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A bacterial view of the periodic table: genes and proteins for toxic inorganic ions

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Abstract Essentially all bacteria have genes for toxic metal ion resistances and these include those for Ag^{+}, AsO_2^{-}, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, TeO_3^{2-}, Tl^{+} and Zn^{2+}. The largest group of resistance systems functions by energy-dependent efflux of toxic ions. Fewer involve enzymatic transformations (oxidation, reduction, methylation, and demethylation) or metal-binding proteins (for example, metallothionein SmtA, chaperone CopZ and periplasmic silver binding protein SilE). Some of the efflux resistance systems are ATPases and others are chemiosmotic ion/proton exchangers. For example, Cd^{2+}-efflux pumps of bacteria are either inner membrane P-type ATPases or three polypeptide RND chemiosmotic complexes consisting of an inner membrane pump, a periplasmic-bridging protein and an outer membrane channel. In addition to the best studied three-polypeptide chemiosmotic system, Czc (Cd^{2+}, Zn^{2+}, and Co^{2}), others are known that efflux Ag^{+}, Cu^{+}, Ni^{2+}, and Zn^{2+}. Resistance to inorganic mercury, Hg^{2+} (and to organomercurials, such as CH_3Hg^{+} and phenylmercury) involve a series of metal-binding and membrane transport proteins as well as the enzymes mercuric reductase and organomercurial lyase, which overall convert more toxic to less toxic forms. Arsenic resistance and metabolizing systems occur in three patterns, the widely-found *ars* operon that is present in most bacterial genomes and many plasmids, the more recently recognized *arr* genes for the periplasmic arsenate reductase that functions in anaerobic respiration as a terminal electron acceptor, and the *aso* genes for the periplasmic arsenite oxidase that functions as an initial electron donor in aerobic resistance to arsenite.

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Introduction

Toxic heavy metals have been abundant on the planet Earth and microbes have been exposed to them (for example the cations of Hg and the oxyanions of As) since basically the beginning of life, nearly 4 billion years ago. Thus the question of whether toxic metal (and oxyanions of some soft metals) resistance systems evolved in microbes in response to human pollution in the last few hundreds or thousands of years is easily answered in the negative. These resistance determinants have been here for billions of years; and the arguments supporting such a broad and untestable assertion needs to be addressed element by element. The primary basis for this conclusion is the abundance and wide spread occurrence of such resistance systems, from bacterial type to type and with frequencies ranging from a few percent in ''pristine'' environments to nearly all isolates from heavily polluted environments. The DNA and amino acid sequences and structures of the genes and proteins concerned with arsenic resistance indicate an ancient origin [56], although it is premature to conclude whether early ''bio-available'' As occurred as As(III) or as As(V).

Genetic and mechanistic studies of toxic metal ion resistance systems have been reviewed frequently over the last 30 years. We will not cite earlier reviews and reports of all toxic metal resistances (before [96, 98, 101]), but rather cite newer metal-by-metal reviews, best found now in a focused issue of *FEMS Microbiol. Rev.* [17] plus newer major findings that expand or complete our picture of how bacteria cope with toxic metal stress. However, some elements have been chosen for deeper consideration within space limits, as they provide models for study of resistance to other elements, while others are only considered in passing, usually because we lack the needed understanding or because little progress has

been made since earlier reports such as [101]. Other viewpoints (with emphasis on chemistry rather than on genetics and microbiology) of the Periodic Table and life appear occasionally [40, 59, 63], but the differences are such that there is basically no overlap. A 43rd monograph in the "Metal Ions in Biological Systems" series [95] appeared recently with quite different (more environmental) perspectives on some of the toxic ions considered here, as well as with separate chapters on major "good elements" that are required for growth of all cells (including H, C, O, N, P, Fe, and S) [97]. Space limits preclude consideration of these here.

The most frequent mechanism of toxic divalent cation resistance is energy-dependent pumping out, that is membrane efflux pumps (Fig. 1).

Mercury and organomercurial resistance genes

Resistance to mercury and organomercurials was the first studied and is still the best understood of toxic metal resistance systems. Other than that for arsenic, it might be the most widely found toxic ion resistance system and occurs in all bacterial divisions where it has been sought. Mercury resistance is frequently found in new microbial total genome sequences. The current best overview of bacterial mercury resistance is found in Barkay et al. [9]. Mercury resistance occurs widely with Gram negative and Gram positive bacteria, in environmental, clinical and industrial isolates, and frequently mercury resistance genes are found on plasmids and encoded by transposons. The mercury-resistance transposon Tn *21* occupies about 8 kb of the 94 kb plasmid R100 (NCBI accessions NC_002134; gi:9507549), the first multidrug resistance plasmid found in Japan 50 years ago, together with several antibiotic resistance determinants. However, chromosomal mercury (organomercurial) resistance is also common, for example in *Bacillus* isolates [79].

The protein gene products and their functions for the range of genes found in mercury resistance *mer* operons in Gram negative bacteria is shown in Fig. 2. Starting with Hg^{2+} (or organomercurials) outside the cell surface, the problem for the bacteria is to bring the toxic compound in to the cytoplasmic enzyme where it is detoxified, without allowing the toxic mercury to be free, causing damage. Counter-intuitively, the bacteria have evolved a Hg^{2+} -transport system that binds extracellular Hg^{2+} and moves it along in a cascade of cysteines on different proteins (called a thiol "bucket brigade") to the active sites on mercuric reductase. No outer membrane protein has been associated with this transport process (Fig. 2). However, most *mer* operons systems of Gram negative bacteria encode the small MerP protein that binds Hg^{2+} in the periplasm. This protein (approximately 72 amino acids length after processing of a leader peptide) has had its structure solved, with 4

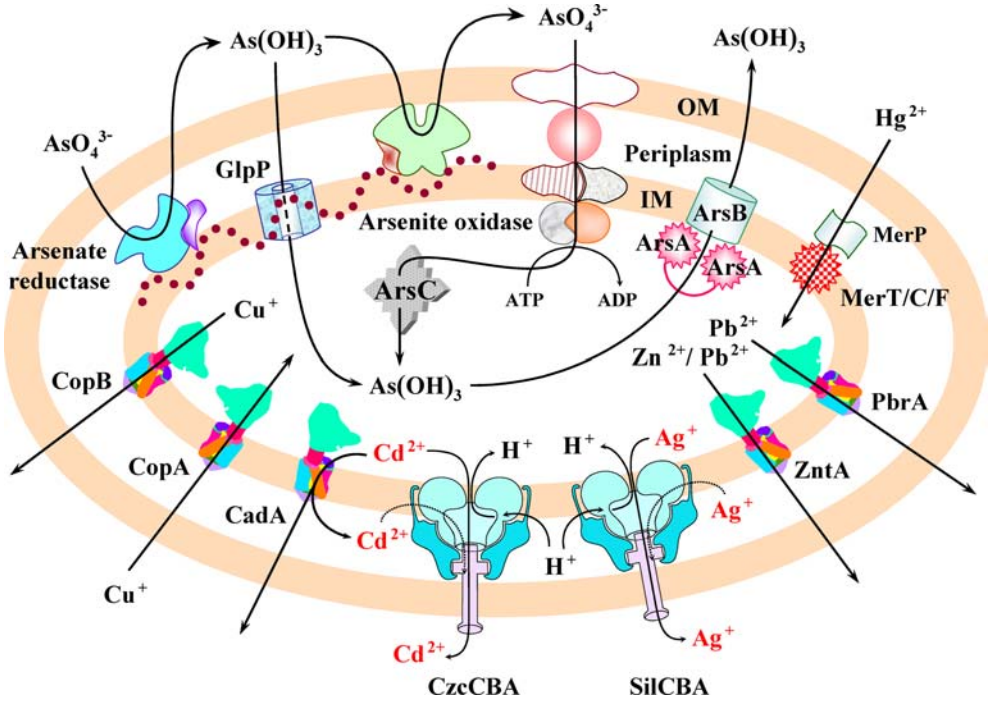

Fig. 1 Overview of membrane associated uptake, efflux, reduction and oxidation of metal ions. The complexity of several examples of some families (such as CopA/B and CadA P-type ATPases and three component chemiosmotic RND systems (CzcCBA and SilCBA) preclude a useful detailed listing here and readers are referred to the separate sections concerning each element. Arsenic is given more emphasis, as enzymes in the periplasm and cytoplasm are included as well as three classes of transporters (GlpP, the aquaglyceroporin; a multicomponent Pst-like ABC ATPase uptake system; and a two component ArsA/B ATPase efflux pump). The CBA efflux transport systems extend from the cytoplasm across the outer membrane of Gram negative bacteria. How the substrates of transport systems (influx and efflux) that do not have indicated associated outer membrane proteins function is not known

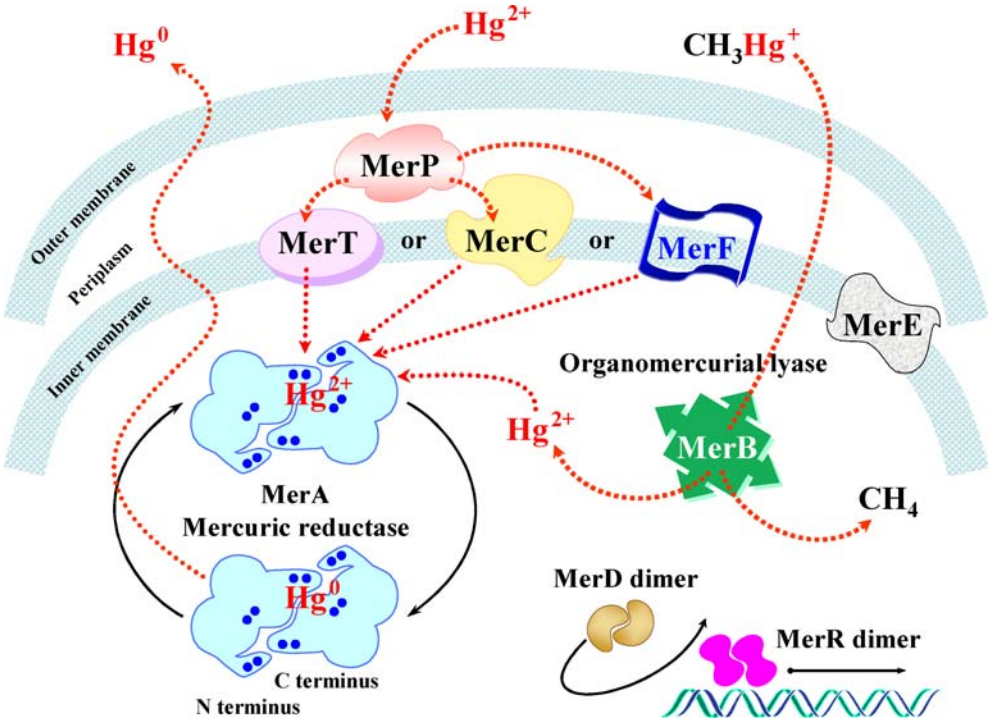

Fig. 2 The products of the *mer* operon(s). MerR is the transcriptional regulatory protein; MerA is mercuric reductase enzyme; MerB is organomercurial lyase enzyme; MerC is an alternative (to MerT) membrane uptake protein; MerD is a co-repressor or "chaperone"s involved in transcriptional regulation; MerE is a hypothesized membrane protein that has not been studied; MerF is an additional alternative (to MerT) membrane uptake protein. Transport across the inner and outer membranes without indicted proteins are either by diffusion through the lipid bilayer or via yet unidentified proteins

anti-parallel beta sheets plus two short alpha helical regions and a "loop" containing the Cys14-X2-Cys17 motif that binds Hg^{2+} in a linear S–Hg–S manner [71, 108]. MerP is thought to pass Hg^{2+} in rapid exchange with two of the four cysteines in the inner membrane protein MerT (Fig. 2). Since there is no ATP-binding motif, Hg^{2+} uptake across the inner cell membrane MerT protein may involve cysteine to cysteine transfer without overall energy-coupling, or be driven by the membrane potential. MerT is modeled as passing across the membrane three times as alpha helical regions [9] and with a cysteine pair in the first membrane helix thought to accept Hg^{2+} initially from MerP. Then a cytoplasmic cysteine pair may receive Hg^{2+} from the first pair. Experimental evidence supports this model [119]. While MerT is found most often in *mer* operon products, alternative membrane transport proteins MerC or MerF are also known. MerC crosses the membrane four times and MerF just twice, but all three of these proteins are thought to function in similar transport processes [119]. A single cell (or operon) can contain more than one membrane transport protein (determinant). MerE is an additional membrane protein of currently unknown function (Fig. 2).

Once at the inner surface of the inner membrane, Hg^{2+} is thought to be transferred in still another cysteine pair-to-cysteine pair exchange to the N-terminal cysteine pair of the large homo-dimeric enzyme mercuric reductase, MerA (Fig. 2). All recognized MerA sequences (with the single exception of that from *Streptomyces*) have this N-terminal domain once (or twice in *Bacillus*) that is closely homologous in sequence and thought to function similarly to MerP. The N-terminal Hg^{2+} binding domain of mercuric reductase is often lost from proteolytic activity during purification of the enzyme. It also is missing in the one available crystal structure of mercuric reductase [94], suggesting that the domain lacked a fixed position within the crystal. The *Bacillus* enzyme that was purified and solved for crystal structure has two fused back-to-back MerP-like sequences at its N-terminus. Yet the processed MerA lacking the N-terminal domain functions enzymatically in vitro, and the "donor" function of the N-terminus can only be seen in careful kinetic experiments [35]. It is hypothesized that the Hg^{2+} is next transferred by still another cysteine-pair to cysteine-pair exchange to the C-terminal cysteine pair (C558 C559 in Tn *21*-determined MerA numbering) of the MerA subunit. These cysteines are needed for cellular enzyme activity [9], but can be bypassed with some Hg^{2+} adduct substrates [36].

