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Review Article

Efflux Mechanisms of Resistance to Cadmium, Arsenic and Antimony in Prokaryotes and Eukaryotes

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ABSTRACT

Kan-Jen Tsai, Ching-Mei Hsu and Barry P. Rosen (1997) Efflux mechanisms of resistance to cadmium, arsenic and antimony in prokaryotes and eukaryotes. *Zoological Studies* **36**(1): 1-16. Resistance to toxic metals is ubiquitously found in prokaryotes, both gram positive and gram negative, and in all types of eukaryotes, including fungi, plants, protozoans, and animals. In both prokaryotes and eukaryotes, toxic metals are extruded from cells through efflux transport systems to confer this resistance. Although the chemical substrates recognized by each transport system vary considerably, many heavy metal and metalloid translocating ATPases have been identified by evolutionarily unrelated pumps that have evolved the same function. Among these efflux mechanisms of resistance to heavy metals and metalloids, bacterial cadmium resistance is mediated by a primary transporter while the thiol-linked efflux systems have developed for cadmium resistance in eukaryotes. In contrast, bacterial resistance to arsenicals and antimonials is mediated by a primary ATP-coupled pump in association with a catalytic subunit, whereas an ATP-coupled As-thiol pump has evolved to confer the resistance in eukaryotic systems. The biochemical aspects of the efflux mechanisms related to cadmium, arsenic, and antimony resistance in prokaryotes and eukaryotes are discussed in detail in the present review.

Key words: Efflux, Resistance, Cadmium, Arsenic, Antimony.

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INTRODUCTION

In both prokaryotes and eukaryotes there are transport systems that produce resistance to drugs and toxic metals by extrusion from the cell, reducing the intracellular concentration to subtoxic levels (Dey and Rosen 1995a). To treat clinical drug resistance we must first know the molecular mechanisms by which resistance occurs. This chapter will briefly review biochemical aspects of resistance to the toxic metals cadmium, arsenic, and antimony.

Arsenicals and antimonials were among the 1st chemotherapeutic agents used to treat infectious diseases; nearly a century ago Paul Ehrlich introduced into clinical use his so-called "silver bullet", Salvarsan, for the treatment of syphilis and trypanosomal diseases. For his development of this organic arsenical drug Ehrlich was awarded the Nobel Prize in 1908. He noted that Salvarsan resistance rapidly arose in previously arsenic sensitive cells. Ehrlich hypothesized that arsenicals must be taken up by cells via a cell surface arsenical receptor in order to be effective. He postulated that resistant organisms might no longer take up the toxic arsenical, surviving the drug. In our respective laboratories we are investigating the mechanisms of resistance to cadmium, arsenic, and antimony in prokaryotes and eukaryotes. Interestingly, although both kingdoms have evolved efflux pumps for toxic metals, the specific mechanisms and the proteins that produce them appear to be the results of independent, convergent evolution (Fig. 1).

Bacterial metal resistances evolved originally from exposure to natural sources, and only more recently from our use of drugs (Hughs and Datta 1983). Toxic metal ions are ubiquitously found in the environment (Foye 1977, Knowles et al. 1983). In eubacteria, plasmid-mediated resistances to inorganic salts, such as arsenate, arsenite, antimony, lead, cadmium, zinc, bismuth, and mercury have been previously reported (Novick et al. 1968, Foster 1983). Bacterial cells bearing these resistance determinants can evade metal toxicity, suggesting that there might be specific mechanisms encoded by these genetic elements to exclude metal ions. Of these, two have been characterized in sufficient biochemical detail to warrant review: the CadA ATPase that produces cadmium resistance in *Staphyloccocus aureus* (Nucifora et al. 1989), and the arsenite-translocating ATPase that provides resistance to arsenicals and antimonials in *Escherichia coli* (for recent reviews, see Kaur

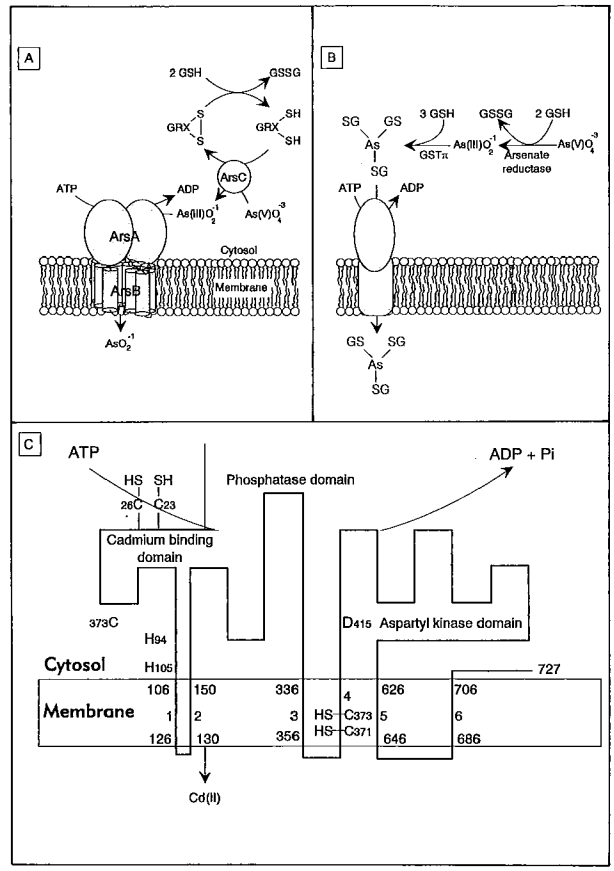

Fig. 1. Prokaryotic and eukaryotic pumps for metals and metalloids. Bacteria and eukaryotes have convergently evolved different resistance pumps for the ATP-coupled extrusion of cadmium, arsenicals, and antimonials from cells. **(A)** In bacteria, ArsA and ArsB form a membrane-bound oxyanion-translocating ATPase for extrusion of arsenite and antimonite. The 63-kDa ArsA ATPase is the catalytic subunit. The 45.5-kDa ArsB subunit, an integral membrane protein located in the inner membrane of *E. coli*, has conductivity for arsenite or antimonite. ArsC is an arsenate reductase that expands the range of resistance by reducing arsenate (As(V)) to arsenite (As(III)). Electrons are transferred from glutaredoxin, which is re-reduced by glutathione. **(B)** Resistance to arsenite and antimonite in eukaryotic cells occurs by the ATP-coupled extrusion of a soft metal-thiol complex, where As(III) or Sb(III) are postulated to react with a physiological thiol such as glutathione in mammalian cells or trypanothione in *Leishmania*. Although arsenate and antimonite are slowly reduced nonenzymatically by glutathione and other thiols, arsenate and the antileishmanial drug Pentostam, which contains Sb(V), may require a reductase to convert them to the trivalent form at physiologically significant rates. Sb(III) and As(III) also react spontaneously with thiols, but enzymatic conjugation may be required for reaction to produce resistance. The metalloid-thiol complex is proposed to be the substrate of a transport system that extrudes the conjugate, thus producing resistance. Similar eukaryotic pumps exist for the transport of Cd(II)-thiol conjugates. **(C)** Resistance to Cd(II) in prokaryotes is catalyzed by the CadA P-type ATPase. The domains in this 727-residue protein are postulated from their position in homologues (Silver and Walderhaug 1992).

et al. 1992, Dey and Rosen 1995a). In addition, the bacterial arsenical resistance will be compared with evolutionarily unrelated eukaryotic pumps that have evolved the same function.

BACTERIAL CADMIUM RESISTANCE AND REGULATION

As a consequence of antibiotic usage, bacterial cadmium resistance has been prevalently found in clinical isolates (Udo et al. 1994 1995). In most cases, cadmium resistance is associated with other genes conferring resistance to 1 or more antibiotics. For example, chromosomally-encoded cadmium resistance was found in 5 methicillin-resistant *S. aureus* strains along with resistance genes to mercury (Witte et al. 1986). Strains with the chromosomally-encoded cadmium resistance determinants exhibit resistance to cadmium only and not to zinc, and they actively extrude cadmium. However, cadmium-resistant genetic elements acquired through transposition and transformation processes are also frequently found as well (Lebrun et al. 1994a,b). One such cadmium resistance determinant was found in the staphylococcal plasmid pI258 (Novick 1963 1968). This penicillinase-producing plasmid, originally isolated from a clinical specimen, also carries resistance to erythromycin, mercury, lead, bismuth, cadmium, zinc, arsenic, and antimony. Cadmium resistance produced by pI258 was shown to be composed of 2 separated loci, *cadA* and *cadB*. The *cadA* resistance determinant confers approximately 100-fold resistance. However, *cadB* gave only a low-level resistance to cadmium (Smith et al. 1972). The *cadB* resistance determinant is found only on some, but not all, penicillinase plasmids in *S. aureus* harboring *cad*A. The mechanism of *cadB*-mediated cadmium resistance has not been determined, but it does not produce cadmium efflux (Perry et al. 1982).

Plasmid-mediated *cad*A cadmium resistance determinant confers resistance to both cadmium and zinc and shows no homology to the chromosomally-encoded cadmium efflux system described above (Witte et al. 1986). Initial reports indicated that *cadA*-mediated cadmium resistance correlated with decreased cadmium uptake in cells (Chopra 1970, Tynecka 1975, Weiss et al. 1978, Tynecka et al. 1981a,b). The effects of energy sources and inhibitors suggested that Cd(II) transport was mediated by an electroneutral $2H^+$/Cd(II) antiporter coupled to the electrochemical proton gradient (Tynecka et al. 1981b). As will be discussed in more detail below, more recently several lines of evidence have suggested that *cad*A cadmium efflux is catalyzed by a primary pump and not a secondary transporter. First, from the amino acid sequence deduced from the DNA sequence of the *cad* operon, the CadA protein is a member for the P-type ATPase family of cation pumps (Nucifora 1989). Second, the protein has been characterized biochemically, and shown to be an ATP-dependent Cd(II) pump (Tsai et al. 1992).

The *cad*A resistance determinant from *S. aureus* plasmid pI258 has been cloned and expressed in *Bacillus subtilis* (Nucifora 1989). A 3.5-kb nucleotide fragment was demonstrated to contain all the genetic elements required for conferring full resistance to cadmium and zinc in *B. subtilis* (Fig. 2A). Two open reading frames (ORFs) were identified. The 1st, *cadC*, encodes a polypeptide of 122 amino acids, which, as discussed below, is the repressor protein of the *cad* operon. The 2nd ORF, *cad*A, encodes a protein of 727 amino acid residues, which, as described below, is the Cd(II)-translocating ATPase.

