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Arsenic Metabolism in Prokaryotic and Eukaryotic Microbes

Hiranmoy Bhattacharjee · Barry P. Rosen (✉)

Department of Biochemistry and Molecular Biology,
Wayne State University School of Medicine,
540 E. Canfield Avenue, Detroit, MI 48201, USA
brosen@med.wayne.edu

Abstract This chapter will focus on recent progress on the mechanisms of metalloid uptake, metabolism, and detoxification in bacteria, archaea, and eukaryotic microbes. One of the initial challenges of the earliest cells would have been the ability to detoxify heavy metal ions, transition metal ions, and metalloids, including arsenic and antimony. The presence of arsenic resistance (*ars*) genes in the genome of by far most living organisms sequenced to date illustrates firstly that *ars* genes must be ancient and secondly that arsenic is still ubiquitous in the environment, providing the selective pressure that maintains these genes in present-day organisms. Some early cells also probably could use arsenite as an electron acceptor, giving selective pressure for the evolution of respi-

ratory arsenate reductase. As atmospheric O_2 levels increased, arsenite was oxidized to arsenate abiotically. This provided an advantage for the evolution of arsenate reductases, some for arsenate respiration and energy production, and others for arsenate detoxification. Present-day selective pressure for metalloid resistance also comes from sources such as natural release of arsenic from volcanic activities, mining activities, the burning of coal, and other human activities. In addition is the use of arsenicals and antimonials as chemotherapeutic drugs for the treatment of parasitic diseases and cancer. Resistance to these drugs is becoming a major dilemma. Thus, an understanding of the molecular details of metalloid transport systems and detoxification enzymes is essential for the rational design of new drugs, and for treating drug-resistant microorganisms and tumor cells. Finally, this chapter will summarize recent identification of novel enzymes for arsenic reduction, oxidation, and methylation that expand the possibilities for metalloid metabolism and transformations.

1 Introduction: The Arsenic Geocycle

Arsenic is widely distributed in the Earth's crust and occurs primarily in four oxidation states: arsenate [As(V)], arsenite [As(III)], elemental arsenic [As(0)], and arsenide [As(–III)]. Volcanic eruptions are a source of human exposure to arsenic. Mining, copper smelting, coal burning, and other combustion processes also bring arsenic into our environment. Anthropogenic sources of arsenic include both inorganic and organic forms. Arsenic serves as an active ingredient in various commonly used herbicides, insecticides, rodenticides, wood preservatives, animal feeds, paints, dyes, and semiconductors.

Microbes play an important role in cycling arsenic between its various oxidation states (Fig. 1) (Mukhopadhyay et al. 2002). Inorganic arsenate entering the microbial cytosol through the phosphate transport system is reduced to arsenite, which is then extruded out of the cell, either through channels or secondary transporters (Rosen 2002). Arsenite is also generated by certain microbes that use arsenate as the terminal electron acceptor in anaerobic respiration (Oremland and Stolz 2003). These arsenate-respiring microbes can release arsenite from arsenate-rich sediments, leading to arsenic contamination of ground water (Oremland and Stolz 2005). Arsenite-oxidizing microbes utilize the reducing power from As(III) oxidation to gain energy for cell growth (Stolz et al. 2006). Microbes can also convert inorganic arsenic into gaseous methylated arsenide (Bentley and Chasteen 2002; Qin et al. 2006). However, whether microbes can metabolize arsenic salts to elemental arsenic remains to be determined. Marine microorganisms can convert inorganic arsenicals to various water- or lipid-soluble organic arsenic species. These include generation of di- and trimethylated arsenic derivatives (DMA, TMA), arsenocholine, arsenobetaine, arsenosugars, and arsenolipids. Arsenobetaine can be degraded to inorganic arsenic by microbial metabolism, completing the arsenic cycle in marine ecosystems (Dembitsky and Levitsky 2004).

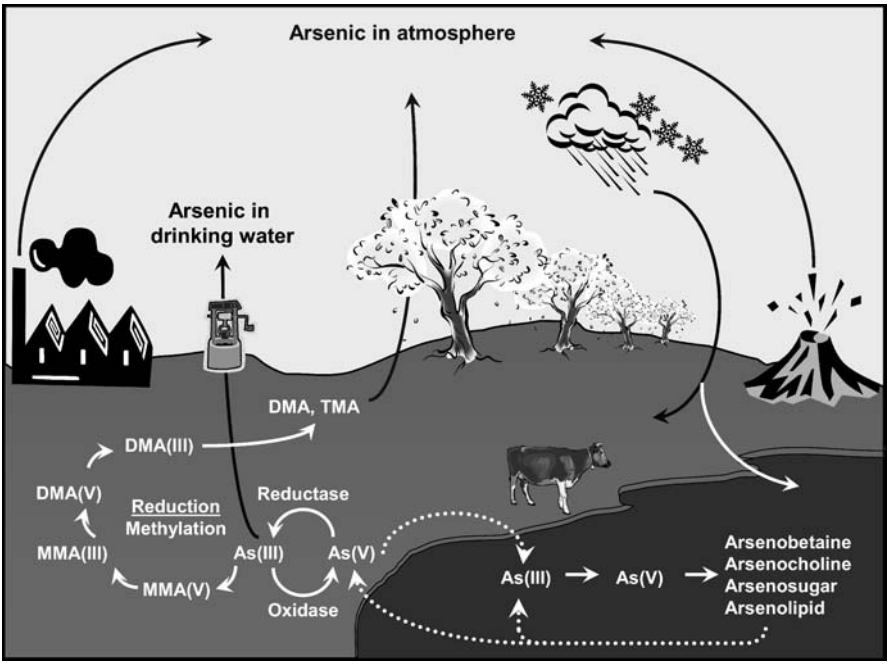

Fig. 1 The arsenic geocycle (see text for details)

2 Uptake Systems for Metalloid Oxyanions and Oxyacids: Phosphate Permeases, Aquaglyceroporin Channels, and Glucose Permeases

Inorganic arsenic has two biologically important oxidation states: pentavalent (As(V)) and trivalent (As(III)). In solution the pentavalent form, H_3AsO_4, exists as the oxyanion arsenate. As a solid, the unhydrated trivalent form is arsenic trioxide (As_2O_3). Reflecting a pK_a of 9.2, in solution arsenic trioxide is the undissociated acid, $As(OH)_3$. Even though it is not an oxyanion in solution, As(III) is frequently called arsenite, and so this term will be used interchangeably with arsenic trioxide in this chapter. Inorganic arsenic in both the pentavalent and trivalent oxidation states uses uptake systems that bring these toxic compounds into cells adventitiously.

Arsenate is an analogue of phosphate, and most organisms take up arsenate via phosphate transporters. In *Escherichia coli* there are two phosphate transporters, Pit and Pst (Rosenberg et al. 1977), both of which catalyze uptake of arsenate. Of the two, the Pit system is the major arsenate uptake system (Willsky and Malamy 1980a,b). In eukaryotic microbes arsenate is also taken up by phosphate transporters, although these are unrelated to the bacterial systems. For example, in *Saccharomyces cerevisiae*, several phosphate transporters catalyze arsenate uptake (Bun-ya et al. 1996).

Two pathways for cellular uptake of trivalent metalloids As(III) and Sb(III) have been identified (Fig. 2). In *E. coli*, GlpF was the first uptake system for As(III) and Sb(III) to be identified (Sanders et al. 1997; Meng et al. 2004). GlpF is the glycerol facilitator of *E. coli* (Heller et al. 1980). This gene was identified using a genetic screen mutant resistant to Sb(III) (Sanders et al. 1997). The assumption was that mutants unable to take up metalloids would become resistant, and the *glpF* mutant is highly resistant to Sb(III). Although As(III) transport is reduced by approximately 80% in the mutant (Meng et al. 2004), enough As(III) gets into the cells by as yet unidentified transporter(s) to keep the cells sensitive to As(III). GlpF was the first identified member of the aquaporin superfamily. The superfamily has two branches, the aquaporin channels that have small pores sufficient only to allow water conduction (Agre et al. 2002), and the aquaglyceroporins, which have larger pores of sufficient diameter to conduct neutral organic solutes such as glycerol and

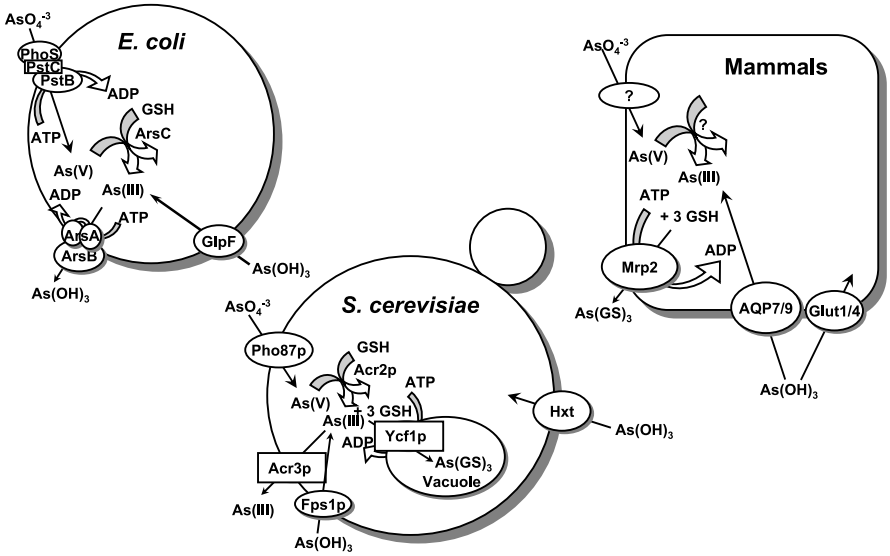

Fig. 2 Pathways of arsenical uptake and detoxification in prokaryotes and eukaryotes. Arsenate (As(V)) is taken up by phosphate transporters, while As(III) is taken up by aquaglyceroporins (GlpF in *E. coli*, Fps1p in yeast, and AQP7 and AQP9 in mammals) and hexose permeases (HXT1, HXT3, HXT4, HXT5, HXT7, or HXT9 in yeast, and GLUT1 and GLUT4 in mammals). In both *E. coli* and *S. cerevisiae*, arsenate is reduced to arsenite by the bacterial ArsC or yeast Acr2p enzymes. In both organisms, glutathione and glutaredoxin serve as the source of reducing potential. The proteins responsible for arsenate uptake and reduction in mammals have not yet been identified. In *E. coli*, arsenite is extruded from the cells by ArsB alone or by the ArsAB ATPase. In yeast, Acr3p is a plasma membrane arsenite efflux protein, and Ycf1p, which is a member of the MRP family of the ABC superfamily of drug-resistance pumps, transports $As(GS)_3$ into the vacuole. In mammals, Mrp isoforms, such as Mrp2, pump $As(GS)_3$ out of cells