The intracellular Hg^{2+} is thought to be transferred directly from the membrane MerT protein to mercuric reductase rather than from small intercellular thiols such as glutathione (or mercaptoethanol which is used for cell-free enzyme assays). Hg^{2+} binds to mercuric reductase on the carboxyl-terminal Cys557 Cys558 of one subunit and is then transferred by rapid thiol/thiol exchange to the Cys135 Cys140 thiol pair of the other monomer (Fig. 3a) [9]. After, the Hg^{2+} is passed on to this active site cysteine pair, it is reduced by electron transfer from FAD cofactor. The four Cys residues are closely together in the protein structure [94] and additionally a Tyr residue is involved in the binding of Hg^{2+}. The enzymatic mechanism of mercuric reductase has been studied by Engst and Miller [36]. Mercuric reductase is an FAD-containing yellow flavoprotein, func-

tionally related to glutathione reductase and lipoamide dehydrogenase (of wide occurrence in prokaryote and eukaryote cells).

Organomercurial lyase is the small mono-meric enzyme that cleaves the Hg–C covalent bond releasing Hg^{2+} (the substrate of mercuric reductase) and reduced organic compounds such as methane from methyl mercury or benzene from phenyl mercury (Fig. 2, 3b) [9, 101]. Organomercurial lyase is determined by the *merB* gene found only with few *mer* operon-containing plasmids in *Escherichia coli*, but perhaps 50% in *Pseudomonas*, and in all *Staphylococcus aureus* "penicillinase" plasmids [9, 101]. *Mer* operons with the *merB* gene for organomercurial lyase are also found on the chromosome of MRSA (methicillin-resistant *S. aureus*) and on the chromosomes of *Bacillus* isolates from as different sources as North American polluted estuaries, Russian mine soil and the sediment from Minamata Bay Japan. Some Gram negative bacteria have two versions of the *merB* gene and the Bacilli frequently have three *merB* genes in close chromosomal proximity. The MerB primary sequence is unusual, having no paralog enzymes with related sequences but different substrates [86, 101]. A wide range of MerB sequences have been identified (accessible in GenBank and a wide range of organomercurial substrates known, although we do not know in any case how sequence here determines substrate specificity. The enzymatic reaction for organomercurial lyase was shown to be a concerted proton attack on the Hg–C bond by an SE2 reaction mechanism [9, 10], after the organomercurial is initially bound to a cysteine thiol (Fig. 3b). It is unclear whether a dicarboxylic acid residue [10], a tyrosine or a second cysteine thiol is the source of proton that adds for example to methylmercury forming methane or to phenylmercury forming benzene. Recent mutagenesis and structural studies of organomercurial lyase [11, 30, 31, 86] have led to the suggestion that a cysteine thiol might be the source of the proton that attacks the Hg–C bond. Thus, Fig. 3b is more explicit than in previous models [9, 10], but still tentative. Three cysteines (Cys96, Cys117 and Cys159 in the MerB from plasmid R831 numbering) are conserved among organomercurial lyase sequences. Cys96 and Cys159 (but not Cys117) are required for full in vitro activity [86], leading to the proposal that Cys96 and Cys159 play essential roles in the reaction mechanism (Fig. 3b) [11, 86] but that Cys117 (although completely conserved) may play a non-catalytic role [9, 11]. The non-essential Cys160 is found in organomercurial lyases of Gram negative bacteria (forming a vicinal cysteine pair with Cys159) but not Gram positive bacteria and Cys215 Cys216 (*Bacillus* RC607 MerB1 numbering) form a C-terminal vicinal cysteine pair in organomercurial lyases of Gram positive bacteria. Four steps are pictured (Fig. 3b), with the organomercurial (methylmercury as an example, although experimental work is generally done with less toxic mercurials) initially forming a thiol-mercury covalent bond with the invariant Cys159. A second cysteine thiol, from Cys96, possibly forms a bond with the Hg and the proton from Cys96 or from Tyr93 or from a conserved aspartate or glutamate (Asp98 is conserved in organomercurial lyases) attacking the Hg–C bond releasing methane (or another alkyl or aryl organic product) [9, 11]. The Hg is then bound to the organomercurial lyase (step 3) by the two thiols and the Hg^{2+} released in cell-free assays generally to added small thiols such as glutathione and in intact cells either to soluble thiols or perhaps directly transferred to the C-terminal vicinal cysteines of mercuric reductase (Fig. 3b) [11]. Surprisingly with the wide variety of available MerB sequences and genes, there has been little effort to determine differences in organomercurial substrate specificity among the wide range of aliphatic and aromatic organomercurials known to be substrates for the enzyme.

Arsenic enzymatic redox reactions and resistance

Resistance to both arsenite (As[III], $As(OH)_3$) and arsenate (As[V], AsO_4^{3-}) is widely found among both Gram-negative and Gram-positive bacteria. Usually this is in the form of an *ars* operon with a minimum of three co-transcribed genes, *arsR* (determining the regulatory repressor), *arsB* (determining the membrane transport pump) and *arsC* (the determinant of a small intracellular arsenate reductase). Indeed, the *ars* operon occurs more widely in newly sequenced bacterial genomes (those with over 1,000 or 2,000 genes) than do the genes for tryptophan biosynthesis; and it has been argued partially because of this that arsenic resistance (both with arsenate reductase and arsenite oxidase) is a very ancient system probably found in early cells [56, 72]. The argument for a resistance mechanism being present near the time of the origin of life because of a range of toxic inorganic chemicals present at that time is not subject to experimental proof. The ArsR regulatory protein and ArsB efflux pump protein provide resistance to, recognize and function with antimony, Sb(III), as well as arsenite, As(III) [101]. The *ars* operon confers resistance to Sb(III) as well as to AS(III) and As(V) [19].

Occasionally two additional genes, *arsA* and *arsD*, are found in *ars* operons of Gram negative bacteria, so the gene order is *arsRDABC*. ArsA is an intracellular ATPase protein that binds as a dimer to the membrane ArsB protein, converting its energy coupling from the membrane potential to ATP [110]. The arsenite membrane efflux pump is unique in that it can function either chemisomotically (with ArsB alone) or as an ATPase (with the ArsAB complex). The *ars* operon systems have been thoroughly reviewed recently [72, 103] and will not be described in detail here.

ArsD is thought to be a trans-acting co-repressor [61] of the *arsRDABC* operon, in addition to ArsR (the well-defined repressor) [18, 101]. However, the role of ArsD is currently unclear. It appears to both bind to the gene transcriptional complex controlling maximum mRNA synthesis and to function as a chaperone protein that

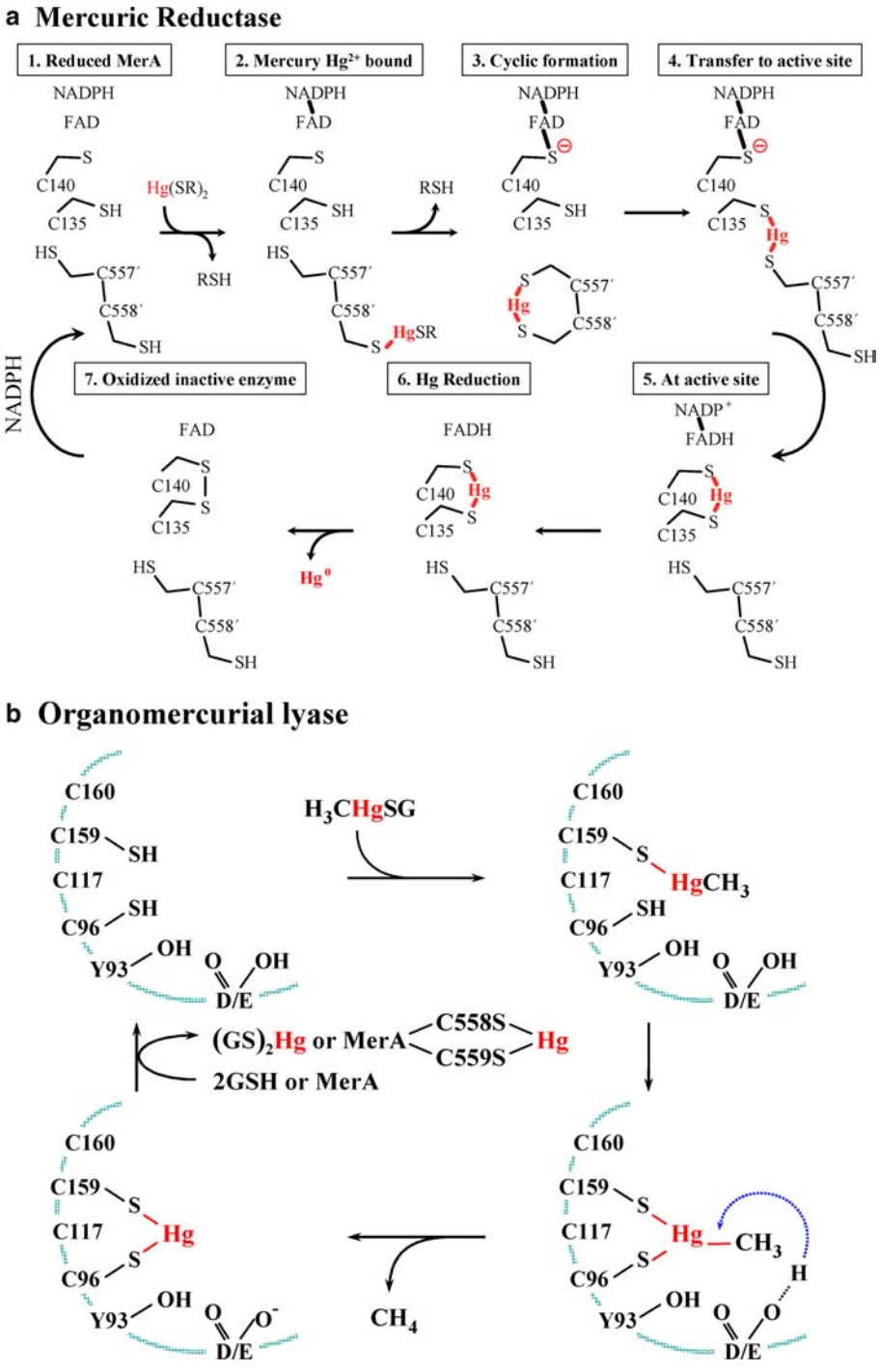

Fig. 3 The reaction of **a** mercuric reductase and **b** organomercurial lyase (modified after [9–11, 71]

binds As(III) and delivers it to the ArsA ATPase subunit (B.P. Rosen, personal communication). ArsD contains two-pairs of adjacent thiol residues, Cys12-Cys13 and Cys112-Cys113, which bind As(III) or Sb(III), thus making four binding pairs per homo-dimer DNA-binding complex.

In recent years, additional and totally different enzymes involved in arsenic resistance and redox chemistry have been isolated and their genetic basis studied [72, 84, 102]. These are the periplasmic respiratory arsenite oxidase [4, 5] and the respiratory arsenate reductase [2]. The arsenite oxidase is considered a resistance mechanism converting highly toxic high arsenite to relatively less toxic arsenate [73, 102], while respiratory arsenate reductase functions as a terminal electron acceptor, allowing heterotrophic anaerobic growth in the absence of oxygen. The oxidase and reductase contain homologous molybdopterin centers and [Fe–S] cages (Fig. 4) and both are coupled to inner membrane respiratory chains, with the oxidase as an initial electron donor (Fig. 4a) while the reductase is the terminal electron acceptor (Fig. 4b) in an anaerobic respiratory process [2,

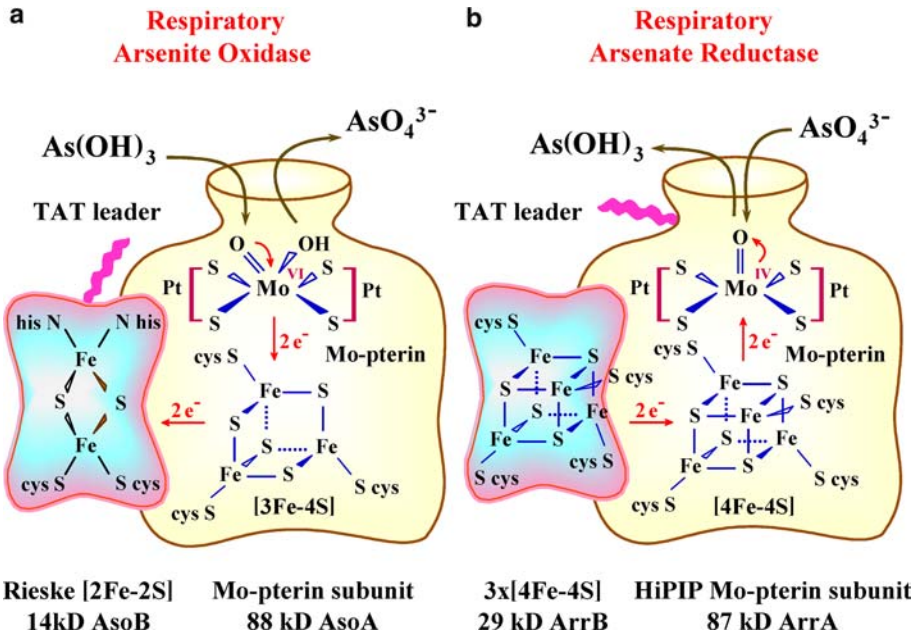

Fig. 4 Model of respiratory arsenite oxidase and arsenate reductase (modified from [102], with permission)