CadA

As mentioned above, from its predicted amino acid sequence, CadA is a member of the family of P-type cation-translocating ATPases. Members of this group of enzymes include eukaryotic proteins such as the Na^+/K^+-ATPase of animal cells, the Ca^{2+}-ATPase from sarcoplasmic reticulum (MacLennan et al. 1985, Shull et al. 1985, Brandl et al. 1986), and the H^+-ATPase of *Neurospora* (Addison 1986, Hager et al. 1986, Serrano et al. 1986). Bacterial members of this family include the Cu^{2+}-ATPase of *Enterococcus hirae* (formerly *Streptococcus faecalis*) (Solioz et al. 1987, Walderhaug et al. 1987, Odermatt et al. 1993), the Ca^{2+}-ATPase of *Flavobacterium odoratum* (Gambel et al. 1992), the K^+-translocating ATPase of *E. coli* (Hesse et al. 1984, Walderhaug et al. 1987), and the Mg^{2+}-ATPase of *Salmonella typhimurium* (Snavely et al. 1991). Based on the generalized structure of P-type ATPases, a model of CadA protein with 6 transmembrane α-helices was proposed (Fig. 1C) (Silver et al.1989, Silver and Walderhaug 1992). Putative domains include the ATP-binding domain and the 7-residue phosphorylation site (DKTGTLT) from residues 415 to 421, which are conserved in all P-type ATPases. D415 corresponds to the residue phosphorylated in P-type ATPases (Puppe et al. 1992). In support of this

model, CadA, which was identified as a Cd(II)-inducible membrane protein on sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tsai et al. 1992), was shown to form a phosphorylated intermediate during the catalytic cycle (Tsai and Linet 1993). The phosphoenzyme intermediate formed in the presence of cadmium and γ-[^{32}P]-ATP was hydroxylamine sensitive, consistent with an acylphosphate bond.

Using everted membrane vesicles prepared from cells of *B. subtilis* expressing the *cad* operon, CadA was shown to catalyze ATP-dependent ^{109}Cd(II) transport (Tsai et al. 1992). No uptake was observed in the presence of NADH or reduced phenazine methosulfate, both of which generate a pH gradient (Tsai et al. 1992). These results indicate that ATP, but not the proton motive force, is the energy source utilized to drive cadmium transport. Therefore, the transporter is clearly a primary pump and not a secondary proton exchanger. However, the possibility that CadA catalyzes ATP-coupled Cd(II)/H$^+$ exchange cannot be excluded.

CadA has recently been functionally expressed in *E. coli* under control of the T7 phage promoter (Tsai and Linet, unpublished data). Since *E. coli* is intrinsically cadmium resistant, this property could not be examined in vivo. However, in vitro assays demonstrated its function. First, CadA could be visualized as an inducible band on sodium dodecyl sulfate polyacrylamide gel electrophoresis. Second, intact cells extruded ^{109}Cd(II). Third, everted membrane vesicles had ATP-dependent ^{109}Cd(II) transport. Site-directed mutagenesis of the *cadA* gene was performed using *E. coli* expression, focusing on the 2 cysteine residues, Cys23 and Cys26, found at the amino terminus of CadA (Fig. 1C). These 2 cysteine residues have been postulated to be the Cd(II) recognition site (Silver and Walderhaug 1992). This domain of CadA shows sequence similarity with mercuric reductases and with the R100 periplasmic mercury binding protein (Nucifora et al. 1989, Silver et al. 1989). Recently 2 human Cu(II)-ATPases, the Menkes and the Wilson gene products include the similar amino terminal sequences. The sequences shown in these 2 gene products are believed to play a parallel role to the initial binding of metal ions (Bull et al. 1993, Silver et al. 1993). However, the latter 2 proteins repeat the cysteine

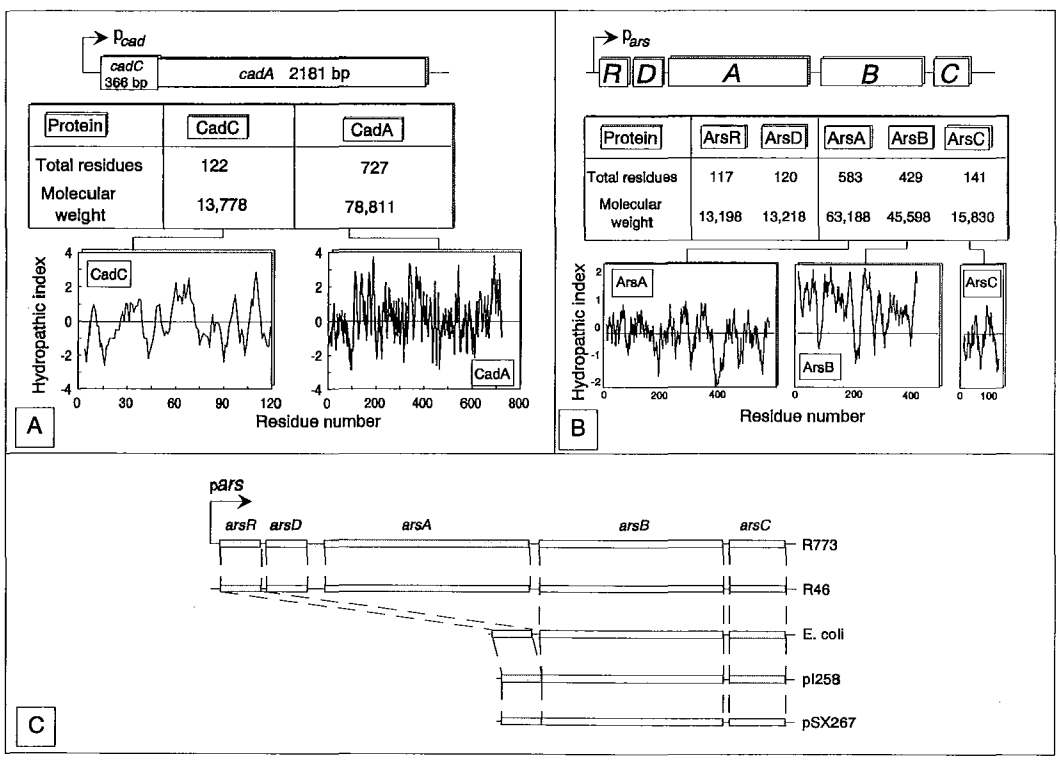

Fig. 2. Bacterial operons and their gene products that produce resistance to Cd(II)/Zn(II) or As(III)/Sb(III). (A) The *S. aureus* plasmid pI258 *cad* operon. (B) The *E. coli* plasmid R773 *ars* operon. (C) Comparison of bacterial *ars* operons.

motif 6 times, compared with the single one in CadA. The homologous regions in mercury resistance proteins are believed to be sites of initial recognition and chelation of mercuric ions.

To elucidate the roles of these cysteine residues in CadA, the site-directed mutants C23S, C23G, C26S, and C26G were constructed (Tsai and Linet, unpublished data). Although these cysteine mutants had reduced cadmium transport activity in vivo, they still retained efflux activity. In vitro assays of cadmium uptake in everted membrane vesicles showed a more drastic effect of the mutations, but, again, some activity remained. These results suggest that these 2 cysteines participate in but are not individually essential for Cd(II) recognition. A more detailed genetic analysis will be necessary to elucidate the phenomena.

CadC

CadC, a 122 residue protein (Fig 2A), amino acids was identified as a 13.8-kDa [^{35}S]-methionine-labeled band by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Yoon and Silver 1991). From the deduced amino acid sequence, CadC is a member of the ArsR family of metalloregulatory proteins (San Francisco et al. 1990, Shi et al. 1994) (Fig. 3). As discussed below, ArsR is the As(III)-responsive repressor of the arsenical resistance operon. This information strongly suggested that CadC protein is the Cd(II)-responsive repressor of the *cad* operon (Nucifora et al. 1989). Initial studies did not show an involvement of CadC in in vivo regulation (Yoon et al. 1991). More recently, using gel shift assays and DNase I footprinting, Endo and Silver (1995) have shown that purified CadC binds to *cad* DNA. CadC was dissociated from the *cad* operator DNA region by the inducers Cd(II), Bi(III), and Pb(II). Furthermore, CadC inhibited in vitro transcription. These results strongly indicate that CadC is the transcriptional regulator of the *cad* operon.

BACTERIAL RESISTANCE TO ARSENICALS AND ANTIMONIALS

Perhaps due to the wide use of arsenic compounds as antimicrobials, feed supplements, and herbicides, plasmid-mediated resistance to toxic anion arsenite and arsenate has been found in both gram-positive and gram-negative bacteria (Silver et al. 1981, Kaur and Rosen 1992a). The 1st report on plasmid-mediated arsenical resistance was the pI258 isolated from the gram-positive bacterium *Staphylococcus aureus* (Novick and Roth 1968). Similarly the *Escherichia coli* plasmid R773 confers high-level resistance against arsenite and arsenate (Hedges and Baumberg 1973). Resistance results from the active extrusion of oxyanions (Mobley and Rosen 1982, Silver and Keach 1982).

