondria [187]. The sequence similarity of CzcD, ZRC1, and COT1 is so high that earlier authors were tempted to forward some working hypothesis about the function of these proteins: the CDF proteins are membrane-bound proteins involved in zinc, cobalt, and cadmium transport. That would lead to the assumption that CzcD functions as a sensor by a slow uptake of the zinc, cobalt, and cadmium cations.

Many of the regulatory systems by which cells sense and then respond to environment signals are called *two-component* systems. Such systems are characterized by having two different proteins: (1) a specific *sensor* protein located in the cell membrane; and (2) a cognate *response regulator* protein. The sensor protein has kinase activity and is often referred to as a sensor kinase. Sensor kinases detect a signal and in response phosphorylate themselves (autophosphorylation) at a specific histidine residue on their cytoplasmic surface. This phosphoryl group is then transmitted to another protein inside the cell, the response regulator.

The response regulator is a DNA-binding protein that regulates transcription. Such a two component regulatory system is involved in transcription control of heavy metal homoeostasis in *Ralstonia* [340]. A conserved domain search with a CzcS query sequence produced significant alignments with COG0642, BaeS, signal transduction histidine kinase (signal transduction mechanism); COG2205, KdpD, osmosensitive $K^+$ channel histidine kinase (signal transduction mechanism); and COG5002, VicK, signal transduction histidine kinase (signal transduction mechanism). Thus, it can be inferred that CzcS is a cation-sensing sensor kinase.

A conserved domain search with the CzcR query sequence revealed that it is a DNA-binding protein/transcriptional regulatory protein. PSSMs producing significant alignments (with high score bits) were COG0745, cd00156, smart0048, and pfam00072. The attributes of these notations are as follows: COG0745, 0mpR (transcription), cd00156, REC (homo dimer), smart00048 (receiver domain), pfam00072 (effector domain). COG0745, OmpR, response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain (signal transduction mechanisms/transcription); cd00156, REC, signal receiver domain (originally thought to be unique to bacteria [CheY, OmpR, NtrC, and PhoB], now recently identified in eukaryotes ETR1 *Arabidopsis*

*thaliana*; this domain receives the signal from the sensor partner in two-component systems; contains a phosphoacceptor site that is phosphorylated by histidine kinase homologs; usually found N-terminal to a DNA-binding effector domain; forms homodimers); smart00448, REC, cheY-homologous receiver domain; and pfam00072, response_reg, response regulator receiver domain. (This domain receives the signal from the sensor partner in bacterial two-component systems. It is usually found N-terminal to a DNA-binding effector domain.)

The mechanism used by the response regulator to control transcription depends on the system being described. In *E. coli*, the osmolarity of the environment controls which of two proteins, OmpC or OmpF, is synthesized as part of the outer membrane. The response regulator of this system is OmpR. When OmpR is phosphorylated, it acts as an *activator* of transcription of the *ompC* gene and a *repressor* of transcription of the *ompF* gene. In the Czc system, CzcD is the possible transducer, a transmembrane protein that can sense the cations. This CzcD should be in contact with CzcS. When CzcD binds to the cations, it probably changes conformation and causes a change in the autophosphorylation of CzcS. Phosphorylated CzcS (CzcS-P) then phosphorylates CzcR (forming CzcR-P), a *response regulator*, which is by its nature a transcriptional regulatory protein.

Mutagenesis, or the production of changes in the DNA sequence that affect the expression or structure of gene products, is one of the best methods for understanding gene function. Studying the phenotype of the mutants has become logically the subject of functional genomics. At the molecular level, for example, phenotype includes all temporal and spatial aspects of gene expression as well as related aspects of the expression, structure, function and spatial localization of proteins. In a recent study, comparison of the efflux systems of 63 sequenced prokaryotes with that of *Ralstonia metallidurans* indicated that heavy metal resistance is the result of multiple layers of resistance systems with overlapping substrate specificities, but unique functions [340]. The comparative genomics lead to the conclusion that the creation of the outstanding heavy metal resistance of *R. metallidurans* was because of gene multiplication by duplication and horizontal transfer, stepped up to the differentiation of function and, finally, combining the genes into highly efficient operons [340].