4]. Understanding of these two enzymes (and their genes) was recently summarized [102]. The genes for the two polypeptide respiratory arsenite oxidase are called *asoAB* (although alternative nomenclature *aoxBA* and *aroAB* have been proposed; the genes for the respiratory arsenate reductase are called *arrAB* [102]. For the respiratory arsenite oxidase (Fig. 4a), an X-ray diffraction-based protein structure is available [34]. The oxyanion substrate is thought to enter a shallow conical pit in the enzyme surface, contacting the embedded Mo(VI) directly. Concerted two electron transfer occurs [50] and arsenate is released from the pit and molybdenum reduced to Mo(IV). Then electrons are transferred first to an [3Fe–4S] cage located about 15 Å distance in the protein, and from the [3Fe–4S] in the large Mo-pterin subunit to an [2Fe–2S] cluster in the small subunit (Fig. 4a) [4, 34]. From the small subunit, the electrons are transferred to the inner membrane respiratory chain, possibly initially to an azurin or cytochrome and eventually to oxygen, the terminal electron acceptor. For the functionally-related respiratory arsenate reductase (Fig. 4b), the electrons are transferred in the opposite direction, from the respiratory electron transport chain to the enzyme and finally from the Mo(IV)-pterin cofactor to the substrate arsenate (Fig. 4b). This means that the mid-point reduction potentials of the [Fe–S] centers in both subunits [50] of both enzymes must be quite different.

Respiratory arsenite oxidase and arsenate reductase (Fig. 4) are both considered to be periplasmic proteins (found outside the inner cell membrane but associated with it) and have in their gene-derived amino acid sequences canonical TAT (Twin Arginine Transporter) signal sequences that guide the hetero-dimeric folded protein with embedded cofactors from the cytoplasm to the periplasmic space [102]. Notably, the TAT leader is found at the N-terminus of the small subunit for arsenite oxidase but of the large subunit for arsenate reductase (Fig. 4).

There is an additional aspect of microbial arsenic biochemistry concerning which much less is known. That is the methylation and demethylation of inorganic arsenic and the incorporation of inorganic arsenic in to small organic compounds such as arsenobetaine, arsenolipids and arsenosugars [39, 72]. Little is known about the synthesis of the more complex organoarsenical compounds and this may be limited to eukaryotic organisms. The demethylation enzymes (and their genes) are also unidentified, while such a cycle must occur for completion of the cycle [72]. However, microbial methylation of inorganic arsenic has been recognized for over a century, as poisonous volatile arsenic compounds were released from ‘‘moldy wall paper’’ [39, 72]. Similar methylation by prokaryotes and animal tissues has come more recently [72, 109]. Still today, rather little is known about these processes. However, a major step forward occurred with the isolation and sequencing of the first arsenic methylase gene, from mammals [109]. Sequence homology searches then identified apparently the same gene in humans and other animals and also in prokaryotes [109] (C. Rensing, personal communication). The mammalian enzyme occurs in the liver and thus is not a product of bacteria living within animals. A limitation of these homology searches is that the arsenic methylase is similar to ubiquinone E methylases, whose chemistry is based on multiple cysteines residues within the sequence [109]. Direct experimental evidence [109] has shown that the methyl donor is *S*-adenosyl methionine (Fig. 5) and that methylation of arsenite to dimethylarsinic acid (Fig. 5) requires also the reduced small cytoplasmic protein reduced thioredoxin, the same cofactor as required by one group of intracellular arsenic

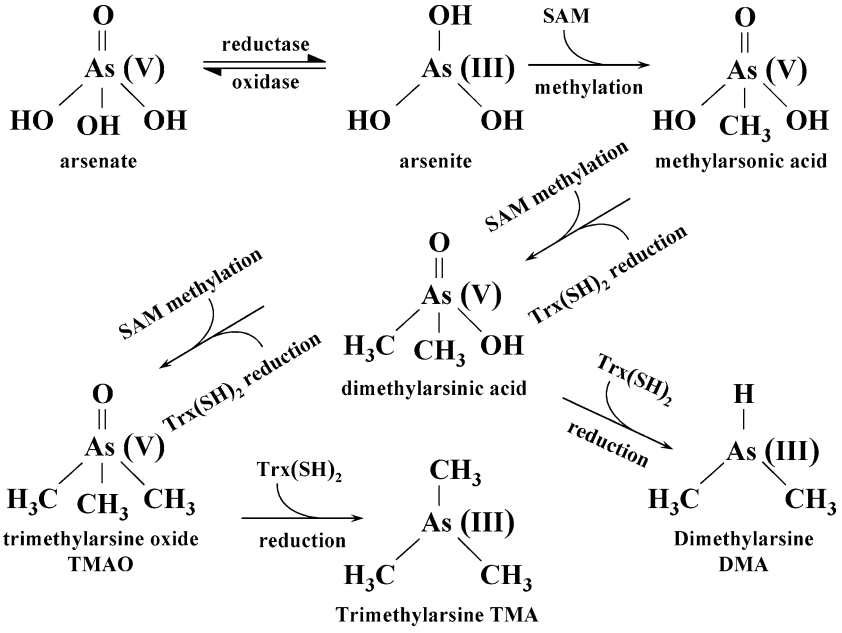

Fig. 5 Predicted steps in methylation of inorganic arsenic (modified after [72] with results from [109]. Methylation is concomitant with oxidation from As(III) to As(V) and reduction back to As(III) by thioredoxin ($Trx(SH)_2$) precedes the next round of methylation from *S*-adenosylmethionine (*SAM*)

reductases [69, 72]. The mammalian enzyme in vitro produces mostly dimethylarsinic acid, very little of the monomethyl product and no detectible trimethylarsine oxide or reduced dimethylarsine and trimethylarsine (the volatile compounds, whose production can be considered a microbial resistance mechanism [39]). Clearly the availability of this first arsenic methylase gene and purified enzyme, although from mammalian sources, opens the possibility of rapid progress in genetic and structural biochemical study of these important transformations.

ATPase membrane pumps and chemiosmotic membrane pumps

The remaining toxic inorganic ion resistance systems that are described here function as cation or oxyanion efflux systems, generally without coincident covalent or redox chemistry. Efflux pumps are the most commonly-found mechanism of microbial resistance to inorganic ions. These systems fall into a small number of patterns and are all distinct and not homologous to the mercury uptake and arsenic efflux systems described above. Seven major types of efflux pumps are known: Two are ATPases, (1) P-type ATPases (generally single polypeptide determinants, with a covalently phosphorylated—from ATP—intermediate) and (2) ABC ATPases (ATP binding cassette without phosphorylated intermediates and consisting of a cytoplasmic membrane-associated ATPase subunit, one or two membrane embedded pump channel subunits, a periplasmic space ion-binding protein, and an outer membrane porin protein. The ABC ATPase family include both uptake pumps and efflux pumps, although the efflux pumps today do not include those for metal ions (see the Internet site for membrane transporter taxonomy maintained by M. Saier http://www.biology.ucsd.edu/~msaier/transport/ or http://www.tcdb.org/tcdb/index.php?tc=3.A.1 for an up-to-date listing of these systems.

The additional three efflux pump classes are chemiosmotic ion/proton exchangers, (3) the single membrane polypeptides of the Major Facilitator Superfamily (MFS; generally a single polypeptide that transverses the membrane 12 or 14 times primarily in an alpha helical structure [93]), (4) the Cation Diffusion Facilitator family (CDF) [46] including CzcD for cadmium, zinc and cobalt), and (5) the CBA family of three polypeptide chemiosmotic antiporters such as CzcCBA with an inner membrane (A) protein of over 1000 amino acids in length, and outer membrane (C) protein and a coupling (B) protein connecting the two in the periplasmic space. We are calling these CBA for the order of the genes on the chromosome and to distinguish them from ABC ATPase systems. However, the name Resistance-Nodulation-Cell Division (RND) Superfamily is widely used for these efflux transporters as different examples are involved in resistance to such toxic compounds as cations and small organic compounds, nodulation in *Rhizobium*, or cell division such as in *E. coli* [115]. Two additional families of chemiosmotic transport systems that are more narrow in substrate range are the (6) ChrA (CHR) chromate efflux system and (7) ArsB, the arsenite [As (III)] and antimonite [Sb (III)] efflux system that is currently unique in that it can function alone as a chemiosmotic efflux system or with a second ArsA subunit functions with energy from ATP. An encyclopedic listing of these and other transport families is found at http://www.tcdb.org/. Examples of some of these transporters are summarized in Fig. 1.

Three patterns are found for cadmium efflux (the next toxic cation to be considered) and these are (with the

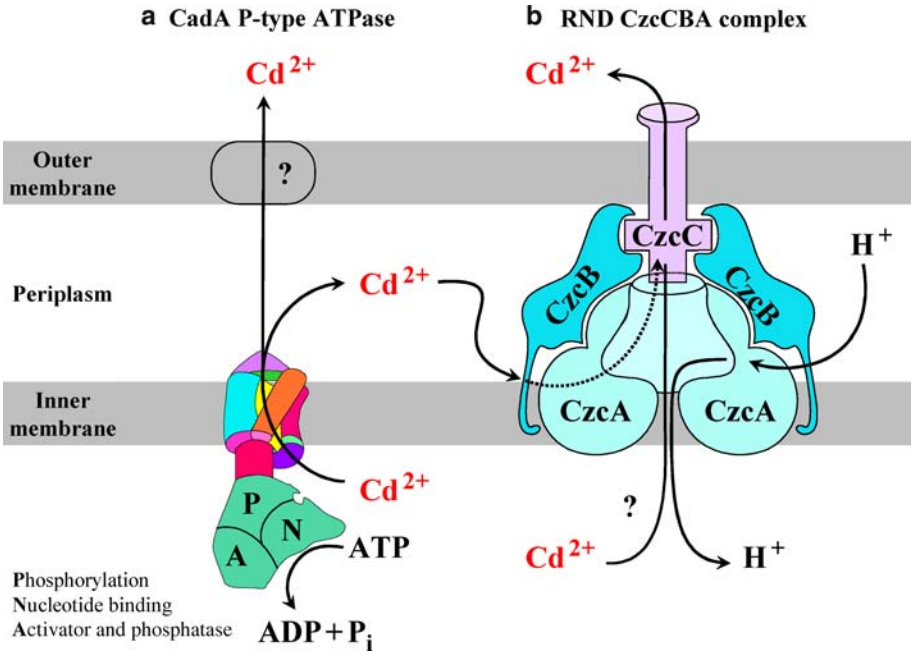

Fig. 6 Molecular models of efflux **a** P-type ATPase and **b** RND chemiosmotic antiporter

names of examples) CzcD, a single membrane polypeptide chemiosmotic efflux pump, CzcCBA, a three polypeptide chemiosmotic complex with CzcA (a large inner membrane protein), CzcC (a smaller outer membrane protein) and CzcB (a periplasmic coupling protein that connects CzcA and CzcB and forms a continuous channel from the cytoplasm to the outside of the cell (Figs. 1, 6b) [80, 81], and CadA, a large single polypeptide P-type ATPase (Fig. 6a). All three of these proteins are membrane embedded Cd^{2+} efflux pumps. Three polypeptide chemiosmotic CzcCBA complex that function as an ion/proton exchanger to efflux Cd^{2+}, Zn^{2+} and Co^{2+} is a member of the metal-resistance family within the larger superfamily of chemiosmotic pumps are called RND (for Resistance, Nodulation and Division, since some members are involved in bacterial nodulation formation by *Rhizobium* and others are involved in cell division, for example in *E. coli*). They have also been called CBA transporters to distinguish them from the ABC (ATPase Binding Cassette) family of ATPases. Genes for related RND divalent cation efflux systems have been isolated in *R. metallidurans* strains CH34 and 31A, *czr* (Cd^{2+} Zn^{2+} resistance), *cnr* (Co^{2+} Ni^{2+} resistance), and *ncc* (Ni^{2+} Co^{2+} resistance) [47, 58, 68, 81].

Bacterial cadmium resistance

Cadmium resistance is found widely in environmental and clinical bacteria. This differs from Hg and As resistances in that no oxidation/reduction chemistry or other enzyme transformations are involved and in that there are different efflux pump mechanisms, a P-type ATPase and also chemiosmotic pumps in both Gram-positive and Gram-negative bacteria. For the best-studied examples, the CadA ATPase of *S. aureus* (encoded by an early "antibiotic resistance plasmid" and the CzcCBA complex of *R. metallidurans* CH34 (isolated from a zinc waste discard basin) [68], these were the initially studied examples of more general families of genes and proteins that pump cations other than Cd^{2+} out of bacteria as well. There are now known P-type efflux ATPases for Ag^{+}, Cu^{+}, Zn^{2+}, Ni^{2+}, and Pb^{2+} and three polypeptide RND (CBA) systems that pump out Cd^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} [68, 81].