urea (Borgnia et al. 1999). Most bacteria have GlpF homologues that render them sensitive to arsenite. Again, this is chiefly adventitious uptake of a toxic compound by a transporter with a physiological role in normal metabolism. However, in some organisms the gene for an aquaglyceroporin homologue, *aqpS*, is found in *ars* operons and appears to have evolved to confer arsenate resistance (Yang et al. 2005), as described in more detail below. As mentioned, arsenate is an analogue of phosphate, so it is easy to understand why it should be able to get into cells through phosphate transporters. It is not as obvious why arsenite should be taken up by channels for organic solutes such as glycerol. From the extended X-ray absorption fine structure (EXAFS) spectrum at neutral pH, the primary solution form of arsenic trioxide is $As(OH)_3$ (Ramirez-Solis et al. 2004) (Fig. 3). This polyhydroxylated arsenical is an inorganic analogue of glycerol, which indicates how it is recognized by GlpF.

In eukaryotic microbes aquaglyceroporin channels also conduct uptake of As(III) and Sb(III). Fps1p, the yeast homologue of GlpF, conducts arsenite uptake in *S. cerevisiae* (Wysocki et al. 2001). By functional complementation in yeast, we showed that mammalian aquaglyceroporins AQP7 and AQP9 catalyze uptake of trivalent metalloids (Liu et al. 2002). The capped RNA for the four human aquaglyceroporins was expressed in frog oocytes, and the results showed that they conduct As(III) in order of effectiveness as AQP9 > AQP7 $\gg$ AQP3 $\gg$ AQP10 (Liu et al. 2004b). This has importance for human health and disease. First, arsenic contamination of drinking water is a health problem in countries such as India and Bangladesh, and considerable individual variability in sensitivity to arsenic is found (Acharyya et al. 1999). One reason for this variability could be differential expression of AQP9, the liver isoform, in those individuals. Also, there appears to be a relationship between arsenic sensitivity and nutrition, and expression of AQP9 has been shown to be elevated by nutritional restriction (Carbrey et al. 2003). We propose that these two facts are linked: inhabitants of villages in West Bengal and Bangladesh often have poor diets, which leads to elevation in AQP9 in the liver and hence higher rates of uptake of arsenite. Thus, these individuals are more sensitive to arsenite than inhabitants of cities who have better nutrition. Arsenic trioxide (Trisenox) is used clinically as a chemotherapeutic agent for the treatment of acute promyelocytic leukemia (Soignet et al. 1998; Soignet 2001). Expression of AQP9 in leukemia cells sensitizes the cells to Trisenox, leading to the proposal that differential expression of AQP9 in leukemia patients could lead to variable effectiveness of Trisenox (Bhattacharjee et al. 2004). The discovery of pharmacological agents that selectively increase expression of AQP9 in leukemia cells could make the drug more effective and selective.

Another eukaryotic microbe, the human pathogen *Leishmania major*, also takes up As(III) and Sb(III) by an aquaglyceroporin, LmAQP1 (Gourbal et al. 2004). This is also clinically relevant because the drug of choice for treatment

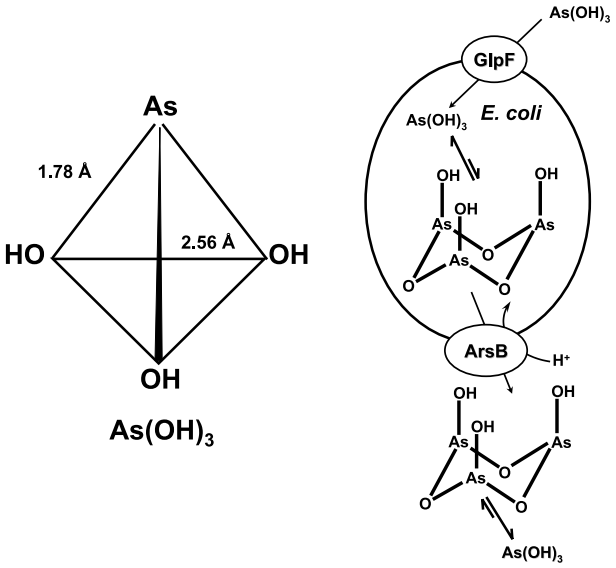

Fig. 3 The substrates of GlpF and ArsB. *Left*: Solution structure of arsenic trioxide. In solution at physiological pH, arsenic trioxide (As_2O_3) dissolves to form $As(OH)_3$, a trigonal pyramidal structure with three As – O bonds of 1.78 Å. *Right*: Postulated structures of the metalloid substrates of GlpF and ArsB. In *E. coli*, $As(OH)_3$ (or $Sb(OH)_3$) uptake is facilitated by the GlpF channel. Polymerization of three $As(OH)_3$ units is predicted to form a six-membered, oxo-bridged metalloid ring, with molecular similarity to a hexose. The ArsB antiporter exchanges this metalloid ring with positively charged H^+, thereby coupling efflux to the electrochemical proton gradient

of leishmaniasis is the pentavalent antimonial Pentostam. At least a portion of the drug appears to be reduced to Sb(III), the active form of the drug, in macrophages. The *Leishmania* amastigote, which resides in the phagolysosome of the infected macrophage, takes up Sb(III) by LmAQP1. Thus, it appears that arsenite gets into cells of most, if not all, organisms by aquaglyceroporins.

A second family of membrane transporters that catalyze arsenite uptake is the family of glucose permeases (Liu et al. 2004a). In the presence of glucose, most $As(OH)_3$ is taken up by yeast by the aquaglyceroporin Fps1p. However, in the absence of glucose, the Δ*fps1* strain exhibits only a 25% reduction in $^{73}As(OH)_3$ uptake compared to its parent, suggesting that the majority of $As(OH)_3$ accumulation in *S. cerevisiae* is via hexose permeases. The family of hexose permeases in *S. cerevisiae* is quite large, including 18 hexose transporters, Hxt1p to Hxt17p and Gal2p, and two glucose sensors, Snf3p and Rgt2p (Boles and Hollenberg 1997). These membrane proteins are members of the major facilitator superfamily (Marger and Saier 1993). A strain lacking *FPS1* and the genes for all 18 hexose permeases exhibits only residual up-

take of arsenic trioxide, and expression of *HXT1, HXT3, HXT4, HXT5, HXT7*, or *HXT9* restored transport, which indicates that many of the hexose permeases can transport As(III). These results clearly demonstrate that glucose carrier proteins catalyze transport of trivalent arsenic. More recently mammalian GLUT1 and GLUT4, which are homologues of the yeast HXT hexose permeases, have also been shown to transport As(III) (Liu et al. 2006). Thus, hexose transporters are responsible for a portion of the uptake and most likely the toxicity of inorganic trivalent arsenic trioxide in mammals, including humans.

How do hexose permeases transport As(III)? Even though the majority species in solution is $As(OH)_3$, other forms may be present in lower amounts. One form may be a six-membered ring composed of a trimer of $As(OH)_3$ that has molecular similarity to hexoses (Meng et al. 2004) (Fig. 3). The Cambridge Structural Database contains 109 oxo-bridged As – O – As compounds, including ten with six-membered $(As-O)_3$ rings. In addition, the crystal structure of arsenious oxide, As_4O_6, is also a six-membered $(As-O)_3$ ring with the fourth As(III) coordinated to the three axial oxygens (Hamson and Stosick 1938). Thus, it is reasonable to consider that $As(OH)_3$ forms a cyclic trimer that is an inorganic analogue of glucose.

3
Arsenic Respiration: Arsenate Reductases and Arsenite Oxidases

Arsenate may serve as electron acceptor for anaerobic respiration processes, while arsenite may donate electrons to chemolithotrophic bacteria. The next two sections describe the enzymes responsible for the respective chemical reactions.

3.1
Arsenate Reductases

Anaerobic cell growth in several bacterial species is linked to energy generation during the reduction of arsenate to arsenite. For example, the bacterium *Chrysiogenes arsenatis* respires anaerobically using arsenate as the terminal electron acceptor and acetate as the respiratory electron donor (Krafft and Macy 1998). During growth, arsenate is reduced to arsenite, the reduction being catalyzed by an arsenate respiratory reductase (Arr). It is believed that energy is conserved via electron-transport-linked phosphorylation, with the arsenate reductase functioning as a terminal reductase, which is coupled to an electron-transport chain in the membrane. The soluble *C. arsenatis* arsenate reductase is a periplasmic, heterodimeric protein that consists of two subunits with molecular masses of 87 kDa (ArrA) and 29 kDa (ArrB). Sequence analysis indicates that ArrA is a Mo/Fe protein that shows sequence homology

to the large dimethyl sulfoxide (DMSO) reductase class of proteins. ArrB is believed to contain a [4Fe – 4S] cluster that may be involved in the transfer of electrons to the molybdenum cofactor of the ArrA subunit. The K_m for arsenate is 0.3 μM, and the V_{max} is 7 millimoles arsenate reduced per minute per milligram of protein. Fumarate, nitrate, sulfate, and selenate could not serve as alternative electron acceptors for the arsenate reductase. The molecular details and mechanism of arsenate reduction and energy coupling remain to be determined.