Genetic and molecular biological analyses of the arsenical resistance genes on plasmids of gram-negative and gram-positive bacteria revealed

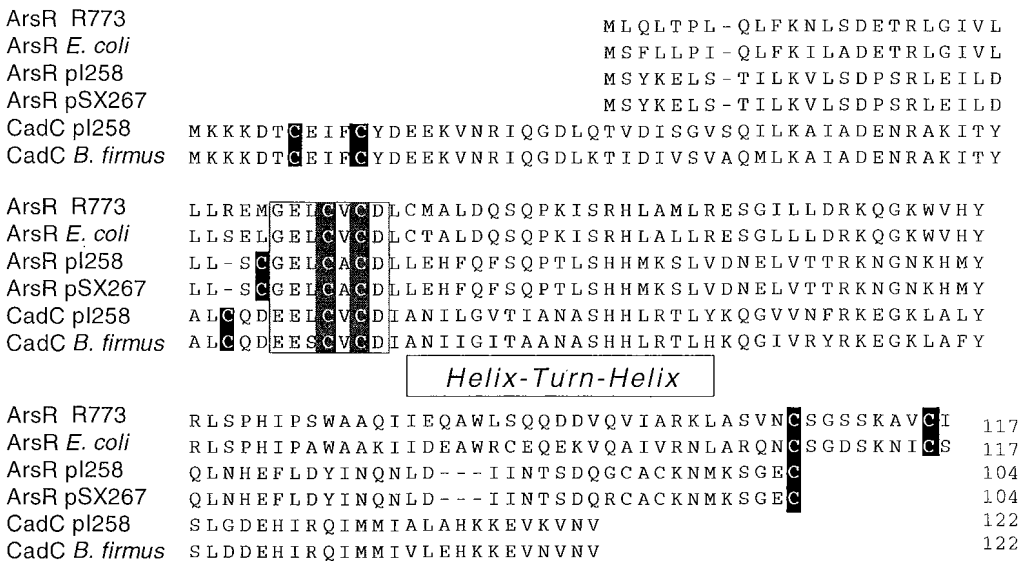

Fig. 3. The ArsR family of metalloregulatory proteins. The primary sequences of 4 As(III)/Sb(III) repressors are aligned with 2 Cd(II)/Zn(II) repressors. In the shaded box is the metal binding domain, including Cys32 and Cys34 of the R773 ArsR. Other cysteine residues are identified. The open box shows the location of the DNA binding helix-turn-helix domain.

that a single cluster of genes, designated the *ars* operon, was responsible for the resistance phenotype. The *ars* operon of plasmids R773 and R46 each have 5 genes: *arsR*, *arsD, arsA*, *arsB*, and *arsC* (Chen et al. 1986, Bruhn et al. 1996) (Fig. 2B). Recently a chromosomal *ars* operon of *E. coli* has been identified (Sofia et al. 1994) and shown to confer a low level of arsenical resistance in *E. coli* (Carlin et al. 1995). The chromosomal *ars* operon encodes only 3 proteins, ArsR, ArsB, and ArsC. Two *ars* operons on staphylococcal plasmids pI258 (Ji and Silver 1992) and pSX267 (Rosenstein et al. 1992) have also been sequenced, and those also have only 3 genes, *arsR*, *arsB*, and *arsC*. The physical maps of these operons are illustrated in Fig. 2C.

The arsenical resistance carried on plasmid R773 is the best characterized and will be described in detail. In the *ars* operon of plasmid R773, the *arsR* and *arsD* genes encode regulatory proteins (Tisa and Rosen 1991, Wu and Rosen 1991 1993a). The *arsA* and *arsB* gene products form an ATP-driven oxyanion pump (Dey et al. 1994). A model of the oxyanion pump is shown in Fig. 1A. ArsA is the catalytic subunit of the pump (Hsu and Rosen 1989a), while ArsB is the membrane anchor for ArsA and forms the anion-conducting pathway (Tisa and Rosen 1990a, Dey et al. 1994). Arsenate resistance requires the product of the *arsC* gene to reduce arsenate to arsenite, which is then extruded by the pump (Gladysheva et al. 1994, Oden et al. 1994).

ArsA

Analysis of the predicted amino acid sequence of ArsA indicated that the N-terminal A1 half of the protein exhibits similarity to the C-terminal A2 half (Chen et al. 1986) (Fig. 4A). Each half of the ArsA protein contains the nucleotide-binding consensus sequence termed the A1 and A2 sites (Walker et al. 1992). As predicted from the in vivo energetics and the protein sequence, the purified ArsA protein is an oxyanion-stimulated ATPase (Rosen et al. 1988). Characterization of the activity of purified ArsA protein (Hsu and Rosen 1989a) suggested that the oxyanion pump is evolutionarily unrelated to any known classes of ion-translocating ATPases (Pedersen and Carafoli 1987).

The role of the 2 similar but not identical nucleotide binding sites has been investigated by several approaches. Using a fluorescent ATP analogue, TNP-ATP, it was shown that both sites are true ATP binding sites (Karkaria et al. 1991). However, binding of ATP to both sites can be differentiated by their ability to form a photoadduct with α-[^{32}P]ATP upon UV radiation (Rosen et al. 1988). Although both sites are functional for ATP binding, only the A1 site can be photolabeled. Subsequently, the A1 and A2 sites have been cloned and expressed as separate polypeptides, the A1 peptide forms a photoadduct but the A2 peptide does not. These results reflect differences in the local environment of the 2 ATP binding sites, and the functions of the 2 sites may differ (Kaur and Rosen 1994).

Mutational analysis demonstrated that both nucleotide binding sequences are required for the catalytic activity of the ArsA protein and resistance (Karkaria et al. 1990, Kaur and Rosen 1992b). However, genetic complementation assays showed that the 2 functional ATP binding sites need not be contained in the same polypeptide (Kaur and Rosen 1993). Thus 2 defective ArsA proteins, one with the altered A1 site and the other with the altered A2 site together can form a functional ArsA complex. This suggests that the A1 and A2 sites of ArsA are independent to each other in fulfilling their functions. Recently in vivo reconstitutions were performed with peptides containing portions of the A1 and A2 half of ArsA (Kaur and Rosen 1994). The reconstituted complexes exhibit ATPase activity. These results are consistent with the observation that ArsA is a functional dimer (Hsu and Rosen 1989b, Hsu et al. 1991). It is proposed that there are 2 catalytic sites in the dimer, each composed of interacting A1 and A2 sites from opposing ArsA monomers. Although ATP is bound to both sites in a monomer (Karkaria and Rosen 1991), it is not known whether both sites are catalytic or one is regulatory.

What is the mechanism of activation of the ArsA ATPase activity by the effectors arsenite or antimonite? Dimerization of ArsA could be demonstrated by chemical crosslinking and light scattering methods (Hsu et al. 1991). The results suggest that in the absence of substrate, the ArsA protein is mostly monomer, while in the presence of the metalloids arsenite or antimonite, the protein is primarily dimer. A model of the monomer-dimer equilibrium has been proposed (Fig. 4B). ArsA exists in a monomer and dimer equilibrium. Binding of the effector shifts the equilibrium toward the catalytically competent dimer. If the role of the metalloid is to induce dimer formation, the dimer would have higher affinity for ATP than the monomer. More recently, increased affinity of the A1 site for ATP in the presence of antimonite

has been demonstrated by photolabeling with α-[^{32}P]ATP (Kaur and Rosen, unpublished data). Thus the metalloids are the allosteric activators of the ATPase activity. Although the primary sequence of ArsA did not reveal the presence of any conserved metal binding motifs, the inhibition of ATPase activity by maleimides suggests involvement of cysteines in catalysis (Ksenzenko et al. 1993). The ArsA protein contains 4 cysteines: Cys26, Cys113, Cys172, and Cys422 (Chen et al. 1986). To investigate the role of cysteines in the activation of the ArsA protein, each of the 4 cysteinyl residues was altered to a seryl residue (Bhattacharjee et al. 1995). Cells expressing the mutants altered at Cys113, Cys172, or Cys422 lost arsenite resistance, and all the $ArsA_{C113S}$, $ArsA_{C172S}$, and $ArsA_{C422S}$ proteins exhibited reduced antimonite-stimulated ATPase activity due to a decreased affinity for the metalloid. The results suggest that Cys113, Cys172, and Cys422 are involved in the metalloactivation of the ArsA ATPase. The activator interacts not as an oxyanion but as a soft metal through formation of a 3 coordinate complex with Cys113, Cys172, and Cys422, forming a novel As(III)- or Sb(III)-thiol cage (Fig. 4C).

Functionally ArsA forms a complex with ArsB in the inner membrane of *E. coli* (Tisa and Rosen 1990b). Using membranes from cells expressing only the *arsB* gene, reconstitution of purified ArsA

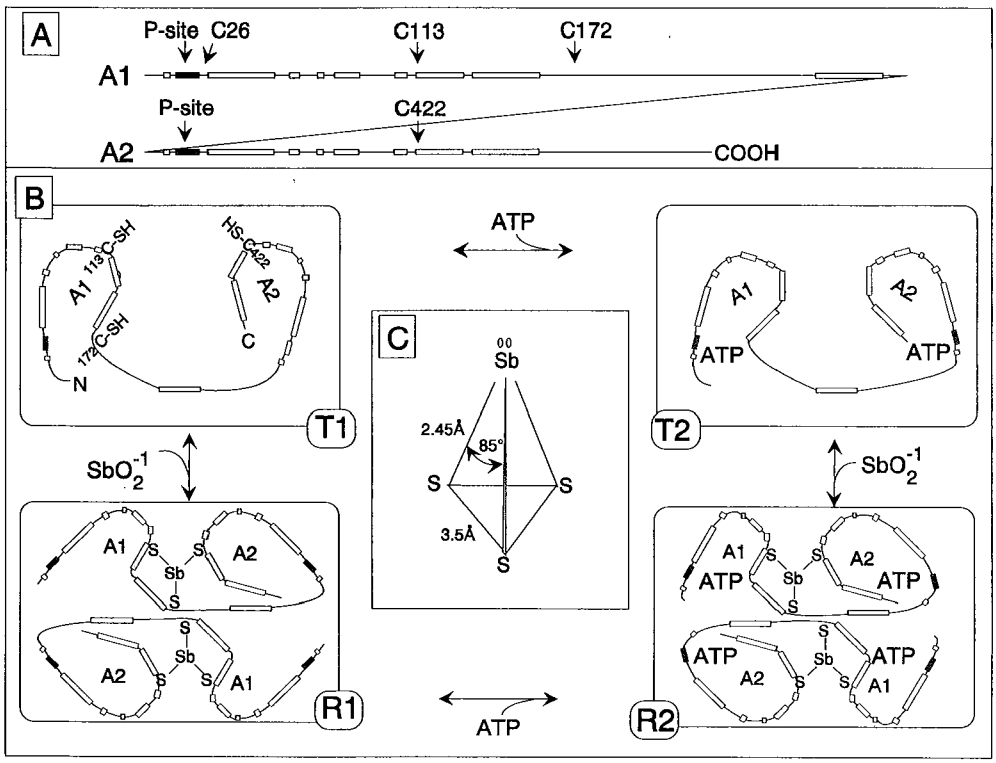

Fig. 4. Metalloregulation of the ArsA ATPase. **(A)** ArsA has 2 homologous halves, A1 and A2. The boxes in the 2 halves indicate the regions of greatest sequence similarity. Each half has a consensus sequence for the binding site of phosphoryl groups of ATP (P-site). The A-site is the region that interacts with adenine of ATP bound to the A1 half. The location of the 4 cysteine residues of the ArsA protein are indicated. **(B)** ArsA ATPase activity is allosterically regulated by binding of As(III) or Sb(III). The 63-kDa ArsA has independent binding sites for metalloids and ATP. In the absence of either a nucleotide or metalloid the monomer exists in an inactive T1 state. Binding of either ATP or a semi-metal each produces a unique and different conformational change in the protein to inactive forms R1 and T2. In solution, the monomer (T form) exists in equilibrium with the dimer (R form). Sb(III) binds as soft metals to the thiolates of Cys113 and Cys172 in the A1 half and to Cys422 in the A2 half of each monomer, producing a more compact structure that favors the subunit interactions that stabilize the catalytically active R2 state. When the ArsA protein is membrane-bound as a subunit of the pump, it is postulated to be at all times a dimer, and the effect of substrate binding is to produce the conformational change from the inactive T state to the active R conformation, promoting catalysis. **(C)** The structure of the Sb(III)-thiol cage in the ArsA protein is postulated from the bond angles and distances found by crystallographic analysis of small molecules containing Sb-S bonds. An As(III)-thiol cage would have a similar structure with slightly different bond angles and distances.