## 11.12 DEVELOPMENT OF PROMISING ANALYTICAL DEVICES FOR TRACE METAL DETECTION IN THE ENVIRONMENT: BIOSENSORS

The massive interest and commitment of resources in the public and private sectors flows from the generally held perception that genomics will be the single most fruitful approach to the acquisition of new information in basic and applied biology in the next several decades. Among many rewards in applied biology, the promise of facile new approaches for development of biosensors is one of the important inclusions.

### 11.12.1 Protein-Based Biosensors

The low molecular weight proteins rich in cysteine residues, the metallothioneins, can bind heavy metal ions nonspecifically in metal-thiolate clusters. Metallothioneins, ubiquitously present in eukaryotes and prokaryotes, are synthesized in response to elevated concentrations of silver, bismuth, cadmium, cobalt, copper, mercury, nickel, or zinc metal ions. The cyanobacterium *Synechococcus* sp. synthesizes metallothionein SmtA in greater quantities when exposed to the elevated concentrations of $Cd^{2+}$ or $Zn^{2+}$. The operon also encodes a divergently transcribed repressor, SmtB, which is a trans-acting repressor of expression from the *smt*A operator–promoter region. Metallothionein protein expression depends upon the interaction between the metal ions and the repressor protein that regulates the expression of metallothinein mRNA. Loss of the repressor gene, *smt*B,

and subsequent unregulated transcription of *smt*A, has been shown to be advantageous to organisms constantly stressed with changing levels of cadmium, copper, lead, nickel, zinc, or arsenate [68].

Again, a mutation rendering inability to synthesize SmtA protein leads to the metal-sensitive phenotype. The purified recombinant SmtA-fusion protein, overexpressed in *E. coli* as a carboxy-terminal extension of glutathione S-transferase, was immobilized in different ways to a self-assembled thiol layer on a gold electrode placed as the working electrode in a potentiometric arrangement in a flow analysis system. This allowed detection of copper, cadmium, mercury, and zinc ions at femtomolar concentrations [341]. Similarly, the regulatory protein MerR encoded by the *mer* operon of Tn*501* in *Pseudomonas aeruginosa*, which controls the expression of itself as well as other *mer* gene products for mercury detoxification (by changing its conformation on binding to $Hg^{2+}$ to align contacts at the promoter region of the operon-activating transcription of the *mer* genes) was also used as the recognition component of a biosensor. In both of these protein-based biosensors, GST-SmtA and MerR, a capacitive signal transducer was used to measure the conformational change following binding [341].

## 11.13 WHOLE-CELL-BASED BIOSENSORS FOR DETECTION OF BIOAVAILABLE HEAVY METALS

The quantitation of bioavailable metal is difficult with traditional analytical methods. However, the bioavailability of metals is an important factor in the determination of metal toxicity and therefore the detection of bioavailable metals is of interest. The new concept of analyzing bioavailability of heavy metals by creating microbial strains capable of sensing the environment will rely largely upon the functional genomics of a metal resistance genetic system. The greatest advantage is the ability of biosensors to detect the bioavailable fraction of the contaminant, as opposed to the total concentration. Such whole-cell bacterial biosensors will create a clearer picture by providing physiologically relevant data in response to a contaminant.

The essence of all metal resistance genetic systems is the specificity of genetic regulatory elements so that the corresponding metal controls the expression of the uptake or resistance gene products. For example, cobalt–zinc–cadmium resistance operon (*czc* operon) in *Ralstonia* sp. CH34 is regulated by a two-component regulatory system composed of the sensor histidine kinase CzcS and the response activator CzcR. Regulatory genes are arranged in an upstream as well as downsteam regulatory region. Genomics revealed the presence of the *czc*R and *czc*S together with *czc*D constituting the downstream regulatory region. Functional genomics with *czc*D::*lac*Z translation fusion and *czc*S::*lux* transcriptional fusion enabled the regulation of both genes by heavy metals to be understood [339].