Arsenate respiratory reductases have also been purified and characterized from *Shewanella* sp. strain ANA-3 (Saltikov and Newman 2003) and *Bacillus selenitireducens* (Afkar et al. 2003), although their structural details are still unknown. *Shewanella* ArrA is predicted to be a Mo/Fe protein with a molecular mass of 95.2 kDa, which is homologous to the DMSO reductase family, and ArrB is predicted to encode a 25.7-kDa iron–sulfur protein. A gene-encoded Tat (twin arginine translocation) motif at the N-terminus of ArrA suggests that the arsenate reductase complex is exported to the periplasm (Saltikov and Newman 2003). The arsenate respiratory reductase from *B. selenitireducens* strain MLS10 is a heterodimer of 150 kDa and is composed of ArrA (110 kDa) and ArrB (34 kDa), again with a putative Tat signal in the gene for ArrA (Afkar et al. 2003), similar to what has been found in *Shewanella* ArrA (Saltikov and Newman 2003), indicating that the enzyme may have an extracytoplasmic location. These arsenate respiratory reductases show significant sequence homology with each other.

While the periplasmic arsenate respiratory reductases are linked to energy metabolism, they are quite different from the cytosolic ArsC arsenate reductases, which play an important role in arsenic detoxification. Both *arr* and *ars* systems are expressed in *Shewanella* sp. strain ANA-3 under different growth conditions (Saltikov et al. 2003). While *arr* is only expressed anaerobically, *ars* is expressed under both aerobic and anaerobic conditions. The *arr* system was activated by a 1000-fold lower concentration of As(III) than that required for the *arsC* system. Under anaerobic conditions, *arr* transcription is activated at nanomolar concentrations of either As(V) or As(III). During the early phases of growth, *Shewanella* preferentially couples the reduction of As(V) to growth, instead of detoxification by the *ars* system. Over the course of growth, as As(III) accumulates to toxic levels, *Shewanella* switches on the *ars* operon to facilitate arsenic resistance.

The arsenate respiratory reductases play an important role in arsenic geochemistry and may lead to arsenic contamination of drinking water supplies. As(V) can bind to minerals commonly found in the environment, such as ferrihydrite and alumina, which can limit its mobility and bioavailability. However, microbial reduction of As(V) to As(III) can facilitate the release and transport of this toxic metal into aquatic environments and into drinking water (Harvey et al. 2002; Islam et al. 2004).

3.2 Arsenite Oxidases

Arsenite oxidase activity has been reported in heterotrophic as well as in chemoautotrophic microorganisms. The best-characterized arsenite oxidase is from the soil pseudomonad *Alcaligenes faecalis* (Anderson et al. 1992; Ellis et al. 2001). The *A. faecalis* arsenite oxidase is a 100-kDa heterodimer of a large and a small subunit (Anderson et al. 1992; Ellis et al. 2001). The large catalytic subunit of 825 residues harbors the molybdenum (Mo) center, the Mo atom being bound to two pterin cofactors and a [3Fe – 4S] cluster, and is structurally related to the DMSO reductase family of molybdenum enzymes and to the Arr arsenate reductases. The small subunit of approximately 134 residues contains a Rieske-type [2Fe – 2S] cluster and is homologous to the Rieske protein domains of cytb complexes and dioxygenases. The protein is likely transported across the cytoplasmic membrane via the Tat protein export pathway, and most likely remains attached to the outer surface of the inner membrane by the N-terminal transmembrane helix of the small Rieske subunit (Lebrun et al. 2003). The catalytically active form of the *A. faecalis* arsenite oxidase has an oxidized Mo(VI) center which is solvent accessible via a hydrophilic channel. Several residues, including His195, Glu203, Arg419, and His423, line the base of the channel and form the arsenite binding site. The minimal mechanism of arsenite oxidation by the *A. faecalis* arsenite oxidase consists of the following steps (Ellis et al. 2001). After arsenite binds at the substrate binding site, it executes a nucleophilic attack on the Mo = O group of the oxidized Mo(VI) center. This reaction yields reduced Mo(IV) coordinated with arsenate. Subsequently, arsenate is released upon its dissociation from the reduced enzyme. Finally, following the addition and deprotonation of water, the reduced enzyme is transformed back to the active form by regenerating the Mo(VI)-oxo group. The reduced electrons generated during the course of the reaction are channeled through the [3Fe – 4S] and [2Fe – 2S] clusters into the periplasmic electron acceptors, perhaps azurin and cytochrome *c*.

Arsenite oxidase genes have also been cloned from the β-proteobacterial strain ULPAs1 (Muller et al. 2003), the heterotrophic *Hydrogenophaga* sp. strain NT-14 (van den Hoven and Santini 2004), and the chemolithoautotrophic bacterium NT-26 (Santini and van den Hoven 2004). Each of these proteins shows significant sequence homology to the *A. faecalis* enzyme. Contrary to ULPAs1 (Muller et al. 2003) and *A. faecalis* arsenite oxidase that are involved in arsenic detoxification (Anderson et al. 1992; Ellis et al. 2001), either of the NT-14 and NT-26 enzymes is implicated in metabolism, as these strains utilize the reducing power from As(III) oxidation to gain energy for cell growth (Santini et al. 2000; Santini and van den Hoven 2004; van den Hoven and Santini 2004).

4
Metalloid Resistances

Arsenate, arsenite, and antimonite are detoxified by an interplay of redox, transport, sequestration, and covalent modification reactions.

4.1
Regulation of Metalloid Detoxification by the ArsR/SmtB Family of Metalloregulatory Proteins

Expression of the genes for resistance to arsenic as well as toxic metals and metalloids is nearly always subject to transcriptional regulation by the toxic ions themselves. One large group is the ArsR/SmtB family of metal(loid)-responsive transcriptional repressors. The 117-residue As(III)/Sb(III)-responsive ArsR repressor encoded by the *arsRDABC* operon of *E. coli* plasmid R773 (Wu and Rosen 1991, 1993; Shi et al. 1994) and the 122-residue Zn(II)-responsive SmtB repressor (Huckle et al. 1993; Morby et al. 1993) were the first identified members of the ArsR/SmtB family of small metalloregulatory proteins. Since the initial reports, the number of identified members of this family has grown to 198, with 192 homologues in Gram-positive and -negative bacteria, and six homologues in archaea. These include proteins that respond to As(III)/Sb(III) (ArsR) (Wu and Rosen 1991), Pb(II)/Cd(II)/Zn(II) (CadC) (Endo and Silver 1995), Cd(II)/Pb(II) (CmtR) (Cavet et al. 2003), Zn(II) (SmtB and ZiaR) (Morby et al. 1993), and Co(II)/Ni(II) (NmtR) (Cavet et al. 2002). ArsR, CadC, and SmtB (and, by extrapolation, all members of the family) are homodimers that repress transcription by binding to DNA in the absence of inducing metal ion. They dissociate from the DNA when metal is bound, resulting in expression of metal ion resistances.

What is the basis of metal ion binding and specificity among members of the ArsR/SmtB family? Selectivity cannot be inferred from genome sequence and annotation, so how can As(III)-responsive regulators be distinguished from those that respond to other metals? We made the assumption that any homologue within or adjacent to putative *ars* operons or arsenic resistance genes responds to As(III), and chose three for further analysis. The first is the well-characterized R773 ArsR, while the other two lack the As(III) binding site of the R773 protein, and yet have been shown to be As(III) responsive. In each subunit of the R773 ArsR repressor there is a metal binding domain consisting of Cys32 and Cys34, which are ligands for As(III) and Sb(III) (Shi et al. 1994). Cys37 is a third ligand, although it is neither required for induction nor conserved in some homologues. This illustrates that a vicinal cysteine pair is sufficient for strong binding of As(III). Consistent with this chemistry, the best inducer of the operon is phenylarsine oxide, which can accommodate only two protein ligands because of the phenyl ring. Note that other types of ligands for As(III) bind much more poorly, so it is unlikely that

ligands such as carboxylates, serine hydroxyls, or histidine imidazole nitrogens, which form only weak As(III) binding sites, could participate. Thus, in As(III)-responsive repressors, it is reasonable to expect that a pair of cysteine residues will form the metalloid binding site.

Although the structure of R773 ArsR has not been determined, the structure of the CadC homologue has been solved (Ye et al. 2005), and this structure was used to model other members of the ArsR/SmtB family. In ArsR, the inducer binding site is located in the α3 helix, which is the first helix of the helix–loop–helix DNA binding domain. We hypothesize that distortion of the DNA binding domain upon inducer binding results in dissociation from the operator/promoter site and transcription of the resistance genes (Fig. 4).

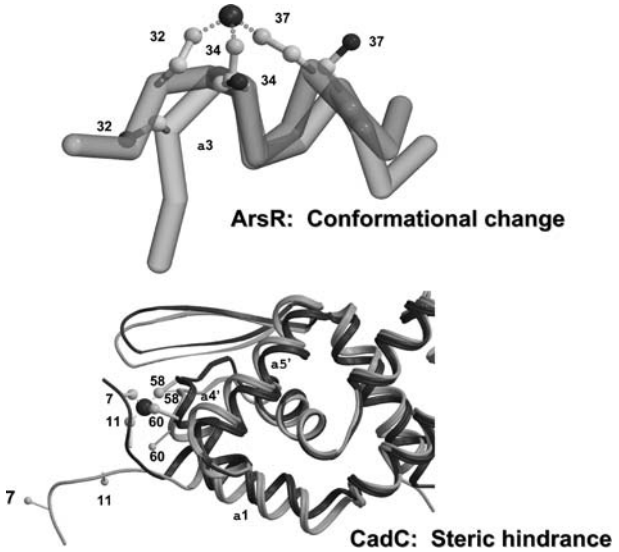

Fig. 4 Models for metalloregulation by the ArsR and CadC repressors. *Top* (ArsR): The apo-ArsR homodimer binds to the operator/promoter region, through a helix–turn–helix domain in each monomer, repressing transcription of the *ars* operon. The sulfur atoms of the three cysteines, Cys32, Cys34, and Cys37, are linearly arrayed along the first helix of the helix–loop–helix DNA binding domain, with more than 10 Å separating Cys32 from Cys37. From EXAFS and biochemical data, As(III) has been shown to bind to Cys32, Cys34, and Cys37 through metal–sulfur bonds, bringing the three sulfur atoms to 2.25 Å from the bound As(III). This is predicted to produce a substantial conformational change in that helix compared with the apo-repressor, resulting in dissociation from the operator/promoter site and transcription of the resistance genes. *Bottom* (CadC): Metalloregulation by CadC is predicted to follow a different mechanism. In this case, binding of Cd(II) to the sulfur atoms of Cys58 and Cys60 from one subunit of CadC, and Cys7 and Cys11 from the other subunit, brings the N-terminus of CadC into close contact with α4, the first part of the helix–loop–helix DNA binding motif, making it inaccessible to the DNA. Thus, binding of Cd(II), Pb(II), or Zn(II) to CadC could sterically block interaction with the *cad* operator/promoter

This idea is based on several lines of evidence. First, from EXAFS analysis, the distance between As(III) and each of the three cysteine thiolates was shown to be 2.25 Å (Shi et al. 1996). From this and the known structures of small-molecule As(III)-thiol compounds, the sulfur-to-sulfur distances would be predicted to be 3.5 Å. In the model of the ArsR aporepressor, the sulfur atoms of the three cysteines are linearly arrayed along the first helix of the DNA binding domain, with more than 10 Å from Cys32 to Cys37 (Fig. 5). To be consistent with the EXAFS results, binding of As(III) must bring the cysteines into proximity, producing a large conformational change in this helix (Fig. 4).