onto the membrane was demonstrated. The reconstituted membrane complex exhibited Sb(III)-stimulated ATPase activity. It is clear that ArsB is the membrane anchor of ArsA. Although ArsA is found as a monomer in cytosol, it functions as a dimer. It is unlikely that ArsA dissociates from the membrane during the catalytic cycle. First, ArsA is so tightly bound to the membrane complex that can only be removed by chaotropic agents (Tisa and Rosen 1990b). Second, binding of ArsA to the membrane exhibits a sigmoidal dependence on the amount of ArsA, suggesting that only the dimer binds to the ArsB protein (Dey and Rosen 1994). Moreover, the sigmoidal binding of ArsA changes to hyperbolic under conditions that favor dimer formation. These results again suggest the effect of the metalloid is to increase the affinity for ATP and to produce a tighter binding between ArsA and ArsB.

ArsB

From its hydropathic profile, ArsB was predicted to be an inner membrane protein that presumably forms the anion-conducting pathway (Chen et al. 1986). The topological arrangement of ArsB within the membrane was determined using a series of gene fusions to *phoA*, *blaM*, and *lacZ* genes (Wu et al. 1992). By analyzing 26 fusions, a topological model was proposed in which the ArsB protein has 12 membrane-spanning α-helices, with the N- and C-termini located in the cytosol, a structure more similar to that of secondary transporters than primary pumps (Maloney 1990).

The mechanism of resistance produced by the 5-gene operons can be compared with that of the 3-gene operons. The *ars* operons of the *E. coli* chromosome and the 2 staphylococcal plasmids do not contain an *arsA* gene, yet they confer arsenite resistance and active extrusion of arsenite, suggesting that ArsB can transport arsenite in the absence of the ArsA protein. Recently cells producing only the R773 ArsB protein were shown to confer an intermediate level of arsenite resistance compared with cells expressing both the *arsA* and *arsB* genes (Dey and Rosen 1995). Resistance in both types of cells was correlated with the exclusion of $^{73}AsO_2^-$. The in vivo energetics of arsenite transport from both types of cells were characterized. The results suggest that ArsB alone can extrude arsenite coupled to electrochemical energy (Fig. 5). When ArsA is present, the ArsA-ArsB complex functions as an obligatory ATP-driven pump, and an inactive ArsA blocked the resistance conferred by ArsB (Dey and Rosen 1995). The F_oF_1 H^+-translocating ATPase also has dual energy coupling (Futai and Kanazawa 1983). As a complex, F_oF_1 is an ATP-dependent proton pump. In the absence of F_1, F_o functions as a electrophoretic proton conducting pathway. Similarly the proton conductivity through F_o is blocked by binding of an inactive F_1. The difference between the 2 types of pumps is that F_oF_1 is always a complex; F_o is never found alone in nature. In contrast, of the 5 *ars* operons identified to date, two have *arsA* and *arsB*, and three have *arsB* alone - a nearly random distribution.

What is the chemistry of arsenite extrusion by ArsB? ArsA is allosterically activated by soft metal-thiol interactions between As(III) and cysteine thiolates. In contrast, the mechanism of ArsB catalysis does not involve thiol chemistry. ArsB contains only a single cysteine residue. The codon for this cysteine was mutated to several other codons with no effect on arsenite resistance or transport (Chen et al. 1996). Recently everted membrane vesicles containing ArsB in the absence of ArsA have been shown to accumulate $^{73}AsO_2^{-1}$ with energy supplied in the form of a membrane potential (M. Kuroda, D. Dey, B.P. Rosen, unpublished data). Thus the oxyanion transport system is either an obligatory ATP-driven pump or a secondary transporter coupled to the electrochemical proton gradient, depending on the subunit composition of the permease (Fig. 5).

If the ArsB protein is sufficient for arsenite resistance, why does the *arsA* gene exist? One possibility is the ArsA-ArsB complex is a more efficient system. If ArsB functions as an electrophoretic anion transporter, it is limited to extruding substrate only to the equilibrium potential (Rosen and Kashket 1978). Thus, an uniporter would put the cell at the mercy of its environment. In contrast, an ATP-coupled pump can pump out far in excess of the proton equilibrium, maintaining low ion concentration in the cytosol independent of the external concentration.

ArsC

Genetic evidence suggested that resistance to arsenate is conferred by the product of the *arsC* gene (Rosen and Borbolla 1984, Chen et al. 1985). ArsC has been purified and crystallized (Rosen et al. 1991), but little is known about the biochemical mechanism of ArsC function until recently.

Although the sequences of the gram-positive and gram-negative ArsC proteins exhibit less than

20% sequence similarity, both confer resistance to arsenate. Resistant cells of *S. aureus* reduced arsenate to arsenite (Ji and Silver 1992b), which was then extruded from cells by the staphylococcal ArsB protein alone. Subsequently in vitro purified ArsC of pI258 was shown to be an arsenate reductase coupled to thioredoxin. In contrast, R773 ArsC exhibited glutathione-coupled arsenate reduction activity (Oden et al. 1994) (Fig. 1A).

If the ArsC proteins are reductases in vivo, the mechanism of electron transfer is not obvious from the protein sequences. The R773 ArsC does not contain any prosthetic group, molybdenum cofactor, or iron-sulfur cage. However, inhibition of reductase activity by N-ethylmaleimide suggests that a redox-active sulfhydryl may participate in the action of ArsC (Gladysheva et al. 1994). The R773 ArsC protein contains 2 cysteines, Cys12 and Cys106 (Chen et al. 1986). Mutational analysis demonstrated that Cys106 is not required for resistance or reductase activity, while an alteration at Cys12 resulted in loss of both (Liu et al. 1995). This suggests that a single thiol group may be sufficient for reductase activity. Those results suggest that an enzyme-substrate complex forms by arsenylating Cys12, with the complex interacting with glutaredoxin with transfer electrons to reduce As(V) to As(III). The resulting arsenite dissociates from the complex, and reduced glutaredoxin is regenerated by the action of glutathione reductase.

Since arsenite is considerably more toxic than arsenate (Knowles and Benson 1983), the reduction within the cytosol is undesirable. It is possible that ArsC is a part of a membrane-bound complex with ArsA and ArsB and catalyzes the reduction of arsenate on the membrane, where it is immediately extruded from the cells. However, there is no data indicating that ArsC interacts with the membrane-bound arsenical pump in *E. coli*.

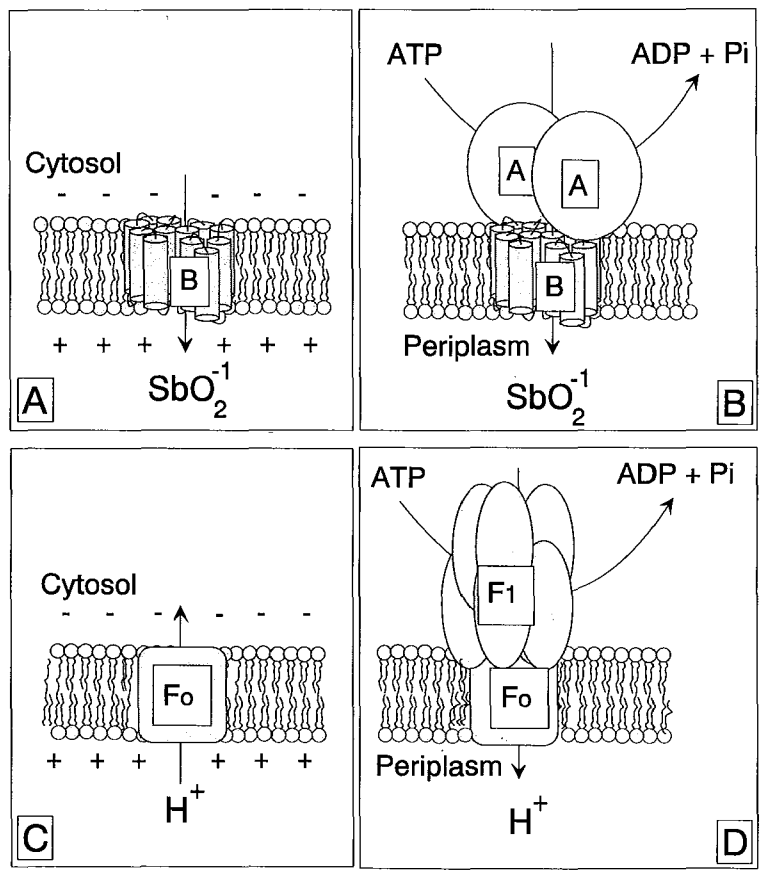

Fig. 5. Dual modes of energy coupling of ion translocating systems. The components of ion transport systems such as the arsenite transporter (A and B) or the H^+-translocating F_oF_1 (C and D) function either as secondary porters (A and C) or primary ATP-driven pumps (B and D), depending on association of the catalytic subunits (ArsA or F_1) with the intrinsic membrane subunits (ArsB or F_o).