These systems can be used as the contaminant-sensing component of the biosensor by detecting the substance for which it is designed to detoxify or excrete. The contaminant-sensing component is combined with the reporter genes to create biosensors that can identify toxic substances at very low levels. When the contaminant-sensing component detects the substance, it triggers the reporter gene. In the development of a mercury-specific biosensor, a hypersensitive clone was constructed using the regulatory sequence along with the mercury ($Hg^{+2}$) uptake genes *merTPC* of the mercury resistance operon. Such a clone was found responsive to $Hg^{+2}$ with as low as 0.5 n*M* — several folds lower than the lowest concentration required to induce the operon without the *merTPC*.

A reporter gene encodes for a mechanism that produces a detectable cellular response. It determines the sensitivity and detection limits of the biosensor. Specific characteristics are needed for the reporter gene to be used in a biosensor. The gene must have an expression or activity that can be measured using a simple assay and reflects the amount of chemical or physical change. Also, the biosensor must be free of any gene expression or activity similar to the desired gene expression or activity being measured. The suitable reporter genes are (1) lacZ (β-galactosidase); (2) firefly (*Photinus pyralis*) luciferase, lucFF; and (3) bacterial luciferase, luxAB [342-43].

## 11.14 LUMINESCENCE-BASED BIOSENSORS

There are few successful reports of successful use of gene-fusion biosensor in the monitoring of a metal toxicity in field application. These strains express a sensitive reporter gene, luciferase, connected to a promoter element responding specifically to various heavy metals. Inserting a heavy metal responsive element directionally cloned in a suitable vector in front of the luciferase gene by using standard recombinant-DNA techniques created all the sensor plasmids. Using a nucleotide database, one can isolate a promoter/operator element generated by a polymerase chain reaction using specific oligonucleotide primers. The plasmids can then be expressed in different hosts, in order to obtain maximal and specific response to each of the metal tested. For instance, Biomet sensors (patented and recognized by OVAM) used a relevant soil bacterium, *Ralstonia metalliduransans*, to modify it to deliver a light reaction when specific heavy metals go into the cell. The quantification of light emissions can easily be done with a microtiter plate luminometer. Freeze-dried sensor cells were also tested used like reagents. Another bioluminescent sensor (*mer-lux* fusion) for detection of mercury was successfully used to examine the feasibility of testing mercury concentration of natural water from a contaminated freshwater pond [343].

### 11.14.1 Development of an *LacZ*-Based Arsenic Biosensor [344]

Understanding the mechanism for arsenic resistance is necessary in order to develop an appropriate biosensor and to better understand its response. Therefore, to develop the biosensor, the arsenic-resistant gene and the reporter gene must be cloned and inserted onto one plasmid, which then is inserted into a host bacterium. The arsenic-sensing biosensor will then be triggered by arsenic, the analyte, entering the biosensor and activating the transcription of the resistant gene, which is to be followed by the transcription of the reporter gene. The entire resistant gene may not be needed, and the biosensor can only use the beginning components, such as the promoter, and should be able to recognize the arsenic and begin the transcription of the plasmid that contains the reporter gene.

The transcription of the reporter gene will produce proteins whose activity will be assessed in direct correlation to the amount of arsenic entering it. Bacterial arsenic resistance genetic operon *ars*RDABC or *ars*RBC present in diverse bacterial species have been well characterized [24,136]. Because the *ars* operator–promoter is inducible by arsenite (or arsenate in the presence of arsC),and inducibility is positively correlated with the concentration of the inducer, the activity of a reporter gene product, if cloned under this promoter, will reflect the availability of the inducer/analyte.