Why does R773 ArsR not bind divalent soft metals such as Cd(II)? From a combination of results from molecular genetics (Sun et al. 2001), biochemistry (Wong et al. 2002), and EXAFS (Busenlehner et al. 2001), the inducer binding domain in CadC was shown to be an S_4 site composed of four cysteine residues, Cys7, Cys11, Cys58, and Cys60, each 2.53 Å from a bound Cd(II) (Fig. 4). Like Cys37 of ArsR, Cys11 is not required for induction. Thus, the location of the inducer binding sites in ArsR and CadC is conserved,

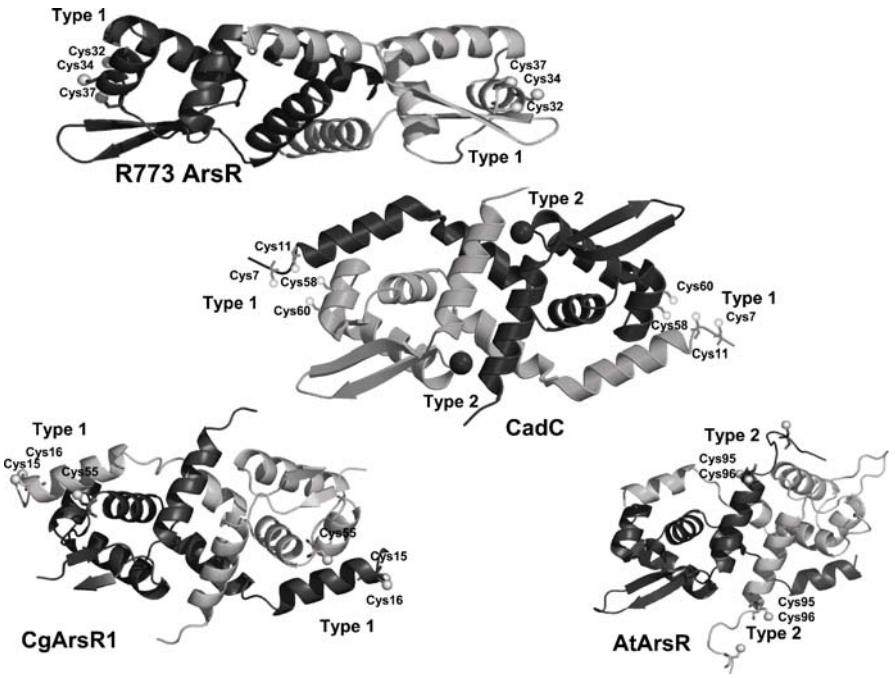

Fig. 5 Model of ArsR aporepressors based on the structure of *Staphylococcus aureus* pI258 CadC. R773 ArsR, CgArsR1, and AtArsR have been modeled based on the structure of pI258 CadC (*center*). The CadC dimer is shown as a ribbon diagram with secondary structural units N-α1-α2-α3-β1-α4-α5-β2-β3-α6-C. Each of the type 1 and type 2 metal binding sites in CadC is composed of two residues from each of the monomers

even though CadC does not respond to As(III) or Sb(III) (Endo and Silver 1995; Tauriainen et al. 1998; Sun et al. 2001) (although it does respond to Bi(III) (Busenlehner et al. 2002)), and ArsR does not respond to Cd(II), Pb(II), or Zn(II) (Wu and Rosen 1993). From modeling the inducer binding sites of ArsR and CadC with bound metals, the mechanism of derepression can be predicted to differ between the two (Fig. 4). While binding of As(III) is proposed to distort the α3 helix of ArsR, causing loss of DNA binding, the binding of Cd(II) to both the α1 and α4 helices of CadC results in the α1 helix sterically hindering the DNA binding site so that the repressor is no longer able to bind to its operator/promoter site.

Both the number and spatial location of available protein ligands are critical for selectivity. ArsR has an S_3 binding contributed by three cysteine residues of a single subunit (Shi et al. 1996), while the site in CadC is S_4, with two of the cysteine thiolates contributed by one subunit and the other two contributed by the other subunit (Busenlehner et al. 2001). CadC has an N-terminal extension that is absent in ArsR. This sequence contains Cys7 and Cys11. The results of cross-linking experiments demonstrated that each of the two binding sites is composed of Cys7 and Cys11 from one subunit and Cys58 and Cys60 from the other (Wong et al. 2002). While this N-terminal sequence is not visible in the SmtB structure, the 1.9-Å structure of CadC extends to residue 10 and clearly shows that the N-terminus of one subunit is located adjacent to α1 of the DNA binding domain of the other subunit (Ye et al. 2005) (Fig. 5). The inducer binding sites located in the DNA binding region have been called type 1 sites, to contrast them with the type 2 sites found in SmtB, which is in a totally different location. These sites have also been called α3N and α5 (VanZile et al. 2000, 2002b), based on a crystal structure of SmtB in which the putative α1 helix is not visible (Cook et al. 1998). If SmtB has an α1 helix, the metal sites would actually be α4N and α6.

The metal binding properties of SmtB have been analyzed in detail by a combination of UV/visible and X-ray absorption spectroscopy (VanZile et al. 2000, 2002a,b). The two metal binding sites in SmtB are at the dimer interface and not in the DNA binding domain, although it has an apparent vestigial type 1 site, with one cysteine corresponding to Cys32 of R773 and another in the other subunit similar to Cys11 of CadC. The type 2 site in SmtB has four ligands, two from one subunit (Asp104 and His106) and two from the other subunit (His117 and Glu120). While there is no trace of a metal binding site at the dimer interface of ArsR, in the CadC structure there are two Zn(II) bound at the dimer interface in sites composed of Asp101 and His103 from one subunit and His114 and Glu117 from the other (Fig. 5). Thus, CadC has the four residues corresponding to the SmtB type 2 site, but this type 2 site is not a physiological inducer binding site (Wong et al. 2002). SmtB has only a type 2 site, while ArsR has only a type 1 site. CadC has both type 1 and type 2 sites. In addition, the CadC type 1 site is more complicated than the type 1 site of R773 ArsR. The two CadC type 1 sites are each formed between

the two subunits of the dimer, while the two ArsR type 1 sites are composed of three residues from a single subunit. It is reasonable to consider that the simple intrasubunit site in the common ancestor of these repressors arose first, and a more complicated intersubunit site arose later. Thus, the ArsR type 1 site is closer to the ancestral site than the CadC type 1 site. ArsR has no type 2 site, CadC has a type 2 site that is not used for metalloregulation, and SmtB has a regulatory type 2 site, suggesting that type 2 sites evolved subsequently to type 1 sites. The presence of a vestigial type 1 site in SmtB is consistent with this concept of sequential evolution of two types of metal sites.

However, the situation appears still more complicated. Two other As(III)-responsive ArsR repressors lack cysteine residues corresponding to Cys32, Cys34, or Cys37 of R773 ArsR, indicating that they do not have a simple type 1 As(III) binding site. The first atypical ArsR controls the *ars* operon of *Acidothiobacillus ferrooxidans* (Butcher and Rawlings 2002). Phylogenetically, this AtArsR is more closely related to CadC and SmtB than to the R773 ArsR and lacks any hint of a type 1 site, with no cysteine residues in the DNA binding domain. It has two cysteine residues, Cys95 and Cys96, at the C-terminal end of each subunit and binds one As(III) per subunit (Qin and Rosen, unpublished results). Mutation of Cys95 and/or Cys96 eliminates metal binding. From EXAFS analysis, As(III) is bound in a mixed S and O/N environment. From this result, we propose that As(III) is bound to the thiolates of Cys95 and Cys96, with the third ligand as a hydroxyl. Modeling AtArsR on CadC, Cys95 and Cys96 are congruent with the type 2 metal binding site of CadC (Fig. 5). However, neither corresponds to the exact residues found in the type 2 site of CadC, indicating that this site is not derived from a CadC-like site simply by two substitutions. More importantly, this type 2 site is composed of residues from a single subunit, and is a simpler type 2 site than the intrasubunit type 2 site of CadC.

In *Corynebacterium glutamicum* ATCC 13032 two *ars* operons are controlled by two closely related ArsRs (Ordonez et al. 2005). Both CgArsRs respond to As(III) (Thiyagarajan and Rosen, unpublished results), but they are quite divergent from the R773 ArsR in primary sequence. They have the N-terminal extension characteristic of CadC-like repressors but do not have cysteine residues corresponding to either the ArsR or CadC type 1 sites. Instead they have two cysteine residues in the first α helix and one cysteine just N-terminal to the putative DNA binding domain. While there are no experimental structure–function analyses yet on the role of these residues in As(III) binding, a model of CgArsR1 built on the CadC structure is quite instructive: the two α1 cysteines from one subunit are juxtaposed to the cysteine in the DNA binding domain of the other subunit (Fig. 5). This intrasubunit type 1 site is exactly what would be predicted for an S_3 As(III) binding site. It may resemble the complex intrasubunit type 1 site of CadC, but the locations of the three cysteines in CgArsR1 are different from those of the four cysteines of the S_4 type 1 site of CadC.