The cation diffusion facilitator family (CDF) of single polypeptide chemiosmotic efflux systems was first described with the CzcD Cd^{2+} and Zn^{2+} efflux system of *R. metallidurans* [46, 81]. Additional members of this family include the Zn^{2+} efflux systems ZitB of *E. coli* and ZntA of *S. aureus* and the FieF ferrous iron efflux system of *E. coli* and *R. metallidurans* [6, 43, 75, 117]. CDF homologs are found encoded on many bacterial genomes and also in Archaea, yeast, plants and animals [46]. Seven CDF proteins are recognized as encoded in the human genome [46, 81]. Thus the CDF family is found widely in many forms of life, as are P-type ATPases, while the RND chemiosmotic proton/divalent cation exchangers are found only in bacteria [81]. Recent kinetic analysis of purified *E. coli* CDF proteins in proteoliposomes indicates functioning as a divalent cation/proton exchange system [22, 23] with these small proteins with six transmembrane alpha-helical regions apparently as a homo-dimer [46].

Although there is cation transport and resistance analysis for various one-polypeptide CDF family pumps, three-polypeptide RND inorganic cation chemiosmotic pumps, and soft metal P-type ATPases, none has been solved by x-ray diffraction or NMR analysis. However, the Ca^{2+} P-type ATPase of animal muscle has a well determined structure [55, 112] (Figs. 1,

6a) and components of the acridine Acr efflux RND resistance system of *E. coli* [37, 121, 122] have good structural understanding. These two models are sufficiently similar in sequence and function as to serve us well today.

In an elegant series of protein structural studies of the Ca^{2+} efflux P-type ATPase from mammalian muscle membrane vesicles, Toyoshima and colleagues [112, 113, 114] established the domain structure of the P-type ATPase and how these domains move relative to one another during function. This is given in detail here as a model for how microbial P-type ATPases will be found to function and since microbiologists rarely follow understanding of this best-studied P-type ATPase. Numerous intermediate steps are recognized in a reaction cycle that includes transport of Ca^{2+} outwards coupled with H^+ moving in to the cytoplasm, driven by a cycle of phosphorylation of an aspartate residue central to all P-type ATPases and later dephosphoryation of that residue. The eight stages of the cycle [55] begin with (1) uptake of 2 Ca^{2+} and release of 2–3 H^+ on the cytoplasmic surface of the membrane embedded component of the ATPase, then (2) the binding of ATP + Mg^{2+} to the nucleotide-binding domain (N; Fig. 6a) of the cytoplasmic region of the ATPase. (3) ATP is hydrolyzed and the gamma phosphate transferred to the active-site aspartate residue, followed by (4) release of the ADP and a massive allosteric change in domain relationships. (5) The phosphorylated ATPase protein takes up 2–3 H^+ at the outer membrane surface and releases the 2 Ca^{2+} cations. This fifth step is rate limiting and irreversible, so that after the cation exchange, the enzyme cannot reversibly synthesize ATP. The protein intermediate states have been traditionally called E1 for high energy Ca^{2+} -binding and E2 for low energy non-Ca^{2+} binding [112]. Next (6) H_20 attacks the phosphorylated aspartate, (7) inorganic phosphate and Mg^{2+} are released; and (8) the ATPase domains refold to the high energy Ca^{2+} binding form.

The P-type Ca^{2+} efflux ATPase has four readily discerned protein domains and five allosteric relationships between domains and substrate-binding motifs [55]. (1) Within the membrane domain are ten predominantly alpha-helical trans-membrane hydrophobic stretches. These move relative to one another during the reaction cycle. The cytoplasmic domains are called (2) nucleotide-binding (N; Figs. 1, 6a) between transmembrane alpha helices 4 and 5, (3) phosphorylation (P) also between helices 4 and 5, and (4) an activator/phosphatase (A) domain between helices 2 and 3 (Fig. 6a).

Each P-type ATPase has highly conserved amino acid sequence motifs shared by all P-type ATPases (bacterial, plant and animal) or at least those with similar cation specificities. Although we still are unable to explain cation specificity in terms of specific amino acid residues at key catalytic locations within the proteins, progress is being made [e.g. 7, 29, 66, 83]. For example, the initial cadmium and copper "soft Lewis acid" divalent cation ATPases for toxic metal ion efflux have eight trans-membrane helices [100, 107] with a conserved Cys Pro Cys or Cys Pro His motif in helix #6. With more sequences available, the Cys is sometimes found following the invariant Pro residue with the His residue preceding it [7, 66]. The "hard Lewis acid" Ca^{2+}/H^+, Mg^{2+}/H^+ and Na^+/K^+ transport ATPases have Glu Pro Val in the equivalent helix #4.

The nucleotide-binding domain includes a shared motif of GDGXNDXP toward the carboxyl-end of the domain sequence. The phosphorylation site TGTKD and the dephosphorylation motif TGES are shared by all P-type ATPases [100, 107].

The five protein structural states of the Ca^{2+} ATPase are (1) the fourth, fifth, sixth and eighth trans-membrane helices form the high affinity inside Ca^{2+}-binding site, with these helices rotating and bending during the reaction cycle. Helix 1 bends to close the Ca^{2+}-binding site. (2) When ATP binds to the N domain, it rotates above the P domain allowing close proximity to the gamma phosphate with the aspartate. (3) ATP hydrolysis and ADP release leads to the 110° rotation of the activator/phosphatase domain so that the TGES phosphatase motif is in contact with the phospho-aspartate, which becomes accessible by outward rotation of the N domain. (4) Helices 4, 5 and 6 rotate and bend, eliminating the tight Ca^{2+}-binding site. Movement of the 4th and fifth trans-membrane helices opens the outside surface for Ca^{2+} egress. After release of the phosphate and Mg^{2+}, (5) movement of the 1st, 2nd and 4th trans-membrane alpha helices allows reformation of the high-affinity Ca^{2+} binding site on the inner membrane surface. The figures (in [55, 112]) that show these kinetic and structural intermediate stages are beyond the scope of this brief review.

There is structural information but less resolution and structure/function understanding of the best-known CzcCBA RND family system, which is AcrAB/TolC that mediates the efflux of acridines and other small organic cations from *E. coli* [37, 49, 51, 62, 76–78, 111, 121, 122] (see http://www.tcdb.org/tcdb/index and http://www.biology.ucsd.edu/faculty/saier.html for current lists of RND systems).

As a basis for understanding how CzcCBA and other homologous systems function, some structural detail is warranted here. Unfortunately, the naming of RND component proteins is different for the two systems, so that the inner membrane protein CzcA is homologous to AcrB, the outer membrane protein CzcC is homologous to TolC and the periplasmic-space bridging protein CzcB is homologous to AcrA. There are no data supporting allosteric domain movements as for the Ca^{2+} P-type ATPase and indeed an open question as to the relationships and locations of the uptake and release sites for substrates such as acridines.

It is possible that the RND systems such as AcrAB/TolC and CzcCBA pick up efflux substrates from the outer lipid leaflet of the cytoplasmic membrane or the periplasmic space (Fig. 6b) and release them through the outer membrane porin proteins, rather than providing

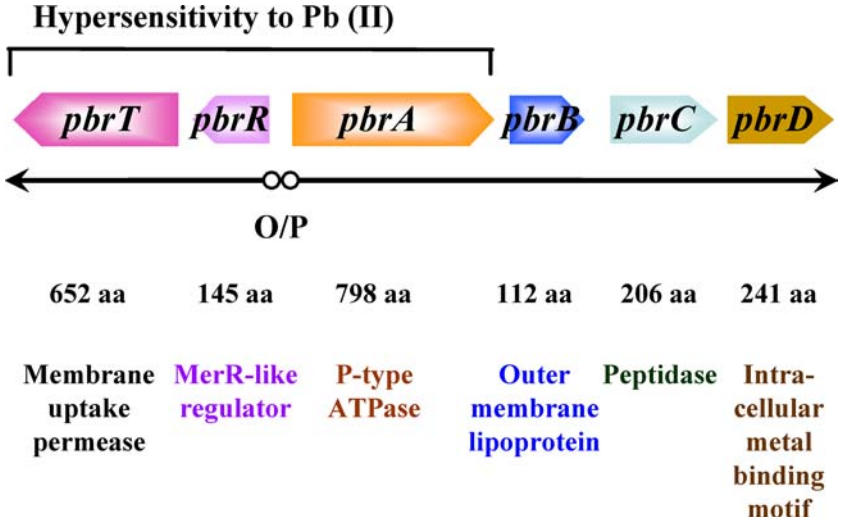

Fig. 7 The lead resistance operons of *R. metallidurans* CH34 (drawn from data of [15])

pathway for the entire distance from cytoplasm to outside the cell (Fig. 6b) [37]. Separate crystal structures of the inner membrane chemiosmotic pump protein AcrB [122], the outer membrane porin protein TolC [37] and as a substitute for the bridging protein AcrA, a homolog from *Pseudomonas* MexA [49] have been solved. This together with data showing the contacts between the three polypeptides in the periplasmic space [111] provide the basis for the model (Fig. 6b) [37, 121]. CzcA (like AcrB) contains four domains, two membrane embedded domains each with 6 membrane-spanning alpha helical regions (that are thought to form the cation and H^+ pathways) and two periplasmic domains of approximately equal size [41]. Three essential residues, Asp402, Asp408 and Glu415 located in the predicted 4th transmembrane helix were essential for cation resistance [41], while Cys and His residues in this region were not required. Asp408 and Glu415 are conserved in AcrB and other homologous sequences. CzcA (like AcrB) might occur in the membrane as a trimer of identical subunits, with relatively little contact between the monomer protein surfaces [122].

Between the intracellular N- and C-terminal sequences of membrane-embedded CzcA (or AcrB), the large periplasmic region, where the two periplasmic domains of three monomers each, form the wall of a large central cavity (Fig. 6b), which is in contact at its top with the opening in the outer membrane protein CzcC (or TolC) and in addition at the monomer interfaces CzcC (or TolC) has three "vestibule" spaces bounded by the outer leaflet of the inner cytoplasmic membrane and the periplasmic space (Fig. 6b). In the currently-accepted model, the substrates of the AcrBA TolC complex are picked up at the vestibule by the complex functioning as a macromolecular "carpet sweeper" and substrate passed through to the funnel cavity and from there to the outer membrane protein TolC (or CzcC). TolC (and probably CzcC) as a monomer bridges from half-way across the periplasmic space where it docks with the CzcA trimer across the outer membrane to the cell surface (Fig. 6b). The pore through TolC (or CzcC) is bordered by a helix of alpha helices in the periplasmic space and a beta barrel structure in the outer membrane. The MexA (homolog of CzcB) structure is less complete with both N- and C-terminal regions lacking fixed positions, when crystallized by itself, without inner or outer membrane components [49]. MexA (CzcB) is thought to be anchored by its N-terminus to the inner cell membrane and to make contact with the periplasmic domains of both AcrB (CzcA) and TolC (CzcC) (Fig. 6b). In the absence of structural data, the functioning of the CzcCBA efflux system demonstrated the efflux of Cd^{2+}, Zn^{2+} and Co^{2+} cations from whole cells or when purified and reconstituted in subcellular membrane vesicles (summarized by Nies [81]). The cross vesicle pH gradient drove divalent cation transport. Aspartate and glutamate residues in the trans-membrane domain of CzcA are considered essential for H^+ movement [41, 81].

Lead resistance

Bacterial lead resistance has long been suspected from environmental microbiology studies, when some bacteria grow at higher concentrations of Pb^{2+} than others. However, it is still unclear whether a single mechanism of Pb^{2+} resistance is commonly found or whether there are different mechanisms in different bacterial types.

Recently the genes that confer Pb^{2+} resistance for a specific microbe, *R. metallidurans* strain CH34, were cloned and sequenced. The DNA sequence of the Pb^{2+} resistance genes provided considerable understanding (Fig. 7) [15]. The six genes in the lead resistance region are organized in probably two divergently transcribed operons (Fig. 7). An operator/promoter region (with 19 nucleotides between the −10 and −35 RNA polymerase-binding motifs) that binds the positively acting (MerR-like) PbrR was found in the middle (Fig. 7), with presumably bidirectional transcription from that position.

The left-to-right transcript shown in Fig. 7 was synthesized inducibly on Pb^{2+} addition [15]. RT-PCR analysis showed that *pbrABCD* were co-transcribed as a single mRNA. The binding of PbrR to the DNA promoter site showed a 1,000-fold preference for Pb^{2+} over other divalent cations including Zn^{2+} [25]. The first gene product PbrT appears to be a membrane protein of the "permease" class that may carry out Pb^{2+} uptake. The question arises as to why one would wish to accumulate a toxic cation such as Pb^{2+}. However, cells with the *pbr* Pb^{2+} resistance system accumulated 3–4 times more Pb^{2+} than cells lacking this system.