In the present study, an arsenic biosensor was created using the *lac*Z reporter gene. The promoterless *lac*Z gene, lacking a ribosome-binding sequence and the first eight nonessential amino acid codons, was coupled with the *ars* promoter along with *ars*R, *ars*D, and a part of *ars*A gene in the translational *lac*Z fusion vector, pMC1871. The recombinant plasmid pASH3, having truncated the *ars* operon, was found hypersensitive to arsenite and arsenate and the phenomenon was found suitable to develop a simple bioassay system for arsenic. However, it is not known whether the hypersensitivity to arsenite rendered by pASH3 is associated with active arsenite uptake like the mercury-hypersensitive clone. Thus far, the hypersensitivity of arsenite observed in *E. coli* cells conferred by pASH3 cannot be explained from the present state of knowledge of arsenic resistance and or uptake in microorganisms. However, the expression of *ars-lac*Z fusion was expected at a concentration lower than the values observed earlier with the complete *ars* operon *ars*RDABC.

The maximum specific activity was obtained with arsenite between 0.5 and 1 μ*M*; however, β-galactosidase activity was found inducible even with 10 n*M* of arsenite. Therefore, the clone pASH3 could serve a biosensor to detect a very low concentration of arsenic, which may not be easily detectable by the standard chemical methods. When the arsenite-responsive regulation unit of the plasmid pI258 was used to express the firefly luciferase gene (*lucFF*), the lowest arsenite concentration required to induce the reporter gene was 100 n*M* and maximum induction was noted at 3.3 m*M*4 4 arsenite. In another method, expression of *arsD-lacZ* fusion was monitored by an electro-

chemical reaction to assay the arsenite-dependent β-galactosidase activity. The detection limit was 100 n*M*, but a 17-h induction period was required.

These two methods are apparently suitable for environmental samples of low bioavailable arsenic content; however, both require relatively costly instruments and may not be suitable in a field study. There are few reports of successful gene-fusion biosensors in the monitoring of a metal toxicity in field application. For instance, a luminescent bacterial biosensor was shown to be effective in the evaluation of arsenic bioavailability of chromated copper arsenate contamination [343]. Detection limit of arsenite by arsenic hypersensitive clone pASH3 was comparable with the earlier claims; the methodology is relatively simple as well. Moreover, the process can easily be improvised to an acceptable arsenic assay kit with a low-cost investment for monitoring a large number of samples for on-site analysis.

## 11.15 CONCLUSION

The creativity and imagination of the researcher are the two important variables that influence the potential application of genome data. It is prudent to follow a logical course when using genomic data. During the initial data acquisition step, in which genomic data are generated experimentally or retrieved from publicly available data sources, simultaneous evaluation of multiple data sets will ensure higher resolution and greater confidences while increasing the likelihood that the genomic elements of interest are represented. It is imperative to include an extensive mutagenic analysis of all the systems involved while studying the metal interactions at the genomic level. It would also be informative to study the regulation of all these genes by transcription profiling with DNA microarrays and proteomic analyses.

It is encouraging that the understanding of the genetic basis of metal resistance has opened up the ability to use these systems for environmental application. Although much still remains to be learned, the future development of a multianalytical biosensor will further improve the applicability of biosensors. It is probable that a multianalytical biosensor to test more than one trace element in the environment could be developed by genetically labeling the bacteria used in the biosensor. Continued research endeavors will produce best procedures and full applicability of whole-cell bacterial biosensors.

> I cannot but wonder how this incredibly complex mechanism is still functioning at all. Thinking of life one clearly sees our science in the poorest and most primitive light. The properties of living matter are most probably predetermined by the fertilized cell in the same way as the prerequisite of life itself consists in the existence of an atom and consequently, the mystery of everything that existing can be found in its lowest stage of development.
>
> **A. Einstein**

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