What can we conclude from this comparative analysis of ArsR repressors? First, the inducer binding site can be located either near the DNA binding domain or the dimer interface. Second, it is formed by placement of pairs or triads of cysteine residues. Third, it can be formed within a single subunit or between subunits. Fourth, and very interestingly, the As(III) binding sites of R773 ArsR, AtArsR, and CgArsR appear to be the result of three independent and relatively recent evolutionary events, building on the same backbone repressor protein. By placement of four cysteines, four coordinate sites such as found in CadC can be formed. Introduction of harder ligands, such as the oxygen and nitrogen sites of the type 2 sites of CadC and SmtB, allows binding sites for harder metals such as Zn(II) to evolve.

4.2 Families of Arsenite Efflux Carriers: ArsBs and Acr3s

The most common mechanism of arsenite resistance is efflux from cells catalyzed by members of two different and unrelated families of permeases, ArsB and Acr3. The first identified arsenite efflux protein is ArsB encoded by the *arsRDABC* operon of the conjugative R-factor R773, which confers resistance to inorganic As(III) and Sb(III) in *E coli* (Chen et al. 1986; Tisa and Rosen 1990). By itself, ArsB is a secondary efflux protein coupled to the proton-motive force and confers a moderate level of arsenite resistance (Kuroda et al. 1997). ArsB associates with the ArsA ATPase to form a pump that confers high-level resistance (Dey et al. 1994), as described in more detail below. Thus, the Ars efflux system is unique in that it exhibits a dual mode of energy coupling depending on the subunit composition (Dey and Rosen 1995).

ArsB is widespread in bacteria and archaea. It has 12 membrane spanning segments, which is similar to many carrier proteins (Wu et al. 1992). It transports As(III) but has higher affinity for Sb(III). ArsB is an antiporter that catalyzes the exchange of trivalent metalloid for protons, coupling arsenite efflux to the electrochemical proton gradient (Meng et al. 2004). Curiously, As(III) inhibits ArsB-mediated Sb(III) transport, yet Sb(III) stimulates ArsB-mediated As(III) transport. One explanation for these results is that the true substrate of ArsB is a polymer of As(III) or Sb(III) or a copolymer of As(III) and Sb(III) (Fig. 3). Complexes with arsenic are transported with lower affinity than the Sb(III) polymer, so trivalent arsenic appears to inhibit trivalent antimony transport. Reciprocally, complexes with antimony are transported with higher affinity than the As(III) polymer, so trivalent antimony appears to stimulate trivalent arsenic transport. Again, the nature of the polymer is not known, but we have proposed cyclic six-membered oxo-bridged rings (Meng et al. 2004).

The Acr3 family includes members found in bacteria, archaea, and fungi. Unfortunately, the literature is confused by the fact that many members of the Acr3 family have been given the name ArsB, even though they exhibit

almost no sequence similarity to ArsB. The first identified member of this family is encoded by the *ars* operon of the skin (*sigK* intervening) element in the chromosome of *B. subtilis* (Sato and Kobayashi 1998). Fungal members of this family include the *S. cerevisiae* Acr3p metalloid efflux protein (Bobrowicz et al. 1997; Ghosh et al. 1999). Interestingly, yeast Acr3p appears to be selective for As(III) over Sb(III). This is unusual because of the similarity in chemical properties between the two metalloids. Recently *C. glutamicum* ATCC 13032 has been shown to have three genes for Acr3 homologues, each of which contributes to the high level of arsenite resistance in this organism (Ordonez et al. 2005). Expression of one of the *acr3* genes in an arsenite-hypersensitive strain of *E. coli* in which all *ars* genes were deleted confers resistance to As(III) (Meng and Rosen, in preparation). This heterologously expressed Acr3 catalyzes efflux of arsenite from *E. coli*. It exhibits significant differences from R773 ArsB. While ArsB exchanges As(III) with protons, Acr3 does not, but how it is coupled to the proton-motive force is unknown at this point. Like the yeast Acr3p, the *C. glutamicum* Acr3 is also more specific for As(III) than Sb(III), in contrast to ArsB, which has higher affinity for Sb(III) than As(III).

4.3 Efflux Pumps: ArsAB ATPases and Multidrug Resistance-Associated Proteins (MRPs)

The most efficient efflux pumps for metalloids are driven by ATP hydrolysis. These are the ArsAB ATPases, mostly in bacteria, and MRPs, members of the ABC family, in eukaryotic organisms.

4.3.1 ArsAB ATPases

The *ars* operon of R-factor R773 encodes an arsenite extrusion pump that confers resistance to the metalloids As(III) and Sb(III) in *E. coli*. This efflux pump has two subunits: ArsA ATPase is the catalytic subunit of the pump and ArsB functions as a membrane anchor for ArsA and also contains the translocation pathway. While ArsB alone can catalyze the extrusion of oxyanions across the membrane by functioning as a secondary transporter (Dey and Rosen 1995), the ArsA ATPase forms the ArsAB pump with the capacity to hydrolyze ATP, and drive active transport of As(III)/Sb(III) against a chemical gradient (Kuroda et al. 1997).

ArsA is normally bound to ArsB in vivo, but it can be overexpressed and purified as a soluble protein from the cytosol (Rosen et al. 1988). The soluble ArsA exhibits ATPase activity which is stimulated by either As(III) or Sb(III). The 583-residue ArsA ATPase has two homologous halves, A1 and A2, connected by a short linker. Each half has a consensus nucleotide binding do-

main (NBD) and both NBDs are required for ATPase activity and oxyanion transport (Karkaria et al. 1990; Kaur and Rosen 1992). The crystal structure of the MgADP bound form of ArsA has been determined (Fig. 6) at 2.3 Å resolution (Zhou et al. 2000). The A1 and A2 halves of the enzyme are related by a pseudo-twofold axis of symmetry. The two NBDs are in close proximity to each other and are located at the A1/A2 interface. In each NBD, Mg^{2+} is octahedrally coordinated with the β-phosphate of ADP, a threonine hydroxyl group, and several water molecules. In the solved structure, the two NBDs appear to be in different conformations despite extensive topological similarities, with NBD1 almost completely closed and the NBD2 fully open (Zhou et al. 2000, 2001). To determine the role of each NBD in substrate binding and catalysis, a thrombin site was introduced into the linker region that connects the A1 and A2 halves. Following covalent radiolabeling with 8-azidoATP and thrombin cleavage, the A1 and A2 halves migrated with different mobilities on sodium dodecyl sulfate polyacrylamide gel electrophoresis. In the absence of metalloid, both NBDs bind and hydrolyze ATP. However, in the presence of metalloid, although both NBDs hydrolyze ATP, hydrolysis in NBD1 is stimulated to a much greater extent than in NBD2. These experiments suggest that the two homologous halves of ArsA are functionally nonequivalent.

A novel metalloid binding domain (MBD) is also located at the A1/A2 interface and is over 20 Å distant from the NBDs. The MBD contains three metalloid atoms: one As(III) or Sb(III) is coordinated to His148 (A1) and Ser420 (A2), a second to Cys113 (A1) and Cys422 (A2), and the third to Cys172 (A1) and His453 (A2). Kinetic studies indicate that the three metalloid atoms bind with different affinities (Walmsley et al. 2001a), suggesting

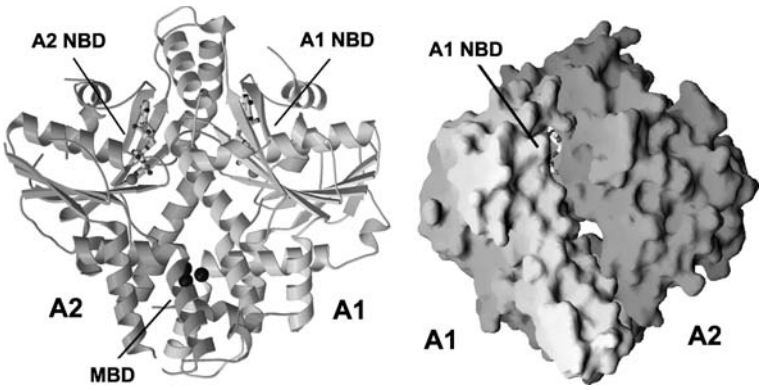

Fig. 6 Structure of R773 ArsA ATPase. *Left*: The overall structure of ArsA is shown as a ribbon diagram. Mg^{2+}ADP is bound to each of the two NBDs in the A1 and A2 halves of ArsA, while three Sb(III) are bound at the single MBD. *Right*: A view of the molecular surface of ArsA showing the relative positions of the A1 and A2 halves and details of ADP bound in the NBD1

that the three metalloid binding sites may allow the pump to operate in different ranges of ion concentration. Each of the amino acids lining the MBD has been altered by site-directed mutagenesis. Alteration of Cys113, Cys172, and Cys422 resulted in substantial loss of metalloid-stimulated ATPase activity (Bhattacharjee et al. 1995; Ruan et al. 2006), while mutation of His148 and His453 showed a modest decrease in allosteric activation of the enzyme (Bhattacharjee and Rosen 2000). Cys113 and Cys422 form the high-affinity metalloid binding site (Ruan et al. 2006). Although, metalloid stimulation of ArsA ATPase activity enhances the ability of the pump to reduce the intracellular concentration of oxyanions, high-affinity binding of metalloid by ArsA is not obligatory for transport or resistance. It appears that binding of metalloid to MBD provides an evolutionary advantage to survive in low levels of arsenite ubiquitously present in the environment (Ruan et al. 2006).

The transfer of information of metal occupancy at MBD to the two NBDs is performed by two signal transduction domains (STDs). Two stretches of residues, D^{142}TAPTGH^{148} in A1 STD and D^{447}TAPTGH^{453} in A2 STD, physically connect the MBD to NBD1 and NBD2, respectively. By introducing tryptophan residues in proximity to the $D^{142/447}$TAPTGH$^{148/453}$ sequence, conformational changes have been observed in response to binding of nucleotide and metalloid at the NBDs and MBD, respectively (Zhou et al. 1995, 2002; Zhou and Rosen 1997).