PbrD appears to be an intracellular Pb^{2+} -binding protein. Its sequence contains a Cys-rich potential metal- binding motif (Cys-X_7-Cys-Cys-X_7-Cys-X_7-His-X_{14}-Cys) and the predicted metal-binding region of PbrD contains a large number of Pro and Ser residues between the Cys residues. Deletion of the *pbrD* gene lowered Pb^{2+} accumulation back to sensitive cell levels [15]. The left-most and right-most genes in Fig. 7 may encode a resistance mechanism based on uptake followed by intracellular sequestration. The first gene in the long *pbrABCD* operon, *pbrA* encodes a large P-type ATPase of the CysProCys soft metal efflux pump family [107]. The PbrA P-type ATPase shares the other general sequence properties of all P-type ATPases including the muscle Ca^{2+} ATPase. PbrB is predicted to be a small outer membrane lipoprotein, suggested to assist in removal of Pb^{2+} that was pumped by PbrA into the periplasmic compartment. PbrC might be the signal peptidase that removes the signal peptide from a lead resistance protein transported to the periplasmic space, probably PbrB. However, at this time, only the DNA sequence is available. Direct experimental tests are needed.

In contrast to the Pbr system of *R. metallidurans*, Levinson et al. [60] found intracellular lead phosphate precipitates in *S. aureus* cells with plasmid-governed Pb^{2+} resistance. Cell-surface Pb-thiol precipitates were obtained with genetically-engineered bacteria [8, 116]. Using cells of *R. metallidurans* strain CH34, Diels et al. [32] obtained practical bio-removal of Cd^{2+} (and other toxic cations) from soil by sequestration apparently by binding on outer cell proteins. Although these (and older) reports are all preliminary, it appears that intracellular and extracellular binding of Pb^{2+} may provide additional mechanism(s) for lead resistance.

Zinc resistance

Zinc differs from the toxic cations described above, in that it is also a nutrient, absolutely required for some enzymes and at low concentrations by all (or most) cells. So the problem for the bacterial cell is "homeostasis", to bring in sufficient Zn^{2+} for nutrition by membrane uptake pumps and to regulate by different membrane efflux pumps for resistance to excess Zn^{2+} [13]. The same pump does not work in both directions depending on concentration, but rather there are separate uptake and efflux pumps. Table 2 has a brief summary of many Zn^{2+} translocating uptake and efflux system. Some are relatively restricted to Zn^{2+} as substrate; others are less restrictive and also pump Cd^{2+}, Co^{2+}, Mn^{2+}, Pb^{2+} and even Mg^{2+}. Unfortunately, sometimes the same 3-letter mnemonic is used for fundamentally different systems in different bacteria (Tables 1, 2). For example, ZntA is the zinc-transporting P-type efflux ATPase found in *E. coli* and in *R. metallidurans* [13, 81], but ZntCBA are the components of the Zn^{2+} effluxing RND system in cyanobacteria [20] homologous to CzcCBA of *Ralstonia*. Generally insufficient transport and genetic experiments have been done to understand the full range of divalent cations for many of these systems. ZitB from *E. coli* and CzcD from *R. metallidurans* are chemiosmotic Zn^{2+} transporters of the cation diffusion facilitator (CDF) protein family [81]. The uptake of radioactive Zn^{2+} by inside-out membrane vesicles containing ZitB, the equivalent of efflux by right side out membranes of whole cells, was recently demonstrated [6], making more mechanistic studies possible.

In addition to membrane transport pumps moving Zn^{2+} in to and out from bacterial cells, a few bacteria encode a metallothionein protein, which is a small poly-

Table 1 Bacterial heavy metal resistance systems and mechanisms

Toxic ions	Gene mnemonic	Protein function
Hg^{2+} and organomercurials	*mer*	Mercuric reductase and transport
AsO_4^{3-} and $As(OH)_3$	*ars*	Arsenate reductase and transport
$As(OH)_3$	*aso*	Arsenite oxidase and transport
AsO_4^{3-}	*arr*	Respiratory arsenate reductase
Cd^{2+}	*cad*	P-type efflux ATPase
Cd^{2+}, Zn^{2+}, Co^{2+}	*czc*	CBA efflux permease
Co^{2+}, Ni^{2+}	*cnr*	CBA efflux permease
Ni^{2+}, Co^{2+}, Cd^{2+}	*ncc*	CBA efflux permease
Ni^{2+}	*nre*	CBA efflux permease
Cu^{2+}, Cu^{+}	*cop* and *pco*	Copper resistance and transport
CrO_4^{2-}	*chr*	Chromate efflux permease
Ag^{+}	*sil*	Silver resistance and binding
Pb^{2+}	*pbr*	Lead resistance and efflux
$TeO3^{2-}$	*tel, teh*,or *kil*	Tellurite resistance

Table 2 Zn^{2+} bacterial transporters: names and mechanisms

Name	Functional family	Transport direction	Bacteria
ZnuABC	ABC ATPase	Uptake	*Escherichia coli*
MntH	Nramp chemiosmotic	Uptake	*E. coli*
ZupT	ZIP chemiosmotic	Uptake	*E. coli*
CorA	Divalent cation chemiosmotic	Uptake	*E. coli*
ZiaA	P-type ATPase	Efflux	cyanobacteria
ZntA	P-type ATPase	Efflux	*E. coli*
CadA	P-type ATPase	Efflux	*Staphylococcus aureus*
CzcCBA	RND chemiosmotic	Efflux	*R. metallidurans*
ZntCBA	RND chemiosmotic	Efflux	cyanobacteria
CzcD	CDF chemiosmotic	Efflux	*R. metallidurans*
ZntA	CDF chemiosmotic	Efflux	*S. aureus*
ZitB	CDF chemiosmotic	Efflux	*E. coli*

Summary from [13, 20, 42, 81].

thiol cation-binding protein [14, 20]. Metallothionein functions as a cytoplasmic metal cation-binding protein, lowering the free concentrations within the cytoplasm and therefore affording a level of toxic cation resistance. Bacterial metallothionein polypeptides are not homologous in sequence and are therefore unrelated by evolution to the metallothionein of animal cells, which contains as many as 20 cysteines per 60 amino acids length. Whereas eukaryote metallothionein is frequently considered a binding reservoir for Cd^{2+} and Cu^{2+} cations, the cyanobacterial metallothionein seems primarily to function in Zn^{2+} homeostasis [20].

The cyanobacterial metallothionein binds 4 Zn^{2+} cations in a structure involving nine cysteine thiols (found in a 56 amino acid polypeptide) and that has been determined by NMR analysis [14, 20]. A related polypeptide found in two *Pseudomonas* strains bound three Zn^{2+} with ten cysteine thiols [14]. Two Zn^{2+} in the cyanobacterial metallothionein coordinate with four cysteines each, while the other two are bound as a Zn^{2+}/3 cysteine/1 histidine binding site.

Nickel transport and resistance

Ni^{2+}, like Zn^{2+}, is both required as a micronutrient and toxic in excess, without being subject to bacterial redox chemistry. Then, there are Ni^{2+} uptake and Ni^{2+} efflux pumps found in bacterial cells [68, 74]. These are frequently shared with regard to substrate and referred to as Ni^{2+} Co^{2+} transporters [48]. Ni^{2+} uptake can occur either by a single polypeptide chemiosmotic carrier or by a five component ABC ATPase [74]. The five gene products for the *E. coli* ABC Ni^{2+} uptake transporter are NikA (a soluble periplasmic Ni^{2+} -binding protein), NikB and NikC (membrane proteins that form the transmembrane channel) and NikD and NikE (which form the intracellular ATPase part of the complex) [74].

Ni^{2+} (together with Co^{2+}) uptake systems (called NiCoT) are also present in other bacteria, Archaea and fungi, especially with microbes known for high nickel requirements [48], such as hydrogen-metabolizing bacteria (for example the HoxN permease of *Ralstonia eutropha* and the NixA permease for urease production by *Helicobacter pylori*) [74]. New nickel/cobalt resistance systems continue to be found, including a single polypeptide efflux protein in *E. coli* [89]. The single polypeptide Ni^{2+} Co^{2+} permease is predicted to have eight transmembrane alpha helices with a $HisX_4AspHis$ region in helix 2 of HoxN thought to form the Ni^{2+} binding motif [28].

Ni^{2+} efflux occurs via the three component chemiosmotic CBA family complex such as the Cnr and Ncc systems of *R. metallidurans* strains CH34 and A31 [68, 81] that are associated with Ni^{2+} resistance. Thus, Ni^{2+} as other micronutrients that are toxic at high levels is closely regulated for both uptake and efflux.

Copper transport and homeostasis

Because of its critical cellular roles and involvement in oxidation/reduction-active enzymes, copper transport and resistances has been extensively studied in recent years, especially in *E. coli* [87] and *Enterococcus hirae* [105, 106]. Copper is different from cations considered above, in that both Cu^{2+} and Cu^{+} occur and redox chemistry is closely linked to transport and homeostatic equilibrium.

Cu^{2+} is more abundant in aerobic environments such as the extracellular milieu and is less toxic than Cu^{+}, which is intracellular (although not as a free cation). It is not known whether reduction from Cu^{2+} to Cu^{+} occurs at the cell surface prior to transport, or during or at the cytoplasmic membrane surface on entry. However it is thought that intra-cellular copper is exclusively Cu^{+}. *E. coli* grows in millimolar range added Cu^{2+}, whereas 1000x-fold lower micromolar concentrations inhibit growth of cyanobacteria, a different class of Gram negative bacteria. We do not know the basis for this difference. It is also unclear in terms of resistance efflux pumps to what extent Cu^{+} and Ag^{+} are shared substrates of single systems or separately functioning Cu^{+}

and Ag^+ resistance systems occur, with some cross recognition of substrates [87, 99].

CopA is the P-type Cu^+ efflux ATPase of *E. coli* (Fig. 8a) [87] responsible for keeping intracellular copper low, so that probably not a single free Cu^+ is present at equilibrium and unidirectional kinetics rather than equilibrium concentrations dominate copper homeostasis. In the extracellular periplasmic space of Gram negative *E. coli*, the CueO multi-copper oxidase [104] and the CusCFBA multicomponent efflux transport system (Fig. 8a) appear to protect the cell [38, 87]. In spite of the research on copper metabolism with the best studied bacterium, *E. coli*, the usual mechanism for copper cation uptake and intracellular copper movement to specific enzymes are not known [87]. Although the CopA P-type ATPase (Fig. 8a) has the typical properties of a soft-metal cation efflux ATPase discussed earlier for the CadA cadmium efflux ATPase (Fig. 6), understanding is lacking at the sequence level of what provides Cu^+ specificity for CopA as contrasted with Cd^{2+} specificity for CadA or uptake versus efflux orientation (Fig. 8a).

The CusCFBA RND efflux chemiosmotic carrier of *E. coli* has been studied in detail [38, 87]. In addition to the CusCBA proteins similar to those of CzcCBA (Fig. 6b), the small CusF protein has been shown to be an essential periplasmic copper binding protein [38]. CusCFBA confers Cu^{2+} resistance only anaerobically or when there is a mutation in *cueO* [38]. Otherwise the CusCFBA system confers Ag^+ resistance but not that to Cu^{2+}. The CusCFBA protein sequences are closely similar to those of the SilCFBA gene products conferring Ag^+ resistance [44], which strengthens the idea that this class of mono-valent cation RND system with the fourth small CusF-like component is recognizably different from divalent cation RND efflux systems such as CzcCBA. The sequences of the CusCBA products are predicted to be the inner membrane efflux pump, the periplasmic membrane coupling protein and the outer membrane porin protein respectively. Six residues A399 D404 D412 M573 M623 M672 that are conserved in the A inner membrane RND protein sequences for monovalent or for monovalent and divalent cations were needed for full resistance [38]. The additional small gene *cusF* encodes a small protein that after removal of the leader sequence was found in the periplasm. CusF is thought to function as a periplasmic "chaperone" protein bringing Cu^+ to the CusCBA efflux pump, with a single copper cation bound with involvement of methionine residues M69 and M71. A high-resolution X-ray diffraction structure for CueO copper oxidase includes T1, T2 and T3 copper-binding motifs [88].

In addition to the chromosomally-determined copper homeostasis gene products just described, some *E. coli* strains have an additional Pco plasmid copper resistance system ([16]; reviewed by Cooksey [26], Rensing and Grass [87]) with homologous genes also found in *R. metallidurans* CH34 [68]. PcoA, PcoE and PcoC are periplasmic proteins that bind copper. PcoA substitutes for chromosomally-determined CueO and therefore is likely to have copper oxidase activity. In sum, the plasmid *pco* determinant is thought to moderate periplasmic binding and oxidation of excess copper cations carrying out transmembrane transport.

In cyanobacteria, there are two copper P-type ATPases, CtaA which brings copper into the cytoplasm, and PacS which pumps copper cations from the cytoplasm to the thylakoid compartment within the cell, where photosynthesis occurs [20]. A third copper transport protein Atx1 functions as a copper ion "chaperone" (similar to that found in yeast cells), bringing copper ions from site to site within the cytoplasm that in practical terms probably has no free copper at any stage [20]. A NMR-determined structure for Atx1 of cyanobacteria shows a β-α-β-β-α fold and a 2 Cys/1 His predicted copper binding motif in a looped turn.

Silver resistance

Silver ions are highly toxic to all microorganisms, perhaps due to poisoning of the respiratory electron transport chains and components of DNA replication. Bacterial Ag^+ resistance has been reported repeatedly but the genetic basis was not understood until recently. This was recently reviewed in depth in the *FEMS Microbiol Rev* "BioMetals issue" [99].

The Sil Ag^+ resistance system studied in most depth is from *Salmonella* plasmid pMG101 which contains nine genes governing Ag^+ resistance in its 180 kb length. Other plasmids [45] and about 10% of a random Chicago hospital enteric bacterial collection have genes for Ag^+ resistance [99].