Regulation of the *ars* operon

Expression of the *ars* operon on plasmid R773 is metalloregulated by salts of arsenic, antimony, and bismuth (Silver et al. 1981, San Francisco et al. 1990). The region in the operon required for regulatory activity has been shown to contain a single promoter sequence and 2 regulatory genes, *arsR* and *arsD* (Wu and Rosen 1991). ArsR is a *trans*-acting repressor that binds as a dimer to an operator and represses operon transcription (Wu and Rosen 1993b). Inducibility results from the release of the repressor from the operator upon binding of As(III) or Sb(III) (Wu and Rosen 1991). Gel retardation assays demonstrated the formation of DNA-ArsR protein complexes (Wu and Rosen 1993b). To identify the inducer binding domain in ArsR, mutants with a noninducible phenotype were isolated (Shi et al. 1994). The mutations affecting inducibility altered the residues Cys32 and Cys34, located just in front of the putative DNA binding domain (Fig. 3). Additional mutations in this region were introduced by site-directed mutagenesis (Shi et al. 1996). Most of the altered ArsRs still bound to the *ars* operator and repressed transcription, but those with substitutions of Cys32 or Cys34 had reduced or absent response to the inducer, both in vivo and in vitro. Mutations in the DNA binding domain produced ArsRs that no longer repressed in vivo nor bound to DNA in vitro. However, while only the 2 cysteines seemed to be required for induction, As(III) and Sb(III) are normally 3 coordinate, suggesting that a 3rd residue might be involved in metal binding in ArsR. The 3rd ligand could potentially be another thiol or the hydroxyl group of a serine or threonine. X-ray absorption spectroscopy of the As(III)-ArsR complex demonstrated that As(III) is coordinated to 3 sulfur atoms and no oxygens; thus serine or threonine cannot be the 3rd ligand. The remaining cysteines were altered by mutagenesis. While none were required for induction, Cys37 was shown to be required for binding. In other words, it is dispensable for function but participates in binding when present. A model for induction is that As(III) or Sb(III) are bound in a metal-thiol cage similar in geometry to that in ArsA. Binding of the semimetal triggers a conformational change within the DNA binding domain, resulting in dissociation of the repressor from DNA (Fig. 6).

The product of the 2nd regulatory gene *arsD* is also a *trans*-acting repressor (Wu and Rosen

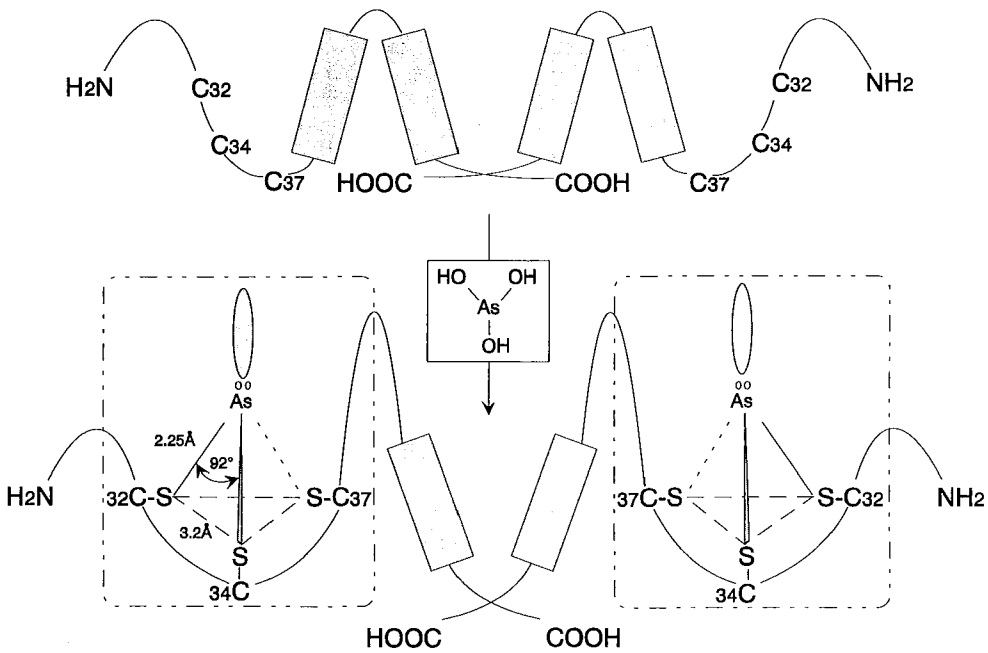

Fig. 6. Metalloregulation by the ArsR repressor. The ArsR homodimer binds to the operator/promoter region through a helix-turn-helix domain in each monomer, repressing transcription of the *ars* operon. As(III) or Sb(III) binds to Cys32 and Cys34 through soft metal-sulfur bonds (solid lines), producing a conformational change in the DNA binding domain such that the repressor dissociates from the DNA. Cys37 is the 3rd ligand for As(III) or Sb(III) (dotted line), although this is not required for induction. Thus As(III) is 3 coordinate in a pyramidal cage with the 3 sulfur thiols as the base.

1993a). ArsD is inducer independent. To elucidate the function of the ArsD protein, a frameshift mutation was introduced into the *arsD* gene. The mutation in *arsD* resulted in increased expression of 3 downstream *ars* genes. Introducing a wild-type *arsD* gene in *trans* reduced the expression of downstream genes to wild-type levels. It appears that the down-regulation of the *ars* operon depends on the amount of the ArsD protein present. Interestingly, cells expressing the *ars* operon with the mutated *arsD* gene were arsenite sensitive, even though the *arsA*, *arsB*, and *arsC* genes were transcribed. This result suggests that the *arsD* gene is required for resistance when the *arsA*, *arsB*, and *arsC* genes are controlled by the *ars* promoter. Thus a regulatory circuit is formed by the action of ArsR and ArsD (Rosen et al. 1993). In the absence of an inducer, constitutively expressed ArsR repressor dimerizes and binds to the *ars* operator, preventing transcription. Upon induction, the ArsR repressor is inactivated and the *ars* operon is derepressed. ArsD is produced along with ArsA, ArsB, and ArsC. When the amount of ArsD reaches a critical level; it presumably binds to a site within the promoter region and prevents further *ars* expression. However interaction between ArsD and the promoter has not been observed in gel retardation assays or in DNase I footprinting assays, suggesting weak affinity of ArsD for the promoter. With its high affinity for the *ars* operator, the inducer-dependent ArsR repressor controls the basal level of operon expression. ArsD controls the upper level of *ars* expression. A likely function for this regulatory circuit is to prevent the overproduction of ArsB, which is toxic in high amounts (San Francisco et al. 1989, Wu and Rosen 1993a).

EUKARYOTIC ARSENITE AND CADMIUM RESISTANCE SYSTEMS

Mammalian arsenite efflux

Tumors rapidly become resistant to the drugs used in cancer chemotherapy, and efflux systems form a major group of such resistances (Gottesman and Pastan 1993). Mammalian cell lines have been selected for arsenic resistance in several laboratories (Lo et al. 1992, Wang and Rossman 1993). Expression of the enzyme glutathione S-transferase π is increased in some arsenite-resistant Chinese hamster cell lines (Lo et al. 1992). Resistance has been shown to involve active extrusion of As(III) (Wang and Lee 1993, Wang et al. 1996). One possibility is that arsenite is conjugated to glutathione by the transferase, followed by extrusion of the conjugate by a transport system (Fig. 1B). We hypothesize that As(III) is conjugated to glutathione by a transferase enzyme, followed by extrusion of the conjugate by a thiol-linked efflux pump (Fig. 1B). Efflux of glutathione S-conjugants has been observed in vesicles from rat heart and liver (Ishikawa 1992). This system is a transport ATPase whose physiological role may be extrusion of xenobiotics such as aflatoxin B1 and release of substances such as leukotrienes. Arsenite and arsenate can react nonenzymatically with glutathione has been characterized (Delnomdedieu et al. 1993 1994). Arsenate is reduced by glutathione to arsenite, and the arsenite reacts further with glutathione to form $(GS)_3As$. Although these reactions occur spontaneously, the reaction is slow, and it is not unreasonable to consider the existence of an eukaryotic arsenate reductase to produce arsenite, which would then be enzymatically conjugated to glutathione. The pump would then extrude the As(III)-thiol conjugate.

Leishmania arsenite efflux

The organic arsenicals Paul Ehrlich synthesized a century ago are related to drugs still used for the treatment of trypanosomal diseases such as the Sb(V)-containing drug sodium stibogluconate (Pentostam). Pentostam is the treatment of choice for the trypanosomatid protozoan parasite *Leishmania*, the causative agent of kala azar and other forms of leishmaniasis. Pentostam resistance occurs in 5% of patients, and resistance rates as high as 70% are found in some endemic areas. In strains of *L. tarentolae* selected in vitro for resistance to trivalent arsenicals and antimonials and cross resistant to Pentostam, we have shown that high-level resistance is related to efflux (Dey et al. 1994c). We have recently identified an ATP-dependent As(III)-thiol pump in plasma membranes (Dey et al. 1996). It is likely that in vivo the As(III)-thiol adduct is extruded. We have recently demonstrated that arsenite-resistant cells have increased levels of intracellular thiols (Mukhopadhyay et al. 1996). The glutathione derivative trypanothione was elevated 40-fold in resistant cells, and the As(III)-trypanothione was transported by the As-thiol pump. We propose that arsenite resistance in eukaryotes has a common theme of As(III) conjugation to a thiol, either glutathione in mammalian cells or trypanothione in parasites, followed by

extrusion of the xenobiotic by the As-thiol pump (Fig. 7).

Yeast cadmium resistance pumps

The theme of drug and metal resistance being associated with thiol-linked efflux systems is true also of cadmium resistance in yeast. In mammalian cells, the P-glycoprotein, the *mdr* gene product, is responsible for resistance to chemotherapeutic drugs (Gottesman and Pastan 1993). When amplified, this protein catalyzes extrusion of a large number of structurally unrelated drugs. The superfamily of proteins that includes the P-glycoprotein includes members found in every organism thus far studied and is related to drug resistance in many cases. One homologue is the YCF1 protein that confers Cd(II) resistance in *Saccharomyces cerevisiae* (Wemmie et al. 1994). YCF1 transports the metal-glutathione complex $Cd(GS)_2$, probably into the vacuole rather than to the outside of the cell, but this still reduces the cytosolic concentration of Cd(II) (Li et al. 1996). Another member of the same superfamily, the HMT1 protein of fission yeast, is unrelated to YCF1 but also transports Cd(II) into the vacuole as a complex with a derivative of glutathione or phytochelatin (Ortiz et al. 1995). Thus the model proposed for As(III) and Sb(III) resistance shown in Fig. 7 most likely can be expanded to include a family of similar resistance pumps including those for Cd(II).