The mechanism of allosteric activation of ArsA ATPase has been elucidated at the molecular level from a combination of biochemical (Bhattacharjee et al. 2000), structural (Zhou et al. 2000, 2001), and kinetic experiments (Walmsley et al. 1999, 2001a,b). To summarize, these studies indicated that ArsA exists in at least two different conformational forms, where bound ATP favors one form and bound metalloids the other. In the absence of activator, the enzyme undergoes a slow conformational change between these forms, leading to a lag in attaining maximal steady-state activity. Binding of oxyanions at MBD acts as molecular glue to bring the A1 and A2 halves of ArsA together. This enhances the steady-state ATPase activity by inducing rapid product release and allowing the protein to adopt a conformation that can bind MgATP for the next catalytic cycle. In the presence of activator, ArsA avoids the rate-limiting isomerization at the end of the ATPase reaction and now ATP hydrolysis becomes rate limiting for the reaction. When ArsA is bound to ArsB, the complex probably functions as a reciprocating engine (Zhou et al. 2001), where cytosolic As(III)/Sb(III) accesses the high-affinity metalloid binding site of ArsA. ATP hydrolysis is linked with stepwise transfer of the metalloid to the low-affinity sites, followed by vectorial transport of metalloid into ArsB.

Homologues of the bacterial ArsA ATPase are widespread in nature and are present in most members of all three domains: bacteria, archaea, and eukaryotes (Bhattacharjee et al. 2001). Similar to *E. coli* R773 ArsA, many bacterial ArsAs also have an A1–A2 arrangement, and are part of *ars* operons that

reside either on chromosomes or plasmids. On the other hand, many bacteria encode for ArsA homologues with only a single "A" structure. The role of the bacterial single A proteins have not been determined. Archaea have either an A1–A2 structure or just a single A arrangement. For example, halobacteria have an A1–A2 organization while methanogenic archaea have a single A structure. All eukaryotic ArsA homologues known to date are single A domain proteins.

To determine the role of eukaryotic ArsA homologues, the yeast homologue (*ARR4*) was disrupted in *S. cerevisiae*. The null mutant shows no growth defects either on synthetic complete media or on rich media (Zuniga et al. 1999). The disrupted strain, however, does not exhibit an arsenic-related phenotype but shows increased sensitivity to heavy metals and temperature, indicating that Arr4p may be part of a complex that is involved in stress tolerance but not arsenic resistance (Shen et al. 2003). In support of this idea, the ATPase activity of purified Arr4p was not stimulated by As(III) or Sb(III). It has been recently shown that, in the presence of Cu(II), Arr4p binds with the yeast intracellular CLC chloride-transport protein, Gef1p (Metz et al. 2006). The Arr4p–Gef1p complex has been implicated to be involved in yeast copper metabolism (Metz et al. 2006). On the other hand, Schuldiner et al. reported that Arr4p is a subunit of the GET complex that is required for the retrieval of HDEL proteins from the Golgi to the endoplasmic reticulum in an ERD2-dependent fashion (Schuldiner et al. 2005).

The mouse homologue of the bacterial ArsA ATPase also shows a single A structure. To determine the physiological role of the mouse homologue, heterozygous Asna1 knockout mice were generated by homologous recombination. The heterozygous Asna1 knockout mice displayed no obvious phenotype. However, early embryonic lethality was observed in homozygous Asna1 knockout embryos, indicating that Asna1 plays a crucial role during early embryonic development (Mukhopadhyay et al. 2006).

Leipe et al. (2002) suggested that ArsA homologues are most likely of archaeal or archaeoeukaryotic origin and spread to bacteria later to provide selective environmental advantage. Although these homologues have common structural motifs, phylogenetic analysis of ArsA homologues suggests separate evolutionary lines for these proteins, resulting in distinct biochemical activities (Gihring et al. 2003).

4.3.2 MRPs

The yeast protein Ycf1p (yeast cadmium factor), a close homologue of the human MRP1 (multidrug resistance-associated protein), catalyzes the vacuolar sequestration of As(III) (Ghosh et al. 1999). MRP1 is an ABC transporter that confers multidrug resistance in human small cell lung carcinoma (Cole et al. 1994). Both MRP1 and Ycf1p are ATPases that pump glutathione S-conjugated

drugs out of the cytosol. MRP1 transports arsenic as a triglutathione conjugate out of the cell (Leslie et al. 2004), while Ycf1p catalyzes the ATP-driven uptake of As(III)–glutathione conjugate into the yeast vacuole (Ghosh et al. 1999).

4.4 ArsD: An As(III) Chaperone

Both prokaryotes and eukaryotes have metallochaperones that sequester metals in the cytoplasm, buffering their concentration, and deliver them to protein targets, such as transporters for extrusion (Rosenzweig 2002). For example, Atx1, the yeast homologue of Atox1, delivers copper to the transport-ATPase Ccc2p in the *trans*-Golgi network for incorporation into the multicopper oxidase Fet3p (Lin et al. 1997).

Although most sequenced bacterial genomes contain an *ars* operon, only a few are five-gene operons such as the *arsRDABC* operon of plasmid R773. The product of the *arsD* gene, a 120-residue polypeptide that is a functional homodimer, is a weak As(III)-responsive transcriptional repressor (Chen and Rosen 1997). Since its regulatory properties are so poor, it is questionable whether that is the primary function of ArsD. Recently, ArsD has been shown to be a metalloid chaperone that delivers As(III) to the ArsAB As(III)-translocating ATPase (Lin et al. 2006). The initial motivation for considering an alternate function for ArsD was analysis of all *ars* operons that contain an *arsD* gene. The *arsD* gene is found in only a small number of operons, 14 to date. Remarkably, in each operon the *arsD* gene precedes an *arsA* gene. This observation suggested that ArsD and ArsA coevolved for a common and related function. A number of lines of evidence support this proposition. (1) When a mixed culture of cells expressing either *arsDAB* or *arsAB* were grown in the presence of a subtoxic concentration of As(III), the cells expressing all three genes took over the culture within 1 week, showing that cells with *arsDAB* have increased fitness in low As(III) compared with cells with only *arsAB*. (2) Expression of ArsD increases the ability of the ArsAB pump to extrude As(III). (3) ArsA and ArsD were shown to interact in vivo by yeast two-hybrid analysis. (4) ArsD and ArsA can be chemically cross-linked in a 1 : 1 complex through the cysteine residues of their metalloid binding sites. (5) The rate of dissociation of metalloid from ArsD is enhanced by four orders of magnitude by interaction with ArsA, consistent with transfer of As(III) from the chaperone to the ATPase. (6) ArsD increases ArsA's affinity for As(III) without altering the V_{max}. This makes the ArsAB pump more effective at low—and environmentally relevant—concentrations of metalloid, a property expected for a metallochaperone. At this time the molecular mechanism of chaperone activity is not known, but we propose that ArsD and ArsB bind to the same site on ArsA sequentially in a cycle of metal transfer from ArsD to ArsA to ArsB concomitant with ATP binding and hydrolysis.

4.5
Arsenate Reductases: ArsCs and Acr2s

In contrast to the membrane-bound respiratory arsenate reductases, enzymes catalyzing arsenate reduction for the purpose of detoxification are soluble proteins.

4.5.1
General Function

Cytosolic arsenate reductases catalyze the two-electron reduction of inorganic As(V) to As(III). These enzymes arose independently at least three times by convergent evolution. One family of arsenate reductases that includes the *E. coli* plasmid R773 ArsC uses glutaredoxin (Grx) and glutathione (GSH) as reductants (Mukhopadhyay and Rosen 2002). A second family, represented by the *Staphylococcus aureus* plasmid pI258 ArsC (Ji and Silver 1992) and the *B. subtilis* chromosomal ArsC, uses thioredoxin (Trx) as a reductant. This bacterial family of arsenate reductases is distantly related to mammalian low molecular weight (LMW) protein tyrosine phosphatases (PTPases) (Messens et al. 1999; Bennett et al. 2001). A third family of eukaryotic arsenate reductases includes Acr2p (Bobrowicz et al. 1997; Mukhopadhyay and Rosen 1998) from the baker's yeast *S. cerevisiae*, and LmAcr2p (Zhou et al. 2004) from the parasitic protozoa *Leishmania major*. The eukaryotic reductases are related to the catalytic domain of the Cdc25 cell-cycle PTPase (Fauman et al. 1998).

4.5.2
R773 Arsenate Reductase

The 141 amino acid R773 ArsC is a monomeric enzyme that catalyzes the reduction of arsenate to arsenite using GSH and Grx as electron donors (Gladysheva et al. 1994). The active site Cys12 is essential for ArsC catalysis (Fig. 7) (Liu et al. 1995) and is surrounded by an arginine triad composed of Arg60, Arg94, and Arg107 (Shi et al. 2003). The arginines are proposed to be involved in arsenate binding and transition-state stabilization (Shi et al. 2003; DeMel et al. 2004). *E. coli* has three glutaredoxins, Grx1, Grx2, and Grx3, each with a classical -Cys-Pro-Tyr-Cys- active-site motif, and Grx2 was found to be the most effective hydrogen donor for the reduction of arsenate by ArsC (Åslund et al. 1994; Shi et al. 1999). Analysis of single and double cysteine-to-serine substitutions in the active site of the three glutaredoxins demonstrated that only the N-terminal cysteine residue is essential for activity. Based on biochemical studies and X-ray crystallographic structures (Martin et al. 2001; DeMel et al. 2004) of the free and ligand-bound forms of ArsC, a mechanism for ArsC-catalyzed arsenate reductase activity has been