The nine gene silver resistance determinant [44, 99] starts with the *silE* gene that determines a small periplasmic metal-binding protein that is unrelated to other metal-binding polypeptide domains, except for the PcoE polypeptide of an *E. coli* copper resistance system [44]. Upstream from *silE*, a gene pair, *silRS*, determining proteins involved in transcriptional gene regulation occur. The predicted regulatory responder protein (SilR) and membrane sensor kinase (SilS) are homologous to other members of the large two-component family. The remaining six open reading frames in the Ag^+ resistance system are transcribed in the opposite orientation from *silRSE* [44, 99] with the *silCBA* genes determining a three-polypeptide RND membrane potential-driven cation/proton exchange complex [99] quite similar to the CzcCBA complex shown in Fig. 6. This complex includes the SilA inner membrane proton/cation pump protein, the SilB membrane fusion protein that is predicted to physically contact SilA and SilC and the SilC outer membrane protein [99]. The product of the last gene of the silver resistance determinant, SilP, is predicted to be a P-type ATPase, similar to CadA shown in Fig. 6 and an additional subclass of differing cation specificity of the large family of P-type efflux ATPases. The silver resistance determinant is unique among resistance systems in encoding both a periplasmic metal-

binding protein and both chemiosmotic and ATPase efflux pumps [99].

Tellurium resistance

There are several known and sequenced tellurite resistance determinants but little is known of how they work. There is evidence of microbial reduction of tellurite to black metallic Te^0, of enzymatic conversion to volatile methyl or hydride compounds. and of membrane transport of tellurite out from microbial cells. However, it is unfortunate that none of these is clearly established as a resistance mechanism and that research has not progressed much beyond previous similar statements [101].

Organotin resistance

Organotin compounds, especially tributyltin, were used for many years in antifouling paints to prevent animal attachment to ships [24, 52, 118]. Inevitably the organotin compounds were released and accumulated in sediments, especially in harbors. At a peak, about 35,000 tonnes of organotin compounds were released per year, with the USA accounting for 30% of that total. Eventually, country by country, organotin-containing marine paints were forbidden, earlier specifically on shorter recreational ships, so that ocean-going commercial ships could continue to use these effective compounds. Tributyltin is massively inhibitory for invertebrate animals and for microbes [27], apparently by inserting within and disrupting membrane function. As expected, microbes with organotin resistance and microbes that degraded organotin compounds to inorganic Sn(IV) were repeatedly found [52, 70, 118, 120]. Although microbial methylation of inorganic Sn has been reported and microbial degradation of organotin compounds is considered essential in bioremediating both water and sediment pollution [52, 118], these processes have been little studied. Microbes can also produce methyltin from inorganic tin compounds [65].

Inorganic stannous fluoride (SnF_2) is frequently the basis for fluoride-containing toothpastes. In addition to effects on the physical structure of the tooth, these preparations are considered to have anti-bacterial activities and we might expect that bacteria resistant to inorganic Sn^{2+} will be found.

Other metal resistances

There are additional inorganic metal resistance mechanisms, some listed in Table 1, for which both space and the absence of in depth understanding preclude more than passing consideration. Toxic chromate [CrO_4^{2-}; Cr(VI)] is taken up by microbial cells via sulfate [SO_4^{2-}] transport mechanisms and chromate is both reduced to Cr(III), which precipitates intracellularly as $Cr(OH)_3$ and pumped out from the cell by the ChrA membrane efflux protein [82]. Cervantes et al. [21] is the most recent summary of understanding of chromate microbiology. Although microbial chromate reduction has been found in many microbial types, this is not basis for known plasmid-determined chromate resistance. Rather the ChrA efflux pump which passes through the membrane 12 times appears to use the membrane potential and proton gradient to remove $Cr0_4^{2-}$ from the cell. ChrA is not related by sequence to other families of membrane efflux proteins but rather appears to be a broadly-found family in bacteria from *Pseudomonas* to cyanobacteria and also in Archaea. The activity of ChrA has been demonstrated in vitro as uptake by inside out membrane vesicles [21]. Recently, a series of random mutant strains isolated as chromate sensitive identified 14 residue positions (of the 416 amino acids in ChrA) that are needed for chromate resistance. The positions affected seemed mostly in the N-terminal half of the protein and occurred both in the membrane segments and cytoplasmic loop regions [1].

Additional microbial metal ion resistances might be anticipated for Al^{3+}, where the importance of acidity in keeping Al^{3+} in solution make acidophilic bacteria good candidates. There are preliminary reports of boron and bismuth resistances, but adequate studies have not been made and bismuth may be associated with As^{3+}, Pb^{2+} or Cd^{2+} resistance systems, as it is well understood that antimony Sb^{3+} resistance is closely associated with As^{3+} resistance [103]. Thalium (Tl^+) resistance has been reported but not studied. One hypothesis might be that Tl^+ functions as a K^+ analog for either uptake or efflux pumps. These are all topics for future work.

Repressors and activators

Basically all bacterial toxic ion resistance systems are determined by clusters of contiguous genes (two to more than ten) functioning as operons (units of mRNA transcription) that are regulated by proteins that bind to the upstream operator DNA regions. In general, four protein classes of metal-responding transcriptional regulators are most frequently found: (a) The MerR family of homo-dimeric activator proteins bind to usually long-10–35 RNA polymerase-binding operator regions (19 or 20 nucleotide spacing). On activation of MerR by Hg^{2+} binding, the DNA bends and twists [9, 17a] to a position that opens the DNA structure allowing mRNA synthesis to initiate. MerR (for Hg^{2+} regulation) is the best understood member of this family [9, 17a] and basically all *mer* operons from Gram negative and Gram positive bacteria function with MerR activator proteins. A Zn^{2+}-responding equivalent ZntR and copper-responding CueR have been studied and others responding to different metal cations and other activators are recognized [17a]. With more genomes analyzed,

more will be found. (b) The second class of metal-responding regulators, ArsR/CadC/SmtB, were the first recognized members of the widely found family of repressors that act negatively, binding to the 17-spaced -10–35 operator region preventing RNA polymerase binding. Upon protein binding of the co-repressor ion, the repressor is released allowing transcription to occur. The ArsR/CadC/SmtB family proteins are homo-dimeric winged helix repressor proteins. When the co-repressor (As[III] for ArsR, Cd^{2+} for CadC, Co^{2+} or Zn^{2+} for *S. aureus* chromosomal CzrA or a range of divalent cations for *Synecococcus* SmtB) bind to the protein, it is released from the DNA allowing transcription of the genes controlling resistance to the metal ions to occur [33]. Busenlehner et al. [18] summarize recent progress in understanding by biophysical analysis of the functioning of these proteins and different modes of inorganic inducer ion binding. The divalent cation Zn^{2+} binds to a pair of tetrahedral, binding sites, with two ligands derived from an alpha helix on one subunit (Asp84 and His86, SmtB numbering) and two derived from an alpha helix of the other subunit (His97 and His100). Formation of the metal protein chelate drives a quaternary structural switch mediated by an intersubunit hydrogen-bonding network that stabilizes the DNA-binding conformation. The structure of the Zn^{2+}-SmtB homo-dimer shows that both metal-binding sites of the dimer must be occupied for the quaternary structural switch to occur.

A third class of transcriptional regulatory proteins has been found in several metal resistance systems. These are the "two component" transmembrane histidine kinase membrane sensor (S) that autophosphorylates at the conserved His residue in response to the extracellular stimulus. The phosphate is then transferred to a conserved aspartate on the second (R) Responder regulatory protein, which is then either activated or inactivated for DNA-binding transcriptional control. Metal-responding RS systems include plasmid PcoRS and chromosomal CusRS copper resistance systems [16, 87], the silver resistance SilRS products [99] and CzcRS of the *Ralstonia* CH34 cadmium/zinc/cobalt resistance system [68]. Homologs of these systems are found in other bacteria, including *Pseudomonas* [90]. The fourth transcriptional regulatory class at this time includes only the YXH complexes involved in control of the Cnr and Ncc divalent cation resistance systems of *Ralstonia* [68]. The H proteins are sigma factor subunits of RNA polymerase and respond to environmental cation addition and the Y proteins are considered to be X-binding anti-sigma factors. Multiple promoter sites are found, one for the regulatory genes and another for genes determining the efflux pump.

There are sometimes what appears to be an additional class of second level regulatory proteins in addition to the primary transcriptional regulators. These include MerD of the mercury-resistance system (Fig. 2), ArsD of the arsenic resistance system and CopZ of copper homeostasis (Fig. 8b). In each of these three cases, there are results interpreted as direct roles in gene regulation and also data supporting roles as "chaperones", that is intracellular carrier proteins whose role is to pick up the toxic ion reducing free cellular levels and to specifically transfer the inorganic ion to the transcriptional regulator or another ion-specific protein [9, 106].

Phytoremediation

Phytoremediation is the use of plants to clean up environmental pollution [85]. Although one promising

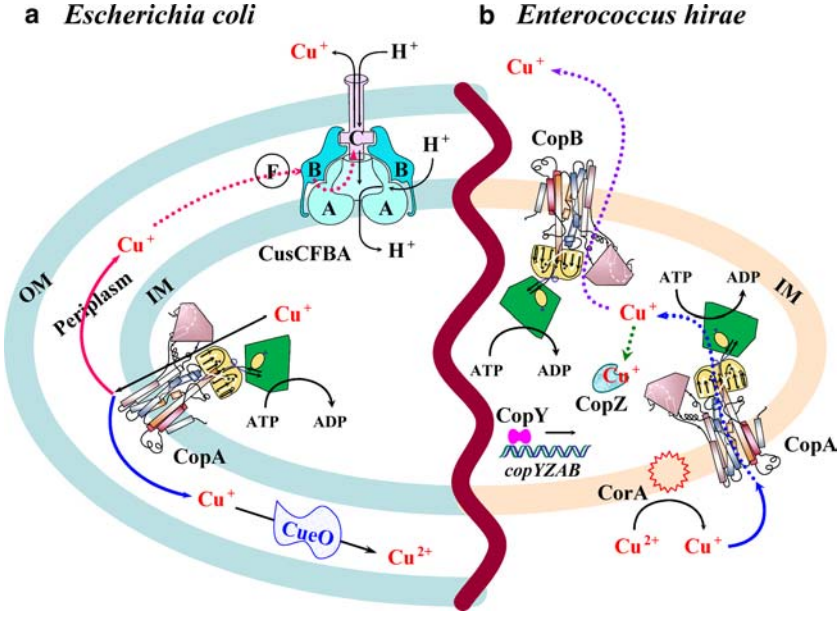

Fig. 8 Current view of copper transport and resistance in **a** *Escherichia coli* and **b** *Enterococcus hirae* (modified from [87, 106])

prospect from the availability of specific genes (and processes for resistances) to toxic heavy metals in microbes is their potential for microbial bioremediation, either with natural isolates [85] or with genetically modified organisms [53, 67], such efforts have not gone far. It seems only that mercury is microbially volatilized from wet soil and aqueous polluted sites such as rivers and sewage works. Arsenic is undoubtedly mobilized by microbial activity and the potential for phytoremediation immobilizing arsenic in soil and water is important [3, 67].

Phytoremediation, however, has greater promise for useful processes, both because of the greater acceptance of plants for environmental enhancement than of microbial additions and because the roots of plants penetrate deeper in soil than we normally would introduce bacteria. Meagher [67] and LeDuc and Terry [57] have provided in depth reports on the promise of Phytoremediation in this issue. Both groups have used microbial genes (*merA, merB* and *arsC*) to produce transgenic plants with enhanced potential [12, 57, 67, 91, 92]. T. Kusano (submitted) has in addition expressed MerC in *Arabidopsis* and obtained enhanced uptake of Hg^{2+}. Although an in depth consideration of this topic is not useful here, it is notable that the potential for transgenic plants expressing bacterial toxic metal resistance genes in phytoremediation is great (Fig. 9). Two different and additional approaches to phytoremediation are under study, firstly the use of naturally-occurring "hyperaccumulating" plants (which are not discussed here, since these do not involve microbes) and secondly the role of microbes in the rhizosphere associated with such plants. Lodewyckx et al. [64] reported a 10x increase to about 80% in the frequency of toxic metal resistance levels of bacteria associated with a Zn^{2+} accumulating plant compared with soil microbe frequency of such resistances.