CONCLUSIONS

Whenever organisms are put under stress, they develop compensatory mechanisms. All organisms, whether prokaryotic or eukaryotic, have evolved resistance mechanisms to metals and metalloids, including Cd(II)/Zn(II) and As(III)/Sb(III). The systems are similar in strategy, with evolution of transport systems for the extrusion of the metals and metalloids. However, at the mechanistic level they are different, as would be expected con-

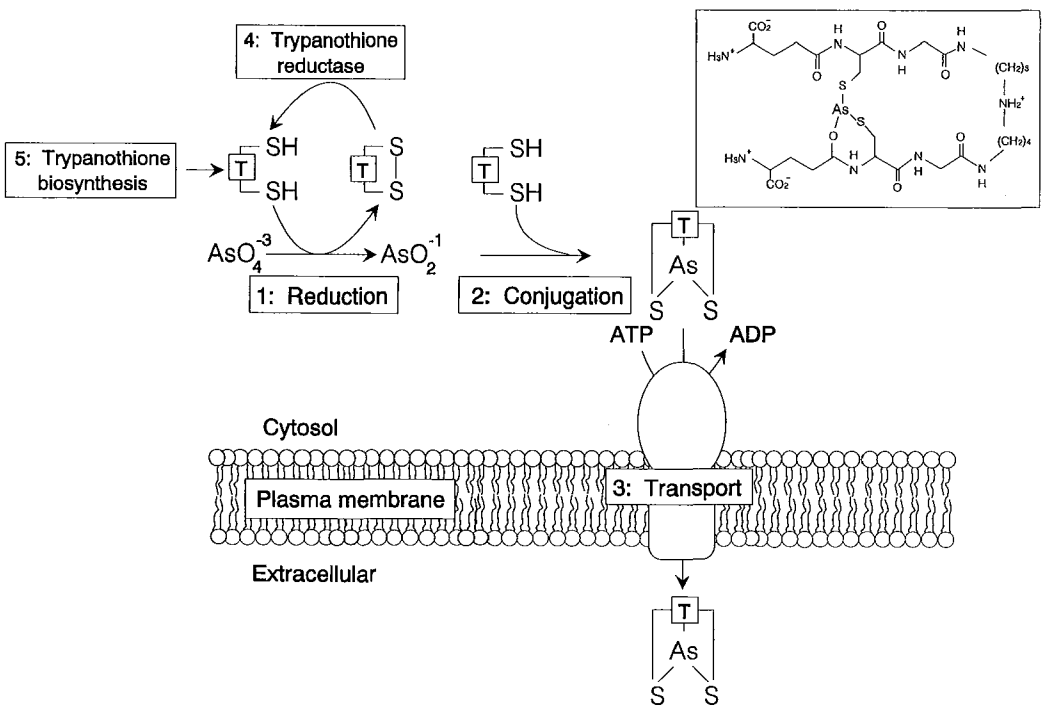

Fig. 7. Model for arsenical/antimonial resistance in *Leishmania*. The 1st step is reduction of Sb(V)/As(V) to Sb(III)/As(III) by trypanothione. It is not known whether this process is enzymatic. The 2nd step is conjugation of the reduced semimetal to trypanothione. While slow spontaneous formation of the As-trypanothione conjugate from arsenate and trypanothione has been observed, it would not be unreasonable to postulate the existence of a trypanothione-conjugating enzyme. The final step in resistance is active extrusion by the ATP-coupled As-thiol pump, lowering the intracellular concentration of the metalloid to subtoxic levels. The rate limiting step in resistance is formation of the As-trypanothione conjugate. Selection for mutations that increases trypanothione biosynthesis thus generates resistance. *Inset*: Proposed structure of the As(III)-trypanothione conjugate based on mass spectroscopic analysis.

sidering that they are the result of convergent evolution. For As(III)/Sb(III), bacteria have evolved a secondary AsO_2^{-1} transporter that can convert to a primary ATP-coupled pump in association with a catalytic subunit. In contrast, *Leishmania* has evolved an ATP-coupled As-thiol pump. For Cd(II)/Zn(II), bacteria have a P-type ATPase, whereas in eukaryotic cells, the cadmium pumps are thiol-linked pumps. Thus the substrates of the prokaryotic and eukaryotic systems are different, but the results are the same: lowering of the intracellular concentration of the toxic metal producing resistance.

As we have discussed in this article, prokaryotic or eukaryotic organisms ingeniously develop various efflux pumps for their resistance to toxic metals or metalloids. It would not be to our surprise that similar efflux mechanisms of resistance to chemotherapeutic agents might be the most frequently employed strategies by both prokaryotic and eukaryotic pathogens each time an inappropriate treatment is made. Thus, an understanding of the molecular details of these transport systems utilized by prokaryotes and eukaryotes is essential to the rational design of drugs targeting the efflux pumps for treating drug-resistant cells and microorganisms.

REFERENCES

Addison R. 1986. Primary structure of *Neurospora* plasma membrane H^+-ATPase deduced from the gene sequence. J. Biol. Chem. **261:** 14896-14910.

Bhattacharjee H, J Li, YK Ksenzenko, BP Rosen. 1995. Role of cysteinyl residues in metalloactivation of the oxyanion-translocating ArsA ATPase. J. Biol. Chem. **270:** 11245-11250.

Bowman BJ, A Siebers, K Altendorf. 1988. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc. Natl. Acad. Sci. USA **85:** 7972-7976.

Bowman BJ, CW Slayman. 1979. The effects of vanadate on the plasma membrane ATPases of *Neurospora crassa*. J. Biol. Chem. **254:** 2928-2934.

Brandl CJ, NM Green, B Korczak, DH MacLennan. 1986. Two Ca^{2+}-ATPase genes: homologies and mechanistic implications of deduced amino acid sequence. Cell **44:** 597-607.

Bruhn DF, J Li, S Silver, F Roberto, BP Rosen. 1996. Arsenic resistance operon of IncN plasmid R46. FEMS Microbiol. Lett. **139:** 149-153.

Bull PC, GR Thomas, JM Rommens, JR Forbes, DW Cox. 1993. The Wilson disease gene is a putative copper transporting P-type ATPase similiar to the Menkes gene. Nature Genetics **5:** 327-337.

Carlin A, W Shi, S Dey, BP Rosen. 1995. The *ars* operon of *Escherichia coli* confers arsenical and antimonical resistance. J. Bacteriol. **177:** 981-986.

Chen CM, T Misra, S Silver, BP Rosen. 1986. Nucleotide sequence of the structural genes for an anion pump: the plasmid-encoded arsenical resistance operon. J. Biol. Chem. **261:** 15030-15038.

Chen CM, HLT Mobley, BP Rosen. 1985. Separate resistances to arsenate and arsenite (antimonate) encoded by the arsenical resistance operon of R-factor R773. J. Bacteriol. **161:** 758-763.

Chen Y, S Dey, BP Rosen. 1996. Soft metal-thiol chemistry is not involved in transport of arsenite by the Ars pump. J. Bacteriol. **178:** 911-913.

Chopra I. 1970. Decreased uptake of cadmium by a resistant strain of *Staphylococcus aureus*. J. Gen. Microbiol. **63:** 265-267.

Chopra I. 1975. Mechanism of plasmid-mediated resistance to cadmium in *Staphylococcus aureus*. Antimicro. Agents Chemother. **7:** 8-14.

Delnomdedieu M, MM Basti, JD Otvos, DJ Thomas. 1993. Transfer of arsenite to dithiols: a model of interaction. Chem. Res. Toxicol. **6:** 598-602.

Delnomdedieu M, MM Basti, JD Otvos, DJ Thomas. 1994. Reduction and binding of arsenate and dimethylarsenate by glutathione: a magnetic resonance study. Chemico-Biological Interact. **90:** 139-155.

Dey S, D Dou, BP Rosen. 1994a. ATP-dependent transport in everted membrane vesicles of *Escherichia coli*. J. Biol. Chem. **269:** 25442-25446.

Dey S, D Dou, LS Tisa, BP Rosen. 1994b. Interaction between the catalytic and the membrane components of an anion-translocating ATPase. Arch. Biochem. Biophys. **311:** 418-424.

Dey S, M Ouellette, J Lightbody, B Papadopoulou, BP Rosen. 1996. An ATP-dependent As(III)-glutathione transport system in membrane vesicles of *Leishmania tarentolae*. Proc. Natl. Acad. Sci. USA **93:** 2192-2197.

Dey S, B Papadopoulou, G Roy, K Grondin, D Dou, BP Rosen, M Ouellette. 1994c. Active efflux of arsenite in oxyanion resistant *Leishmania* is independent of the amplification of the P-glycoprotein related gene *ltpgpA*. Molec. Biol. Parasitol. **67:** 49-57.

Dey S, BP Rosen. 1995a. Mechanisms of drug transport in prokaryotes and eukaryotes. *In* NH Georgopapadakou, ed. Drug transport in antimicrobial and anticancer chemotherapy. New York: Marcel Dekker, pp. 103-132.

Dey S, BP Rosen. 1995b. Dual mode of energy coupling by the oxyanion-translocating ArsB protein. J. Bacteriol. **177:** 385-389.

Endo G, S Silver. 1995. CadC, the transcriptional regulatory protein of the cadmium resistance system of *Staphylococcus aureus* plasmid pI258. J. Bacteriol. **177:** 4437-4441.

Foster TJ. 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol. Rev. **47:** 361-409.

Foye WO. 1977. Antimicrobial activities of mineral elements. *In* ED Weinberg, ed. Microorganisms and minerals. Microbiology series. Vol. 3. New York: Marcel Dekker, 387 pp.

Froehlich JP, PF Heller. 1985. Transient-state kinetics of the ADP-insensitive phosphoenzyme in sarcoplasmic reticulum: implications for transient-state calcium translocation. Biochemistry **24:** 126-136.