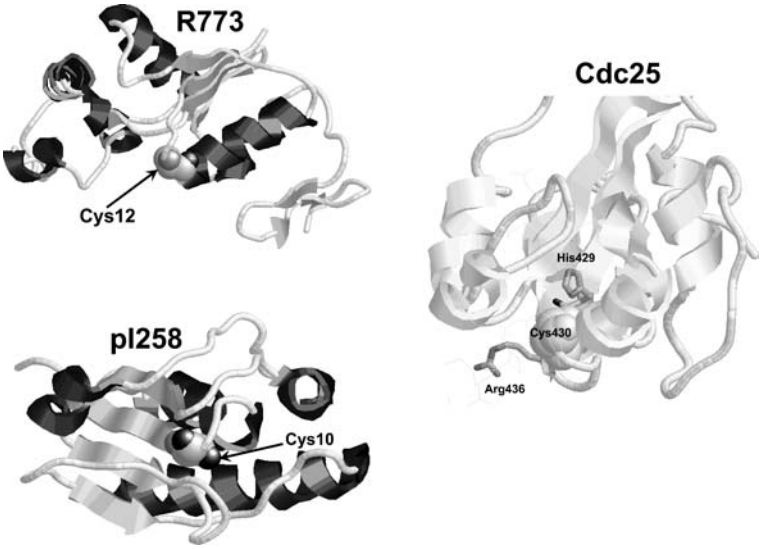

Fig. 7 Structure of arsenate reductase. The structures of R773 and pI258 ArsC are shown, identifying their secondary structural elements. The catalytic cysteines at the active sites of either enzyme complexed with As(V) are also indicated. The yeast arsenate reductase, Acr2p, is predicted to have a similar active site to the human Cdc25a. The structure of the catalytic core of human Cdc25a is shown, identifying the active site Cys430 that charge-pairs with His429, and Arg436, which forms part of the phosphate binding loop

proposed. The reaction starts by a nucleophilic attack by Cys12 on an arsenate that is noncovalently bound at the active site. This leads to the formation of a thioarsenate binary adduct and release of OH^-. In step 2, a nucleophilic attack on the arsenate adduct by GSH results in the formation of an {ArsC-Cys12}S–As(V)–S{glutathione} tertiary complex (Liu and Rosen 1997), and release of water. Step 3 involves binding of Grx, with reduction of arsenate to the dihydroxy monothiol As(III) intermediate, along with release of OH^- and a mixed disulfide complex of glutathione and glutaredoxin. The penultimate step is the formation of a monohydroxy, positively charged As(III), with release of OH^-. In the final step, addition of OH^- releases free arsenite [$As(OH)_3$] and regenerates the free enzyme. Except for the glutathionylated intermediate in step 2, the structures for all other intermediates in the above reaction pathway have been determined (Martin et al. 2001; DeMel et al. 2004).

4.5.3
S. aureus and *B. subtilis* Arsenate Reductases

The ArsC arsenate reductase encoded by *S. aureus* plasmid pI258 and the closely related *B. subtilis* enzyme are 14.8-kDa monomeric proteins that are

unrelated to the R773 ArsC. In contrast to R773 ArsC, which requires GSH and Grx for arsenate reductase activity, the pI258 enzyme is coupled to thioredoxin, thioredoxin reductase, and nicotinamide adenine dinucleotide phosphate (NADPH) (Ji et al. 1994). Mutation of Cys10, Cys82, and Cys89 of pI258 ArsC led to inactive enzymes (Messens et al. 1999), and these cysteines were shown to form a unique intramolecular disulfide cascade, essential for the reduction of arsenate to arsenite (Messens et al. 2002). The enzyme has a LMW PTPase anion binding motif that forms a phosphate binding P-loop and has phosphatase activity, catalyzing hydrolysis of *p*-nitrophenyl phosphate (Zegers et al. 2001), so the single protein has both arsenate reductase and PTPase activity.

On the basis of the structures of the reduced and oxidized forms of pI258 ArsC, along with data from NMR and kinetic studies, a multistep reaction mechanism has been proposed (Zegers et al. 2001; Roos et al. 2006). The first step of pI258 ArsC catalyzed arsenate reduction starts with a nucleophilic displacement reaction by Cys10 (Fig. 7) on arsenate, leading to the formation of a covalent Cys10–arseno adduct (Zegers et al. 2001), which is the equivalent of the Cys12–arseno adduct of R773 ArsC (Martin et al. 2001). In the second step, arsenite is released following a nucleophilic attack by Cys82 on the covalent Cys10–arseno adduct. An oxidized Cys10–Cys82 intermediate is formed (Messens et al. 2002). In the third reaction step, Cys89 attacks the Cys10–Cys82 disulfide, resulting in the formation of oxidized Cys82-Cys89 disulfide and the reduction of Cys10 (Zegers et al. 2001; Messens et al. 2002). Finally, ArsC is regenerated by thioredoxin that reduces the Cys82–Cys89 disulfide.

The X-ray crystal structure for another thioredoxin-linked arsenate reductase has been reported (Bennett et al. 2001) for the enzyme encoded by the *B. subtilis* skin element (Sato and Kobayashi 1998). The *B. subtilis* arsenate reductase contains 139 amino acid residues and is a monomer in solution. Like the pI258 ArsC, the *B. subtilis* ArsC also uses thioredoxin and exhibits PTPase activity, although this rate is many-fold lower than that of the *p*-nitrophenyl phosphate hydrolysis by true PTPase. The crystal (Bennett et al. 2001) and solution structures (Guo et al. 2005) of *B. subtilis* ArsC suggest that the catalytic mechanism for arsenate reduction involves the same triple cysteine redox relay system as the pI258 enzyme (Zegers et al. 2001; Messens et al. 2002).

An interesting variant of arsenate reductase has been described from the cyanobacteria *Synechocystis* sp. strain PCC 6803 (Li et al. 2003). The *Synechocystis* ArsC is homologous to pI258 ArsC and shows both phosphatase and arsenate reductase activities. However, its catalytic activity exhibits a unique combination of both R773 and pI258 ArsC mechanisms. Like the R773 ArsC, *Synechocystis* ArsC requires both GSH and Grx as the source of reducing equivalents for arsenate reduction, rather than thioredoxin, as does the pI258 enzyme. While the R773 enzyme requires only one cysteine for catalysis, the *Synechocystis* ArsC has three essential cysteine residues, like the pI258 *S. au-*

reus enzyme. *Synechocystis* and *S. aureus* enzymes may be the products of two independent evolutionary pathways of LMW PTPase into arsenate reductases (Li et al. 2003).

4.5.4 Eukaryotic Arsenate Reductases

Eukaryotic arsenate reductases that reduce arsenate to arsenite have been identified from *S. cerevisiae* (Bobrowicz et al. 1997; Mukhopadhyay and Rosen 1998), *S. douglasii* (Maciaszczyk et al. 2004), fern (*Pteris vittata*) (Ellis et al. 2006), rice (*Oryza sativa*) (Duan, Rosen, and Zhu 2007) and *L. major* (Zhou et al. 2004). The best-characterized eukaryotic arsenate reductases are Acr2p from *S. cerevisiae* (Mukhopadhyay and Rosen 1998; Mukhopadhyay et al. 2000) and LmACR2 from *L. major* (Zhou et al. 2004).

The 130 amino acid Acr2p exists as a homodimer and is unrelated in sequence to bacterial arsenate reductases. Acr2p is a member of the superfamily of PTPases such as the human cell-cycle dual-specific phosphatase Cdc25a (Fig. 7). Members of this family have a consensus active site $HC(X)_5R$ motif (Denu and Dixon 1995; Fauman et al. 1998). Alteration of either Cys76 or Arg82 at the consensus $C^{76}(X)_5R^{82}$ motif of Acr2p resulted in loss of As(V) resistance in vivo and As(V) reduction in vitro (Mukhopadhyay and Rosen 2001). These results suggest that Cys76 is the equivalent of Cys12 in ArsC and may form As(V) and As(III) intermediates. Similar to the *E. coli* ArsC, Acr2p activity required both GSH and glutaredoxin, but not thioredoxin, to support arsenate reduction. Glutaredoxins from both *S. cerevisiae* and *E. coli* were able to serve as reductants. Analysis of *grx* mutants lacking one or both cysteine residues in the -Cys-Pro-Tyr-Cys- active site demonstrated that only the N-terminal cysteine residue is essential for arsenate reductase activity. This suggests that the catalytic mechanism of Acr2p may follow a similar pathway as *E. coli* ArsC (Mukhopadhyay et al. 2000).

Although Acr2p is a member of the superfamily of PTPases, it does not exhibit phosphatase activity. It has the $C^{76}(X)_5R^{82}$ phosphatase motif but not the glycine-rich phosphate binding motif (GXGXXG) that is found in PTPases. Introduction of glycines at positions 79, 81, and 84 of the Acr2p primary sequence resulted in a gain of PTPase activity and a loss of arsenate reductase activity (Mukhopadhyay et al. 2003). The relative ease with which an arsenate reductase can be converted into a protein–tyrosine phosphatase indicates that selective environmental pressure led to the evolution of an arsenate reductase from a protein–tyrosine phosphatase (or, possibly, the reverse).

An arsenate reductase from *L. major*, LmACR2, has been identified and characterized (Zhou et al. 2004). LmACR2 exhibits 28% sequence identity and 44% similarity with *S. cerevisiae* Acr2p. LmACR2 can functionally complement the arsenate-sensitive phenotypes of the *acr2* deletion strain of *S. cerevisiae* and also the Δ*arsC* strain of *E. coli*. In contrast to yeast Acr2p which

exists as a dimer, the LmACR2 exists as a monomeric species. LmACR2 also requires GSH and Grx as the source of reducing equivalents to catalyze the reduction of arsenate (Zhou et al. 2004). Additionally, LmACR2 can also reduce pentavalent antimony [Sb(V)] compounds (Zhou et al. 2004). This is the first example of an arsenate reductase that also has antimonate reductase activity. Macrophage-infected amastigotes derived from LmACR2-transfected *L. infantum* promastigotes exhibited increased sensitivity to the pentavalent antimony drug, Pentostam. This is because LmACR2 can reduce the pentavalent antimony in Pentostam to the active trivalent form of the drug. Thus, in contrast to the bacterial and yeast arsenate reductases that are components of metalloid detoxification pathways, expression of LmACR2 in *Leishmania* leads to metalloid sensitivity. Why should *Leishmania* evolve an enzyme solely for drug activation as it is detrimental to the pathogen? We have recently shown that LmACR2 also exhibits PTPase activity at similar levels as other known PTPases (Zhou et al. 2006). Most likely, the physiological function of LmACR2 is to dephosphorylate phosphotyrosine residues in leishmanial proteins, and not detoxification of metalloids.