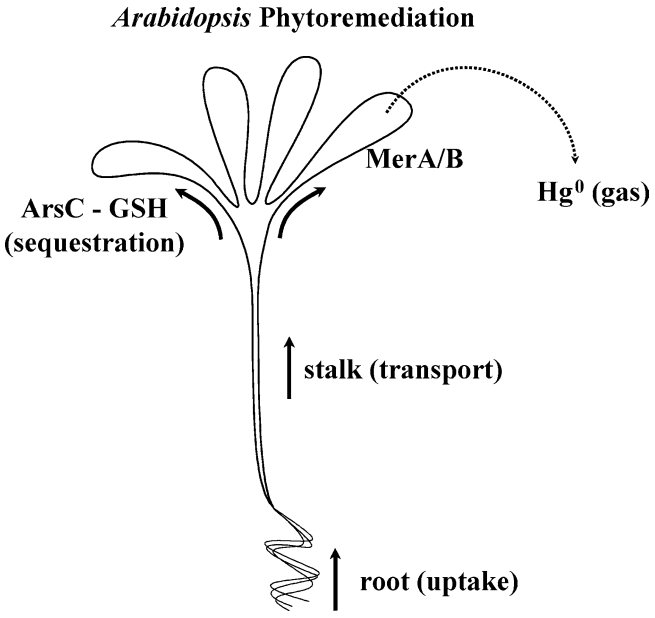

Fig. 9 Phytoremediation by bacterial genes in plants. Three stages of function by bacterial genes in flowering plants are known, including uptake of Hg^{2+} (T. Kusano et al., personal communication), reduction from organomercurials and inorganic mercury to volatile Hg^0 [67] and sequestration of As(III) in vacuoles after reduction from As(V) [67]

Genomics: a view from the top

While cloning and sequencing of specific genes for heavy metal resistances in bacteria has been an active topic for nearly two decades, total genome sequencing projects have more recently uncovered related systems, sometimes functionally equivalent. The rapid increase in available microbial genome data makes specific published references less useful than Internet sites, especially those for GenBank (http://www.ncbi.nlm.nih.gov), The Institute for Genome Research (TIGR) (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl) and JGI (http://genome.jgi-psf.org/mic_home.html) and a recent report on prokaryote genome annotation is available on line at http://www.asm.org/ASM/files/ccLibraryFiles/FILENAME/000000001309/ASM_GenomeAnnotation.pdf. Frequently untested genes provide an additional view of the generality of metal resistance systems and their ancient origin. For example, the 1.8-megabase total genome of *Haemophilus* includes predicted genes for arsenate reductase (*arsC*) and mercury transport (*merT* and *merP*) homologous to those earlier sequenced from bacterial plasmids. Many more genes are assigned functions from computer-based analysis than are these genes experimentally-shown to be functional. Our best guess is that many annotated MerP homologs are not periplasmic mercury-binding proteins, but rather intracellular sensors for detecting and regulating levels of different thiol-binding cations such as copper. The basis for predicting beyond mere sequence homologies is too uncertain to merit detailed discussion. Nevertheless, recent summaries of homologous transport systems in bacterial, Archaea and yeast genomes (see, e.g. http://www.tcdb.org/tcdb/index.php?tc=3.A.1) list a wide range of cation and oxyanion transporters, uptake systems and efflux pumps, plus the wide occurrence of many known families of systems among broadly diverse classes of microbes. The clear conclusion (although requiring caution and without experimental verification) is the ancient origin of inorganic cation and anion transport systems including those for toxic metal ions near the beginning of cellular life on Earth nearly 4 billion years ago [54].

There has been development of the practice of the initial attribution of open reading frames (ORFs) of newly sequenced genomes by attributing the same name as the closest related protein sequence available. Although these attributions are generally reasonable, they are frequently incorrect. The American Society for Microbiology report estimates that up to 5–10% of gene assignments might be wrong (see http://www.asm.org/ASM/files/ccLibraryFiles/FILENAME/000000001309/

ASM_GenomeAnnotation.pdf). Thus our current listings are a basis for gene isolation and further analysis but they have numerous errors and require a thoughtful reading.

References

1. Aguilera S, Aguilar ME, Chavez MP, Lopez-Meza JE, Pedraza-Reyes M, Campos-Garcia J, Cervantes C (2004) Essential residues in the chromate transporter ChrA of *Pseudomonas aeruginosa*. FEMS Microbiol Lett 232:107–112
2. Ahmann D, Roberts AL, Krumholz LR, Morel FMM (1994) Microbe grows by reducing arsenic. Nature 371:750
3. Alkorta I, Hernandez-Allica J, Garbisu C (2004) Plants against the global epidemic of arsenic poisoning. Environ Int 30:949–951
4. Anderson GL, Ellis PJ, Kuhn P, Hille R (2001) Oxidation of arsenite by *Alcaligenes faecalis*. In: Frankenberger WT Jr (ed) Environmental chemistry of arsenic. Marcel Dekker, New York, pp 343–361
5. Anderson GL, Williams J, Hille R (1992) The purification and characterization of arsenite oxidase from *Alcaligenes faecalis*, a molybdenum-containing hydroxylase. J Biol Chem 267:23674–23682
6. Anton A, Weltrowski A, Haney CJ, Franke S, Grass G, Rensing C, Nies DH (2004) Characteristics of zinc transport by two bacterial cation diffusion facilitators from *Ralstonia metallidurans* CH34 and *Escherichia coli*. J Bacteriol 186:7499–7507
7. Arguello JM (2003) Identification of ion-selectivity determinants in heavy-metal transport P_{1B}-type ATPases. J Membr Biol 195:93–108
8. Bang SW, Clark DS, Keasling JD (2000) Engineering hydrogen sulfide production and cadmium removal by expression of the thiosulfate reductase gene (*phsABC*) from *Salmonella enterica* serovar typhimurium in *Escherichia coli*. Appl Environ Microbiol 66:3939–3944
9. Barkay T, Miller SM, Summers AO (2003) Bacterial mercury resistance from atoms to ecosystems. FEMS Microbiol Rev 27:355–384
10. Begley TP, Ealick SE (2004) Enzymatic reactions involving novel mechanisms of carbanion stabilization. Curr Opin Chem Biol 8:508–515
11. Benison GC, Di Lello P, Shokes JE, Cosper NJ, Scott RA, Legault P, Omichinski JG (2004) A stable mercury-containing complex of the organomercurial lyase MerB: catalysis, product release, and direct transfer to MerA. Biochemistry 43:8333–8345
12. Bizily SP, Kim T, Kandasamy MK, Meagher RB (2003) Subcellular targeting of methylmercury lyase enhances its specific activity for organic mercury detoxification in plants. Plant Physiol 131:463–471
13. Blencowe DK, Morby AP (2003) Zn(II) metabolism in prokaryotes. FEMS Microbiol Rev 27:291–311
14. Blindauer CA, Harrison MD, Robinson AK, Parkinson JA, Bowness PW, Sadler PJ, Robinson NJ (2002) Multiple bacteria encode metallothioneins and SmtA-like zinc fingers. Mol Microbiol 45:1421–1432
15. Borremans B, Hobman JL, Provoost A, Brown NL, van der Lelie D (2001) Cloning and functional analysis of the *pbr* lead resistance determinant of *Ralstonia metallidurans* CH34. J Bacteriol 183:5651–5658
16. Brown NL, Barrett SR, Camakaris J, Lee BT, Rouch DA (1995) Molecular genetics and transport analysis of the copper-resistance determinant (*pco*) from *Escherichia coli* plasmid pRJ1004. Mol Microbiol 17:1153–1166
17. Brown NL, Morby AP, Robinson NJ (2003) Thematic issue. Interactions of bacteria with metals. FEMS Microbiol Rev 27:129–447

17a. Brown NL, Stoyanov JV, Kidd SP, Hobman JL (2003) The MerR family of transcriptional regulators. FEMS Microbiol Rev 27:145–163

18. Busenlehner LS, Pennella MA, Giedroc DP (2003) The SmtB/ArsR family of metalloregulatory transcriptional repressors: Structural insights into prokaryotic metal resistance. FEMS Microbiol Rev 27:131–143
19. Carlin A, Shi W, Dey S, Rosen BP (1995) The *ars* operon of *Escherichia coli* confers arsenical and antimonial resistance. J Bacteriol 177:981–986
20. Cavet JS, Borrelly GP, Robinson NJ (2003) Zn, Cu and Co in cyanobacteria: selective control of metal availability. FEMS Microbiol Rev 27:165–181
21. Cervantes C, Campos-Garcia J, Devars S, Gutierrez-Corona F, Loza-Tavera H, Torres-Guzman JC, Moreno-Sanchez R (2001) Interactions of chromium with microorganisms and plants. FEMS Microbiol Rev 25:335–347
22. Chao Y, Fu D (2004a) Kinetic study of the antiport mechanism of an *Escherichia coli* zinc transporter, ZitB. J Biol Chem 279:12043–12050
23. Chao Y, Fu D (2004b) Thermodynamic studies of the mechanism of metal binding to the *Escherichia coli* zinc transporter YiiP. J Biol Chem 279:17173–17180
24. Chau YK, Zhang S, Maguire RJ (1992) Occurrence of butyltin species in sewage and sludge in Canada. Sci Total Environ 121:271–281
25. Chen P, Greenberg B, Taghavi S, Romano C, van der Lelie D, He C (2005) An exceptionally selective lead(II)-regulatory protein from *Ralstonia metallidurans*: development of a fluorescent lead(II) probe. Angew Chem Int Ed Engl 44:2715–2719
26. Cooksey DA (1994) Molecular mechanisms of copper resistance and accumulation in bacteria. FEMS Microbiol Rev 14:381–386
27. Cooney JJ, Wuertz S (1989) Toxic effects of tin compounds on microorganisms. J Ind Microbiol 4:375–402
28. Degen O, Eitinger T (2002) Substrate specificity of nickel/cobalt permeases: insights from mutants altered in transmembrane domains I and II. J Bacteriol 184:3569–3577
29. DeSilva TM, Veglia G, Porcelli F, Prantner AM, Opella SJ (2002) Selectivity in heavy metal-binding to peptides and proteins. Biopolymers 64:189–197
30. Di Lello P, Benison GC, Omichinski JG, Legault P (2004a) ^{1}H, ^{15}N, and ^{13}C resonance assignment of the 23 kDa organomercurial lyase MerB in its free and mercury-bound forms. J Biomol NMR 29:457–458
31. Di Lello P, Benison GC, Valafar H, Pitts KE, Summers AO, Legault P, Omichinski JG (2004b) NMR structural studies reveal a novel protein fold for MerB, the organomercurial lyase involved in the bacterial mercury resistance system. Biochemistry 43:8322–8332
32. Diels L, De Smet M, Hooyberghs L, Corbisier P (1999) Heavy metals bioremediation of soil. Mol Biotechnol 12:149–158. Erratum in Mol Biotechnol 113:171
33. Eicken C, Pennella MA, Chen X, Koshlap KM, VanZile ML, Sacchettini JC, Giedroc DP (2003) A metal-ligand-mediated intersubunit allosteric switch in related SmtB/ArsR zinc sensor proteins. J Mol Biol 333:683–695
34. Ellis PJ, Conrads T, Hille R, Kuhn P (2001) Crystal structure of the 100 kDa arsenite oxidase from *Alcaligenes faecalis* in two crystal forms at 1.64 A and 2.03 A. Struct (Camb) 9:125–132
35. Engst S, Miller SM (1998) Rapid reduction of Hg(II) by mercuric ion reductase does not require the conserved C-terminal cysteine pair using $HgBr_2$ as the substrate. Biochemistry 37:11496–11507
36. Engst S, Miller SM (1999) Alternative routes for entry of HgX_2 into the active site of mercuric ion reductase depend on the nature of the X ligands. Biochemistry 38:3519–3529
37. Eswaran J, Koronakis E, Higgins MK, Hughes C, Koronakis V (2004) Three's company: component structures bring a closer view of tripartite drug efflux pumps. Curr Opin Struct Biol 14:741–747