Futai M, H Kanazawa. 1983. Structure and function of proton-translocating ATPase (F_0F_1): biochemical and molecular biological approaches. Microbiol. Rev. **47:** 285-313.

Gambel AG, MG Desrosiers, DR Menick. 1992. Characterization of a P-type Ca^{2+}-ATPase from *Flavobacterium odoratum*. J. Biol. Chem. **267:** 15923-15931.

Gottesman MM, I Pastan. 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu. Rev. Biochem. **62:** 385-427.

Hager KM, SM Mandala, JW Davenport, DW Speicher, EJ Benz, CW Slayman Jr. 1986. Amino acid sequence of the plasma membrane ATPase of *Neurospora crassa*: deduction from genomic and cDNA sequences. Proc. Natl. Acad. Sci. USA **83:** 7693-7697.

Hedges RW, S Baumberg. 1973. Resistance to arsenic compounds conferred by a plasmid transmissible between strains of *Escherichia coli*. J. Bacteriol. **115:** 459-460.

Hesse JE, L Wieczorek, K Altendorf, AS Reicin, E Dorus, W Epstein. 1984. Sequence homology between 2 membrane transport ATPases, the Kdp-ATPase of *Escherichia coli* and the Ca^{2+}-ATPase of sarcoplasmic reticulum. Proc. Natl. Acad. Sci. USA **81:** 4746-4750.

Himmelweit F, ed. 1960. On the partial function of the cell. *In* Collected papers of Paul Ehrlich. London: Pergammon Press, pp. 183-194.

Hsu CM, P Kaur, CE Karkaria, RF Steiner, BP Rosen. 1991. Substrate-induced dimerization of the ArsA protein, the catalytic component of an anion-translocating ATPase. J. Biol. Chem. **266:** 2327-2332.

Hsu CM, BP Rosen. 1989a. Characterization of the catalytic subunit of an anion pump. J. Biol. Chem. **264:** 17349-17354.

Hsu CM, BP Rosen. 1989b. Structure of the plasmid-encoded anion translocating ATPase. *In* A Kotyk, J Skoda, V Paces, V Kostka, eds. Highlights of modern biochemistry. Zeist: VSP International Science Publishers, pp. 743-751.

Hughes VM, N Datta. 1983. Conjugative plasmids in bacteria of the 'pre-biotic' era. Nature **302:** 725-726.

Hymel L, A Maurer, C Berenski, CY Jung, S Fleischer. 1984. Target size of calcium pump protein from skeletal muscle sarcoplasmic reticulum. J. Biol. Chem. **259:** 4890-4895.

Ikemoto N. 1982. Structure and function of the calcium pump protein of sarcoplasmic reticulum. Ann. Rev. Physiol. **44:** 297-317.

Ishikawa T. 1992. ATP-dependent glutathione S-conjugate export pump. Trends Biochem. Sci. **17:** 463-468.

Ji G, S Silver. 1992a. Regulation and expression of the arsenic resistance operon from *Staphylococcus aureus* plasmid pI258. J. Bacteriol. **174:** 3684-3694.

Ji G, S Silver. 1992b. Reduction of arsenate to arsenite by the ArsC protein of the arsenic resistance operon of the *Staphylococcus aureus* plasmid pI258. Proc. Natl. Acad. Sci. USA **89:** 9474-9478.

Karkaria CE, CM Chen, BP Rosen. 1990. Mutagenesis of a nucleotide binding site of an anion-translocating ATPase. J. Biol. Chem. **265:** 7832-7836.

Karkaria CE, BP Rosen. 1991. Trinitrophenyl-ATP binding to the wild type and mutant ArsA proteins. Arch. Biochem. Biophys. **288:** 107-111.

Kaur P, BP Rosen. 1992a. Plasmid-encoded resistance to arsenic and antimony. Plasmid **27:** 29-40.

Kaur P, BP Rosen. 1992b. Mutagenesis of the second putative nucleotide binding site of an anion-translocating ATPase. J. Biol. Chem. **267:** 19272-19277.

Kaur P, BP Rosen. 1993. Complementation between nucleotide binding domains in an anion translocating ATPase. J. Bacteriol. **175:** 351-357.

Kaur P, BP Rosen. 1994. In vitro assembly of an anion-stimulated ATPase from peptide fragments. J. Biol. Chem. **269:** 1-7.

Knowles FC, AA Benson. 1983. The biochemistry of arsenic. Trends. Biochem. Sci. **8:** 178-180.

Ksenzenko MY, DH Kessel, BP Rosen. 1993. Reaction of the ArsA ATPase with 2-(4'-maleimidylanilino)naphthalene-6-sulfonate. Biochemistry **32:** 13362-13368.

Lebrun M, A Audurier, P Cossart. 1994a. Plasmid-borne cadmium resistance genes in *Listeria monocytogenes* are present on Tn5422, a novel transposon closely related to Tn917. J. Bacteriol. **176:** 3049-3061.

Lebrun M, A Audurier, P Cossart. 1994b. Plasmid-borne cadmium resistance genes in *Listeria monocytogenes* are similar to *cad*A and *cad*C of *Staphylococcus aureus* and are induced by cadmium. J. Bacteriol. **176:** 3040-3048.

Li Z, M Szczypka, Y Lu, DJ Thiele, PA Rea. 1996. The Yeast Cadmium Factor Protein (YCF1): Is a vacuolar glutathione s-conjugate pump. J. Biol. Chem. **271:** 6509-6517.

Liu J, TB Gladysheva, L Lee, BP Rosen. 1995. Identification of an essential cysteinyl residue in the ArsC arsenate reductase of plasmid R773. Biochemistry **34:** 13572-13576.

Lo JF, HF Wang, MF Tam, TC Lee. 1992. Glutathione S-transferase pi in an arsenic-resistant Chinese hamster ovary cell line. Biochem. J. **288:** 977-982.

Lynn AR, BP Rosen. 1994. Transport systems for ions of arsenic, antimony and cadmium encoded by bacterial plasmids. *In* P Foa, MF Walsh, eds. Ion channels and ion pumps. New York: Springer-Verlag, pp. 549-573.

MacLennan DH, CJ Brandl, B Korczak, NM Green. 1985. Amino-acid sequence of a $Ca^{2+} + Mg^{2+}$-dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. Nature **316:** 696-700.

Maloney PC. 1990. A consensus structure for membrane transport. Res. Microbiol. **141:** 374-383.

Mobley HLT, CM Chen, S Silver, BP Rosen. 1983. Cloning and expression of R-factor mediated arsenate resistance in *Escherichia coli*. Mol. Gen. Genet. **191:** 421-426.

Mobley HLT, BP Rosen. 1982. Energetics of plasmid-mediated arsenate resistance in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **79:** 6119-6122.

Morby AP, JS Turner, JW Huckle, NJ Robinson. 1993. SmtB is a metal-dependent repressor of the cyanobacterial metallothionein gene *smtA*: identification of a Zn inhibited DNA-protein complex. Nucleic Acids Res. **21:** 921-925.

Mukhopadhyay R, S Dey, N Xu, D Gage, J Lightbody, M Ouellette, BP Rosen. 1996. Trypanothione overproduction and resistance to antimonials and arsenicals in *Leishmania*. Proc. Natl. Acad. Sci. USA (in press).

Novick RP. 1963. Analysis by transduction of mutations affecting penicillinase formation in *Staphylococcus aureus*. J. Gen. Microbiol. **33:** 121-136.

Novick RP, C Roth. 1968. Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. J. Bacteriol. **95:** 1335-1342.

Nucifora G, L Chu, T Misra, S Silver. 1989. Cadmium resistance from *Staphylococcus aureus* plasmid pI258 *cadA* gene results from a cadmium efflux ATPase. Proc. Natl. Acad. Sci. USA **86:** 3544-3548.

Oden KL, TB Gladysheva, BP Rosen. 1994. Arsenate reduction mediated by the plasmid-encoded ArsC protein is coupled to glutathione. Mol. Microbiol. **12:** 301-306.

Odermatt A, H Suter, R Krap, M Solioz. 1993. Primary structure of 2 P-ATPases involved in copper homeostasis in

Enterococcus hirae. J. Biol. Chem. **268:** 12775-12779.

Ortiz DF, T Ruscitti, KF McCue, DW Ow. 1994. Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. J. Biol. Chem. **270:** 4721-4728.

Owolabi JB, BP Rosen. 1990. Differential mRNA stability controls relative gene expression within the plasmid-encoded arsenical resistance operon. J. Bacteriol. **172:** 2367-2371.

Pedersen PL, E Carafoli. 1987. Ion motive ATPases. I. Ubiquity, properties, and significance to cell function. Trends Biochem. Sci. **12:** 146-150.

Perry RD, S Silver. 1982. Cadmium and manganese transport in *Staphylococcus aureus* membrane vesicles. J. Bacteriol. **150:** 973-976.

Puppe W, A Siebers, K Altendorf. 1992. The phosphorylation site of the Kdp-ATPase of *Escherichia coli*: site directed mutagenesis of the aspartic acid residues 300 and 307 of the Kdp B subunit. Mol. Microbiol. **6:** 3511-3520.

Rosen BP, H Bhattacharjee, W Shi. 1995. Mechanisms of metalloregulation of an anion-translocating ATPase. J. Bioenerg. Biomemb. **27:** 85-91.

Rosen BP, MG Borbolla. 1984. A plasmid-encoded arsenite pump produces arsenite resistance in *Escherichia coli*. Biochem. Biophys. Res. Commun. **124:** 760-765.

Rosen BP, S Dey, D Dou, G Ji, P Kaur, M Ksenzenko, S Silver, J Wu. 1992. Evolution of an ion-translocating ATPase. Ann. New York Acad. Sci. **671:** 257-272.

Rosen BP, ER Kashket. 1987. Energetics of active transport. *In* BP Rosen, ed. Bacterial transport. New York: Marcel Dekker, pp. 559-620.

Rosen BP, U Weigel, C Karkaria, P Gangola. 1988. Molecular characterization of an anion pump. The ArsA gene product is an arsenite (antimonate) stimulated ATPase. J. Biol. Chem. **263:** 3067-3070.

Rosen BP, U Weigel, RA Monticello, BPF Edwards. 1991. Molecular analysis of an anion pump: purification of the ArsC protein. Arch. Biochem. Biophys. **284:** 381-385.