4.6 Arsenite Methylases

The ability to methylate arsenic methylation is widespread: many bacteria, archaea, fungi, plants, and animals do so. The history of arsenic methylation has been described entertainingly (Bentley and Chasteen 2002) and will be only briefly summarized here. Arsenic metabolism produces a garlicky odor. For example, individuals treated with cacodylic acid (dimethylarsinic acid or DMA(V)) have been known to exhale the odor of garlic. Copper–arsenic compounds such as the pigment Paris green were used in the production of wallpaper. Fungi can convert this to a volatile, garlicky, and toxic arsenical, which has been called "Gosio gas" after the Italian physician Bartolomeo Gosio, who described this process in the 1890s. In 1945 Frederick Challenger demonstrated that Gosio gas is trimethylarsine, $(CH_3)_3As$ or TMA(III). Challenger proposed a pathway in which arsenate can be converted to TMA(III) (Fig. 8) by a series of reductions of pentavalent to trivalent arsenicals coupled to oxidative methylations to mono-, di-, and trimethyl species.

Some mammals, including humans and rat, methylate inorganic arsenic and excrete methylated species such as DMA(V) and, to a lesser extent, MMA(V) in the urine. This has led to the proposal that methylation is a detoxification process. However, other species such as chimpanzees do not methylate inorganic arsenic, and do not seem to be any less arsenic sensitive than humans or rat. Additionally, it has been pointed out that the trivalent intermediates in the Challenger pathway, MMA(III), DMA(III), and TMA(III), are considerably more toxic than inorganic arsenate or arsenite (Petrick et al.

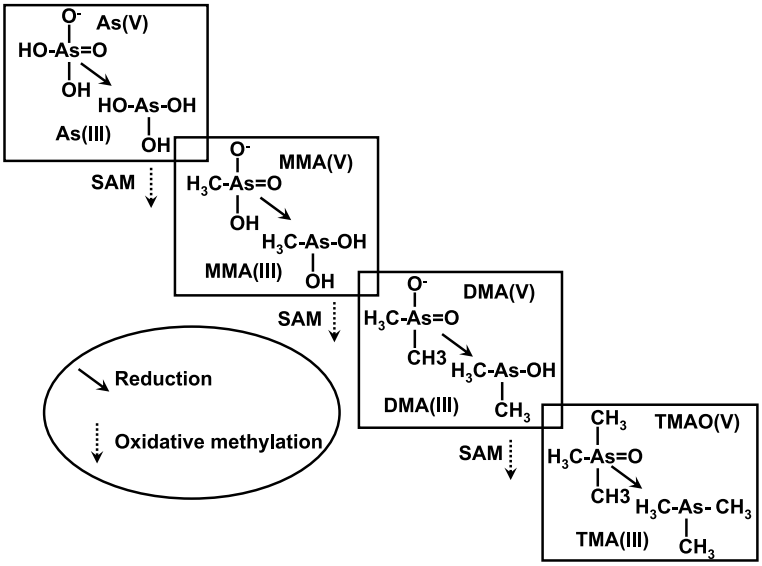

Fig. 8 The Challenger pathway of arsenic methylation. In each step a pentavalent arsenical is reduced to a trivalent arsenical, which is then oxidatively methylated with *S*-adenosylmethionine to form the pentavalent form. The overall scheme involves four reductive steps and three methylations to form the gas TMA(III) from inorganic As(V)

2000; Drobna et al. 2005). In vivo, the toxicity of inorganic and organic arsenicals is DMA(III), MMA(III) > As(III) > As(V) > DMA(V), MMA(V) > TMAO (Akter et al. 2005). The major pentavalent products, DMA(V) and TMAO, are approximately 100-fold and 1000-fold less toxic than As(III), respectively (Hirano et al. 2004). This has led to the proposal that methylation may activate inorganic arsenic to more toxic metabolites. This question remains unresolved to date.

In 2002 Thomas and coworkers cloned the gene for a rat enzyme that methylates As(V) and As(III) using *S*-adenosylmethionine (SAM) as the methyl donor (Lin et al. 2002). The enzyme has been termed Cyt19 or As3MT. It is not known whether arsenic methylation is the primary physiological function of As3MT. However, it does seem likely that this enzyme is the major mammalian arsenic methylase. The enzyme is not made in chimpanzees due to a frame-shift mutation, leading to an aborted protein product, and consequently primates do not methylate inorganic arsenic.

Bacteria and fungi are known to produce volatile and toxic arsines (Bentley and Chasteen 2002), but the physiological roles of arsenic methylation in microorganisms are likewise unclear, as is its biochemical basis. A large number of genes for bacterial and archaeal homologues of As3MT have been identified. To date, 200 homologues have been identified in genome databases, 7 in archaea, 13 in fungi, 17 in metazoa, and 163 in bacteria. A subset of these

genes has been termed *arsM* and their protein product ArsM (arsenite *S*-adenosyl methyltransferase). What sets these homologues apart from As3MT is that they are downstream of an *arsR* gene, encoding the archetypal arsenic-responsive transcriptional repressor that controls expression of *ars* operons (Xu and Rosen 1999), suggesting that these ArsMs evolved to confer arsenic resistance. The gene for the 283-residue ArsM (29 656 Da) (accession number NP_948900.1) was cloned from the soil bacterium *Rhodopseudomonas palustris* and expressed in an arsenic-hypersensitive strain of *E. coli* (Qin et al. 2006). This heterologous expression of *arsM* conferred As(III) resistance to *E. coli* in the absence of any other *ars* genes, demonstrating that methylation is sufficient to detoxify arsenic (Qin et al. 2006). ArsM converted As(III) into a number of methylated intermediates of the Challenger pathway, with volatile trimethylarsine as the end product. MMA(III) and DMA(III) may be more toxic than As(III), but they do not accumulate in cells expressing *arsM*, and, while TMA(III) is more toxic than arsenite, the fact that it is a gas means that it does not accumulate in cells expressing ArsM. TMA(III) volatilization results in loss of arsenic, from both cells and medium. Since ArsM homologues are widespread in nature, this microbial-mediated transformation is proposed to have an important impact on the global arsenic cycle. ArsM was purified and shown to catalyze the transfer of methyl groups from *S*-adenosylmethionine to arsenite, forming di- and trimethylated species, with TMA(III) gas as the final product (Qin et al. 2006). This is the first demonstration that methylation of environmental arsenic by conversion to soluble and gaseous methylated species is a detoxifying process that may contribute to global cycling of arsenic.

4.7
Other Novel Mechanisms of Arsenic Resistance: AqpS and ArsH

In addition to *arsRBC* and *arsRDABC* operons, several bacterial *ars* operons encode for proteins that participate in novel ways to arsenic resistance. For example, the chromosomal *ars* operon of the legume symbiont *Sinorhizobium meliloti* Rm1021 displays a cluster of four genes: *arsR*, *aqpS*, *arsC*, and *arsH*. Although, *S. meliloti* ArsR shows sequence similarity to the ArsR subfamily of helix–turn–helix bacterial transcription regulatory proteins, it does not contain the N-terminal CXCXXC motif, which is required for binding of the inducer in *E. coli* and R773 ArsR (Shi et al. 1996; Xu et al. 1996). Instead, *S. meliloti* ArsR is more closely related to the *A. ferrooxidans* ArsR discussed above, and has two vicinal cysteine pairs located near the C-terminal end of the protein. However, the involvement of these thiols in metalloid binding remains to be determined. The second gene *aqpS* encodes for a membrane protein that belongs to the major intrinsic protein or aquaporin superfamily (King et al. 2004) and shows sequence homology with the bacterial glycerol facilitator (GlpF), yeast aquaglyceroporin Fps1p, and mammalian aquaglyc-

eroporin AQP9. *S. meliloti* ArsC is homologous to *E. coli* ArsC and both show similar levels of arsenate reductase activity. The fourth gene encodes a novel protein, ArsH, which has conserved domains related to the NADPH-dependent flavin mononucleotide reductase class of proteins.

The presence of *aqpS* in lieu of *arsB* in the *S. meliloti ars* operon is interesting, since aquaglyceroporins have been shown to be responsible for the uptake of arsenite (Meng et al. 2004) and antimonite (Sanders et al. 1997), rendering them sensitive to arsenite. To understand the role of *aqpS* in arsenic resistance, *S. meliloti aqpS* and *arsC* were disrupted individually. Disruption of *aqpS* resulted in increased tolerance to arsenite but not arsenate, while cells with an *arsC* disruption showed selective sensitivity to arsenate. Additionally, transport experiments with intact cells showed that AqpS facilitates transport of arsenite. Coexpression of *S. meliloti aqpS* and *arsC* in an *E. coli* Δ*ars* strain complemented arsenate but not arsenite sensitivity. These results indicated that, when *S. meliloti* is exposed to environmental arsenate, arsenate enters the cell through the phosphate transport system and is reduced to arsenite by ArsC. Internally generated arsenite is extruded out of the cell by downhill movement through AqpS. Therefore, AqpS and ArsC together form a novel pathway of As(V) detoxification in *S. meliloti*. This is the only example of an aquaglyceroporin with a physiological role in arsenic resistance (Yang et al. 2005). Several other organisms, for example, *Mesorhizobium loti*, *Caulobacter crescentus*, and *Ralstonia solanacearum*, also have chromosomal *ars* operons that contain the unique combination of *aqpS* and *arsC* genes. This pathway may be widespread in organisms that are exposed primarily to As(V).

The role of ArsH in arsenic resistance is unclear. ArsH is found to be widely distributed in bacteria but sparsely in fungi, plants, and archaea. The *arsH* gene product in *Yersinia enterocolitica* confers resistance to both arsenite and arsenate (Neyt et al. 1997). Similarly, deletion of the *arsH* gene from the IncH12 plasmid R478 resulted in a total loss of resistance to arsenic compounds (Ryan and Colleran 2002). However, *A. ferrooxidans* and *Synechocystis* ArsHs are not required for arsenic resistance (Butcher et al. 2000; Lopez-Maury et al. 2003). We find that inactivation of the *S. meliloti arsH* gene results in increased As(III) sensitivity. Overexpression of ArsH in Δ*ars* strain of *S. meliloti* shows high-level resistance to trivalent arsenicals. This indicates that ArsH can provide resistance to As(III) by a unique mechanism that does not require AqpS and ArsC activities. ArsH has been crystallized (Jun et al., in preparation), and its catalytic mechanism is currently being investigated.

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