38. Franke S, Grass G, Rensing C, Nies DH (2003) Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. J Bacteriol 185:3804–3812
39. Frankenberger WT Jr (ed) (2001) Environmental chemistry of arsenic. Marcel Dekker, New York
40. Frausto Da Silva JJR, Williams RJP (2001) The biological chemistry of the elements: the inorganic chemistry of life. Oxford University Press, Oxford
41. Goldberg M, Pribyl T, Juhnke S, Nies DH (1999) Energetics and topology of CzcA, a cation/proton antiporter of the resistance-nodulation-cell division protein family. J Biol Chem 274:26065–26070
42. Grass G, Franke S, Taudte N, Nies DH, Kucharski LM, Maguire ME, Rensing C (2005a) The metal permease ZupT from *Escherichia coli* is a transporter with a broad substrate spectrum. J Bacteriol 187:1604–1611
43. Grass G, Otto M, Fricke B, Haney CJ, Rensing C, Nies DH, Munkelt D (2005b) FieF (YiiP) from *Escherichia coli* mediates decreased cellular accumulation of iron and relieves iron stress. Arch Microbiol 183:9–18
44. Gupta A, Matsui K, Lo JF, Silver S (1999) Molecular basis for resistance to silver cations in *Salmonella*. Nat Med 5:183–188
45. Gupta A, Phung LT, Taylor DE, Silver S (2001) Diversity of silver resistance genes in IncH incompatibility group plasmids. Microbiology 147:3393–3402
46. Haney CJ, Grass G, Franke S, Rensing C (2005) New developments in the understanding of the cation diffusion facilitator family. J Ind Microbiol Biotechnol 32:215–226
47. Hassan MT, van der Lelie D, Springael D, Romling U, Ahmed N, Mergeay M (1999) Identification of a gene cluster, *czr*, involved in cadmium and zinc resistance in *Pseudomonas aeruginosa*. Gene 238:417–425
48. Hebbeln P, Eitinger T (2004) Heterologous production and characterization of bacterial nickel/cobalt permeases. FEMS Microbiol Lett 230:129–135
49. Higgins MK, Bokma E, Koronakis E, Hughes C, Koronakis V (2004) Structure of the periplasmic component of a acterial drug efflux pump. Proc Natl Acad Sci USA 101:9994–9999
50. Hoke KR, Cobb N, Armstrong FA, Hille R (2004) Electrochemical studies of arsenite oxidase: an unusual example of a highly cooperative two-electron molybdenum center. Biochemistry 43:1667–1674
51. Husain F, Humbard M, Misra R (2004) Interaction between the TolC and AcrA proteins of a multidrug efflux system of *Escherichia coli*. J Bacteriol 186:8533–8536
52. Jude F, Arpin C, Brachet-Castang C, Capdepuy M, Caumette P, Quentin C (2004) TbtABM, a multidrug efflux pump associated with tributyltin resistance in *Pseudomonas stutzeri*. FEMS Microbiol Lett 232:7–14
53. Karenlampi S, Schat H, Vangronsveld J, Verkleij JA, van der Lelie D, Mergeay M, Tervahauta AI (2000) Genetic engineering in the improvement of plants for phytoremediation of metal polluted soils. Environ Pollut 107:225–231
54. Koch AL, Silver S (2005) The first cell. Adv Microb Physiol 50 (in press)
55. Lancaster CRD (2004) Structural biology: ion pump in the movies. Nature 432:286–287
56. Lebrun E, Brugna M, Baymann F, Muller D, Lievremont D, Lett MC, Nitschke W (2003) Arsenite oxidase, an ancient bioenergetic enzyme. Mol Biol Evol 20:686–693
57. LeDuc DL, Terry N (2005) Phytoremediation of toxic trace elements in soil and water. J Ind Microbiol Biotechnol 32 (in press)
58. Legatzki A, Grass G, Anton A, Rensing C, Nies DH (2003) Interplay of the Czc system and two P-type ATPases in conferring metal resistance to *Ralstonia metallidurans*. J Bacteriol 185:4354–4361
59. Levi P (1984) Sistema periodico. English title: the periodic table, translated by Raymond Rosenthal. Schocken, New York
60. Levinson HS, Mahler I, Blackwelder P, Hood T (1996) Lead resistance and sensitivity in *Staphylococcus aureus*. FEMS Microbiol Lett 145:421–425
61. Li S, Rosen BP, Borges-Walmsley MI, Walmsley AR (2002) Evidence for cooperativity between the four binding sites of dimeric ArsD, an As(III)-responsive transcriptional regulator. J Biol Chem 277:25992–26002
62. Li XZ, Nikaido H (2004) Efflux-mediated drug resistance in bacteria. Drugs 64:159–204
63. Lippard SJ, Berg JM (1994) Principles of bioinorganic chemistry. University Science Books, Mill Valley
64. Lodewyckx C, Taghavi S, Mergeay M, Vangronsveld J, Clijsters H, van der Lelie D (2001) The effect of recombinant heavy metal-resistant endophytic bacteria on heavy metal uptake by their host plant. Int J Phytoremediation 3:173–187
65. Makkar NS, Cooney JJ (1990) Methylation of monomethyltin by a bacterial coculture. Geomicrobiol J 8:101–107
66. Mandal AK, Yang Y, Kertesz TM, Arguello JM (2004) Identification of the transmembrane metal binding site in Cu + -transporting P_{1B}-type ATPases. J Biol Chem 279:54802–54807
67. Meagher RB (2005) Strategies for the engineered phytoremediation of toxic element pollution: mercury and arsenic. J Ind Microbiol Bioechnol 32 (in press)
68. Mergeay M, Monchy S, Vallaeys T, Auquier V, Benotmane A, Bertin P, Taghavi S, Dunn J, van der Lelie D, Wattiez R (2003) *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. FEMS Microbiol Rev 27:385–410
69. Messens J, Van Molle I, Vanhaesebrouck P, Van Belle K, Wahni K, Martins JC, Wyns L, Loris R (2004) The structure of a triple mutant of pI258 arsenate reductase from *Staphylococcus aureus* and its 5-thio-2-nitrobenzoic acid adduct. Acta Crystallogr D Biol Crystallogr 60:1180–1184
70. Miller CE, Wuertz S, Cooney JJ, Pfister RM (1995) Plasmids in tributyltin-resistant bacteria from fresh and estuarine waters. J Ind Microbiol 14:337–342
71. Miller SM (1999) Bacterial detoxification of Hg(II) and organomercurials. Essays Biochem 34:17–30
72. Mukhopadhyay R, Rosen BP, Phung LT, Silver S (2002) Microbial arsenic: from geocycles to genes and enzymes. FEMS Microbiol Rev 26:311–325
73. Muller D, Lievremont D, Simeonova DD, Hubert JC, Lett MC (2003) Arsenite oxidase *aox* genes from a metal-resistant *beta-proteobacterium*. J Bacteriol 185:135–141
74. Mulrooney SB, Hausinger RP (2003) Nickel uptake and utilization by microorganisms. FEMS Microbiol Rev 27:239–261
75. Munkelt D, Grass G, Nies DH (2004) The chromosomally encoded cation diffusion facilitator proteins DmeF and FieF from *Wautersia metallidurans* CH34 are transporters of broad metal specificity. J Bacteriol 186:8036–8043
76. Murakami S, Nakashima R, Yamashita E, Yamaguchi A (2002) Crystal structure of bacterial multidrug efflux transporter AcrB. Nature 419:587–593
77. Murakami S, Tamura N, Saito A, Hirata T, Yamaguchi A (2004) Extramembrane central pore of multidrug exporter AcrB in *Escherichia coli* plays an important role in drug transport. J Biol Chem 279:3743–3748
78. Murakami S, Yamaguchi A (2003) Multidrug-exporting secondary transporters. Curr Opin Struct Biol 13:443–452
79. Narita M, Chiba K, Nishizawa H, Ishii H, Huang CC, Kawabata Z, Silver S, Endo G (2003) Diversity of mercury resistance determinants among *Bacillus* strains isolated from sediment of Minamata Bay. FEMS Microbiol Lett 223:73–82. Erratum in FEMS Microbiol Lett 226:415
80. Nies DH (1995) The cobalt, zinc, and cadmium efflux system CzcABC from *Alcaligenes eutrophus* functions as a cation-proton antiporter in *Escherichia coli*. J Bacteriol 177:2707–2712
81. Nies DH (2003) Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev 27:313–339
82. Ohtake H, Silver S (1994) Bacterial detoxification of toxic chromate. In: Chaudhry GR (ed) Biological degradation and bioremediation of toxic chemicals. Chapman and Hall, London, pp 403–415

83. Opella SJ, DeSilva TM, Veglia G (2002) Structural biology of metal-binding sequences. Curr Opin Chem Biol 6:217–223
84. Oremland RS, Stolz JF (2005) Arsenic, microbes and contaminated aquifers. Trends Microbiol 13:45–49
85. Pilon-Smits E (2005) Phytoremediation. Annu Rev Plant Biol 56:15–39
86. Pitts KE, Summers AO (2002) The roles of thiols in the bacterial organomercurial lyase (MerB). Biochemistry 41:10287–10296
87. Rensing C, Grass G (2003) *Escherichia coli* mechanisms of copper homeostasis in a changing environment. FEMS Microbiol Rev 27:197–213
88. Roberts SA, Wildner GF, Grass G, Weichsel A, Ambrus A, Rensing C, Montfort WR (2003) A labile regulatory copper ion lies near the T1 copper site in the multicopper oxidase CueO. J Biol Chem 278:31958–31963
89. Rodrigue A, Effantin G, Mandrand-Berthelot MA (2005) Identification of *rcnA* (*yohM*), a nickel and cobalt resistance gene in *Escherichia coli*. J Bacteriol 187:2912–2916
90. Rossbach S, Kukuk ML, Wilson TL, Feng SF, Pearson MM, Fisher MA (2000) Cadmium-regulated gene fusions in *Pseudomonas fluorescens*. Environ Microbiol 2:373–382
91. Rugh CL, Senecoff JF, Meagher RB, Merkle SA (1998) Development of transgenic yellow poplar for mercury phytoremediation. Nat Biotechnol 16:925–928
92. Ruiz ON, Hussein HS, Terry N, Daniell H (2003) Phytoremediation of organomercurial compounds via chloroplast genetic engineering. Plant Physiol 132:1344–1352
93. Saier MH Jr, Beatty JT, Goffeau A, Harley KT, Heijne WH, Huang SC, Jack DL, Jahn PS, Lew K, Liu J, Pao SS, Paulsen IT, Tseng TT, Virk PS (1999) The major facilitator superfamily. J Mol Microbiol Biotechnol 1:257–279. Erratum in J Mol Microbiol Biotechnol 252:255
94. Schiering N, Kabsch W, Moore MJ, Distefano MD, Walsh CT, Pai EF (1991) Structure of the detoxification catalyst mercuric ion reductase from *Bacillus* sp. strain RC607. Nature 352:168–172
95. Sigel A, Sigel H, Sigel RKO (eds) (2005) Biogeochemical cycles of elements, vol 43. Taylor & Francis Group, Boca Raton
96. Silver S (1996) Bacterial resistances to toxic metal ions—a review. Gene 179:9–19
97. Silver S (1996) Transport of inorganic cations. In: Neidhardt FC et al (ed) *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol 1. ASM, Washington, pp 1091–1102
98. Silver S (1998) Genes for all metals–a bacterial view of the periodic table. The 1996 Thom Award Lecture. J Ind Microbiol Biotechnol 20:1–12
99. Silver S (2003) Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. FEMS Microbiol Rev 27:341–353
100. Silver S, Nucifora G, Phung LT (1993) Human Menkes X-chromosome disease and the staphylococcal cadmium-resistance ATPase: a remarkable similarity in protein sequences. Mol Microbiol 10:7–12
101. Silver S, Phung LT (1996) Bacterial heavy metal resistance: new surprises. Annu Rev Microbiol 50:753–789
102. Silver S, Phung LT (2005) Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. Appl Environ Microbiol 71:599–608
103. Silver S, Phung LT, Rosen BP (2001) Arsenic metabolism: resistance, reduction, and oxidation. In: Frankenberger WT Jr (ed) Environmental chemistry of arsenic. Marcel Dekker, New York, pp 247–272
104. Singh SK, Grass G, Rensing C, Montfort WR (2004) Cuprous oxidase activity of CueO from *Escherichia coli*. J Bacteriol 186:7815–7817
105. Solioz M, Odermatt A (1995) Copper and silver transport by CopB-ATPase in membrane vesicles of *Enterococcus hirae*. J Biol Chem 270:9217–9221
106. Solioz M, Stoyanov J (2003) Copper homeostasis in *Enterococcus hirae*. FEMS Microbiol Rev 27:183–195
107. Solioz M, Vulpe C (1996) CPX-type ATPases: a class of P-type ATPases that pump heavy metals. Trends Biochem Sci 21:237–241
108. Steele RA, Opella SJ (1997) Structures of the reduced and mercury-bound forms of MerP, the periplasmic protein from the bacterial mercury detoxification system. Biochemistry 36:6885–6895
109. Thomas DJ, Waters SB, Styblo M (2004) Elucidating the pathway for arsenic methylation. Toxicol Appl Pharmacol 198:319–326
110. Tisa LS, Rosen BP (1990) Molecular characterization of an anion pump. The ArsB protein is the membrane anchor for the ArsA protein. J Biol Chem 265:190–194
111. Touze T, Eswaran J, Bokma E, Koronakis E, Hughes C, Koronakis V (2004) Interactions underlying assembly of the *Escherichia coli* AcrAB-TolC multidrug efflux system. Mol Microbiol 53:697–706
112. Toyoshima C, Inesi G (2004) Structural basis of ion pumping by Ca^{2+} -ATPase of the sarcoplasmic reticulum. Annu Rev Biochem 73:269–292
113. Toyoshima C, Mizutani T (2004) Crystal structure of the calcium pump with a bound ATP analogue. Nature 430:529–535
114. Toyoshima C, Nomura H, Tsuda T (2004) Lumenal gating mechanism revealed in calcium pump crystal structures with phosphate analogues. Nature 432:361–368
115. Tseng TT, Gratwick KS, Kollman J, Park D, Nies DH, Goffeau A, Saier MH, Jr. (1999) The RND permease superfamily: an ancient, ubiquitous and diverse family that includes human disease and development proteins. J Mol Microbiol Biotechnol 1:107–125
116. Wang CL, Clark DS, Keasling JD (2001) Analysis of an engineered sulfate reduction pathway and cadmium precipitation on the cell surface. Biotechnol Bioeng 75:285–291
117. Wei Y, Li H, Fu D (2004) Oligomeric state of the *Escherichia coli* metal transporter YiiP. J Biol Chem 279:39251–39259
118. White JS, Tobin JM, Cooney JJ (1999) Organotin compounds and their interactions with microorganisms. Can J Microbiol 45:541–554
119. Wilson JR, Leang C, Morby AP, Hobman JL, Brown NL (2000) MerF is a mercury transport protein: different structures but a common mechanism for mercuric ion transporters? FEBS Lett 472:78–82
120. Wuertz S, Miller CE, Pfister RM, Cooney JJ (1991) Tributyltin-resistant bacteria from estuarine and freshwater sediments. Appl Environ Microbiol 57:2783–2789
121. Yu EW, Aires JR, Nikaido H (2003a) AcrB multidrug efflux pump of *Escherichia coli*: composite substrate-binding cavity of exceptional flexibility generates its extremely wide substrate specificity. J Bacteriol 185:5657–5664
122. Yu EW, McDermott G, Zgurskaya HI, Nikaido H, Koshland DE Jr (2003b) Structural basis of multiple drug-binding capacity of the AcrB multidrug efflux pump. Science 300:976–980