Rosenstein R, K Nikoleit, F Gotz. 1994. Binding of ArsR, the repressor of the *Staphylococcus xylosus* (pSX267) arsenic resistance operon to a sequence with dyad symmetry within the *ars* promoter. Mol. Gen. Genet. **242:** 566-572.

Rosenstein R, P Peschel, B Wieland, F Gotz. 1992. Expression and regulation of the *Staphylococcus xylosus* antimonite, arsenite and arsenate resistance operon. J. Bacteriol. **174:** 3676-3683.

San Francisco MJD, CL Hope, JB Owolabi, LS Tisa, BP Rosen. 1990. Identification of the metalloregulatory element of the plasmid-encoded arsenical resistance operon. Nucleic Acids Res. **18:** 619-624.

San Francisco MJD, LS Tisa, BP Rosen. 1989. Identification of the membrane component of the anion pump encoded by the arsenical resistance operon of R-factor R773. Mol. Microbiol. **3:** 15-21.

Serrano R, MC Kielland-Brandt, GR Fink. 1986. Yeast plasma membrane ATPase is essential for growth and has homology with (Na^+/K^+), K^+-and Ca^{2+}-ATPases. Nature **319:** 689-693.

Shi W, JH Wu, BP Rosen. 1992. Identification of a putative metal binding site in a new family of metalloregulatory proteins. J. Biol. Chem. **269:** 19826-19829.

Shi WP, J Dong, RA Scott, BP Rosen. 1996. The role of arsenic-thiol interactions in metalloregulation of the *ars* operon. J. Biol. Chem. **271:** 9291-9297.

Shull GE, A Schwartz, JB Lingrel. 1985. Amino-acid sequence of the catalytic subunit of the $(Na^+ + K^+)$ATPase deduced from a complementary DNA. Nature **316:** 691-695.

Silver S, K Budd, KM Leahy, WV Shaw, D Hammond, RP Novick, GR Willsky, MH Malamy, H Rosenberg. 1981. Inducible plasmid-determined resistance to arsenate, arsenite and antimony (III) in *Escherichia coli* and *Staphylococcus aureus*. J. Bacteriol. **146:** 983-996.

Silver S, D Keach. 1982. Energy-dependent arsenate efflux: the mechanism of plasmid mediated resistance. Proc. Natl. Acad. Sci. USA **79:** 6114-6118.

Silver S, G Nucifora, L Chu, T Misra. 1989. Bacterial resistance ATPases: primary pumps for exporting toxic cations and anions. Trends Biochem. Sci. **14:** 76-80.

Silver S, G Nucifora, LT Phung. 1993. Human Menkes X-chromosome disease and the staphylococcal cadmium-resistance ATPase: a remarkable similiarity in protein sequences. Mol. Microbiol. **10:** 7-12.

Silver S, M Walderhaug. 1992. Gene regulation of plasmid and chromosomal-determined inorganic ion transport in bacteria. Microbiol. Rev. **56:** 195-228.

Smith K, RP Novick. 1972. Genetic studies on plasmid-linked cadmium resistance in *Staphylococcus aureus*. J. Bacteriol. **112:** 761-772.

Snavely MD, CG Miller, ME Maguire. 1991. The mgtB Mg^{2+} transport locus of *Salmonella typhimurium* encodes a P-type ATPase. J. Biol. Chem. **266:** 815-823.

Sofia HJ, V Burland, DL Daniels, G Plunkett III, FR Blattner. 1994. Analysis of the *Escherichia coli* genome. V. DNA sequence of the region from 76.0 to 81.5 minutes. Nucleic Acids Res. **22:** 2576-2586.

Solioz M, S Mathews, P Fürst. 1987. Cloning of the K^+-ATPase of *Streptococcus faecalis*. J. Biol. Chem. **262:** 7358-7362.

Tisa LS, BP Rosen. 1990a. Plasmid-encoded transport mechanisms. J. Bioenerg. Biomemb. **22:** 493-507.

Tisa LS, BP Rosen. 1990b. Molecular characterization of an anion pump: the ArsB protein is the membrane anchor for the ArsA protein. J. Biol. Chem. **265:** 190-194.

Tsai KJ, A Linet. 1993. Formation of a phosphorylated enzyme intermediate by the *cadA* Cd^{2+}-ATPase. Arch. Biochem. Biophys. **305:** 267-270.

Tsai KJ, KP Yoon, AR Lynn. 1992. ATP-dependent cadmium transport by the *cadA* cadmium resistance determinant in everted membrane vesicles of *Bacillus subtilis*. J. Bacteriol. **174:** 116-121.

Tynecka Z, Z Gos, J Zajac. 1981a. Reduced cadmium transport determined by a resistance plasmid in *Staphylococcus aureus*. J. Bacteriol. **147:** 305-312.

Tynecka Z, Z Gos, J Zajac. 1981b. Energy-dependent efflux of cadmium coded by a plasmid resistance determinant in *Staphylococcus aureus*. J. Bacteriol. **147:** 313-319.

Tynecka Z, T Skwarek, A Malm. 1990. Anaerobic ^{109}Cd accumulation by cadmium-resistant and -sensitive *Staphylococcus aureus*. FEMS Microbiol. Letts. **69:** 159-164.

Tynecka Z, J Zajac, Z Gos. 1975. Plasmid dependent impermeability barrier to cadmium ions in *Staphylococcus aureus*. Acta. Microbiol. Pol. **7:** 11-20.

Udo EE, WB Grubb. 1995. Transfer of plasmid-borne resistance from a multiply-resistant *Staphylococcus aureus* isolate, WBG1022. Curr. Microbiol. **31:** 71-76.

Udo EE, JW Pearman, WB Grubb. 1994. Emergence of high-level mupirocin resistance in methicillin-resistant *Staphylococcus aureus* in Western Australia. J. Hosp. Inf. **26:** 157-165.

Walderhaug MO, DC Dosch, W Epstein. 1987. Potassium transport in bacteria. *In* BP Rosen, S Silver, eds. Ion

transport in Prokaryotes. New York: Academic Press, pp. 85-130.
Walker JE, M Saraste, MJ Runswick, NJ Gay. 1982. Distantly related sequences in the α- and β-subunits of the ATP synthase, myosin kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:** 945-951.
Wang HF, TC Lee. 1993. Glutathione S-transferase facilitates the excretion of arsenic from arsenic-resistant Chinese hamster ovary cells. Biochem. Biophys. Res. Commun. **192:** 1093-1099.
Wang Z, S Dey, BP Rosen, TG Rossman. 1996. Efflux mediated resistance to arsenicals in arsenic-resistant and -hypersensitive Chinese hamster cells. Toxicol. Appl. Pharmacol. **137:** 112-119.
Weiss AA, S Silver, TG Kinscherf. 1978. Cation transport alteration associated with plasmid-determined resistance to cadmium in *Staphylococcus aureus*. Antimicro. Agents Chemother. **14:** 856-865.
Wemmie JA, MS Szczypka, DJ Thiele, WS Moye-Rowley. 1994. Cadmium tolerance mediated by the yeast AP-1 protein requires the presence of an ATP-binding cassette transporter-encoding gene YCF1. J. Biol. Chem. **269:** 32592-32597.
Witte W, L Green, TK Misra, S Silver. 1986. Resistance to mercury and to cadmium in chromosomally resistant *Staphylococcus aureus*. Antimicro. Agents Chemother. **29:** 663-669.
Wu JH, BP Rosen. 1991. Regulation of the *ars* operon: The *arsR* gene product is a negative regulatory protein. Mol. Microbiol. **5:** 1331-1336.
Wu JH, BP Rosen. 1993a. The *arsD* gene encodes a second *trans*-acting regulatory protein of the plasmid-encoded arsenical resistance operon. Mol. Microbiol. **8:** 615-623.
Wu JH, BP Rosen. 1993b. Metalloregulated expression of the *ars* operon. J. Biol. Chem. **268:** 52-58.
Wu JH, LS Tisa, BP Rosen. 1992. Membrane topology of the ArsB protein, the membrane component of an anion-translocating ATPase. J. Biol. Chem. **267:** 12570-12576.
Xu C, W Shi, BP Rosen BP. 1996. The chromosomal *arsR* gene of *Escherichia coli* encodes a trans-acting metalloregulatory protein. J. Biol. Chem. **271:** 2427-2432.
Yoon KP, TK Misra, S Silver. 1991. Regulation of the *cadA* cadmium resistance determinant of *Staphylococcus aureus cadA* cadmium resistance determinant of plasmid pI258. J. Bacteriol. **173:** 7643-7649.
Yoon KP, S Silver. 1991. A second gene in the *Staphylococcus aureus cadA* cadmium resistance determinant of plasmid pI258. J. Bacteriol. **173:** 7636-7642.

原核細胞與眞核細胞之利用排出毒性物質方式之抗鎘、砷、銻機理之探討

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對有毒金屬的抗性，普遍在原核細胞中被發現，不但存在革蘭氏陽性菌株，也在革蘭氏陰性菌株中被發現。同時各種的眞核細胞，譬如黴菌、或是植物細胞，以至於原蟲類生物及動物細胞等，也常可發現此種的抗性存在。在原核細胞與眞核細胞，此類有毒金屬的抗性，經常是藉由細胞主動的將有毒金屬排除體外所達成的。雖然，這些抗有毒金屬系統的受質，具有不同的化學屬性。但是，細胞卻可發展出不同進化來源，但具相似性質的運送有毒金屬，或是類金屬的ATP酵素，而將毒性物質排出。在這些將有毒金屬排除體外，來達成抗有毒金屬，或是類金屬的機理中。細菌對鎘的抗性系統，是經由一種具ATP酵素特性的初級膜轉運蛋白所執行的。然而，眞核細胞則發展出利用與硫基結合的方式，而將鎘排除的抗性系統。不同於鎘的抗性系統，在細菌對砷、及銻的抗性方面，則是利用具有使用ATP能力的唧筒，與具催化能力的次蛋白單元連結，所形成的一種抗性系統來執行的。而眞核細胞也進化出需要ATP的特殊的砷硫基抗性唧筒，以達成排除這些有毒物質的特性。在本文中，茲就原核細胞與眞核細胞的鎘、砷、及銻的一些抗性系統，在其生化機理方面做一比較與探討。

關鍵詞：抗性，鎘，砷，銻。

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