Newer Systems for Bacterial Resistances to Toxic Heavy Metals

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Bacterial plasmids contain specific genes for resistances to toxic heavy metal ions including Ag^+, AsO_2^-, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Sb^{3+}, and Zn^{2+}. Recent progress with plasmid copper-resistance systems in *Escherichia coli* and *Pseudomonas syringae* show a system of four gene products, an inner membrane protein (PcoD), an outer membrane protein (PcoB), and two periplasmic Cu^{2+}-binding proteins (PcoA and PcoC). Synthesis of this system is governed by two regulatory proteins (the membrane sensor PcoS and the soluble responder PcoR, probably a DNA-binding protein), homologous to other bacterial two-component regulatory systems. Chromosomally encoded Cu^{2+} P-type ATPases have recently been recognized in *Enterococcus hirae* and these are closely homologous to the bacterial cadmium efflux ATPase and the human copper-deficiency disease Menkes gene product. The Cd^{2+}-efflux ATPase of gram-positive bacteria is a large P-type ATPase, homologous to the muscle Ca^{2+} ATPase and the Na^+/K^+ ATPases of animals. The arsenic-resistance system of gram-negative bacteria functions as an oxyanion efflux ATPase for arsenite and presumably antimonite. However, the structure of the arsenic ATPase is fundamentally different from that of P-type ATPases. The absence of the *arsA* gene (for the ATPase subunit) in gram-positive bacteria raises questions of energy-coupling for arsenite efflux. The ArsC protein product of the arsenic-resistance operons of both gram-positive and gram-negative bacteria is an intracellular enzyme that reduces arsenate [As(V)] to arsenite [As(III)], the substrate for the transport pump. Newly studied cation efflux systems for Cd^{2+}, Zn^{2+}, and Co^{2+} (Czc) or Co^{2+} and Ni^{2+} resistance (Cnr) lack ATPase motifs in their predicted polypeptide sequences. Therefore, not all plasmid-resistance systems that function through toxic ion efflux are ATPases. The first well-defined bacterial metallothionein was found in the cyanobacterium Synechococcus. Bacterial metallothionein is encoded by the *smtA* gene and contains 56 amino acids, including nine cysteine residues (fewer than animal metallothioneins). The synthesis of Synechococcus metallothionein is regulated by a repressor protein, the product of the adjacent but separately transcribed *smtB* gene. Regulation of metallothionein synthesis occurs at different levels: quickly by derepression of repressor activity, or over a longer time by deletion of the repressor gene at fixed positions and by amplification of the metallothionein DNA region leading to multiple copies of the gene. — Environ Health Perspect 102(Suppl 3):107–113 (1994).

Key words: arsenic, bacterial plasmids, cadmium, copper, mercury, metallothionein, metal resistances

Introduction

Bacterial mechanisms for resistances to toxic heavy metals have become a mature scientific subject over the last 20 years. Highly specific systems exist for most toxic cations and oxyanions (Figure 1). The genes determining these resistances are generally (but not always) found on bacterial plasmids. Heavy-metal resistance mechanisms were recently reviewed in *Plasmid* with articles on mercury resistance (*1*), the most thoroughly understood of bacterial resistance systems (*2,3*) and on resistances to arsenic (*4*), copper (*5*), cadmium (*6*), chromate, silver, and tellurite. Here, we have reviewed five less familiar bacterial heavy-metal resistance systems.

Bacterial Plasmid Copper-Resistance Systems

Bacterial plasmid copper-resistance systems have been found on plasmids in *Escherichia coli* (*5,7*) and *Pseudomonas syringae* (*8*) and on the chromosome of *Xanthomonas campestris* (*8*). It was thought that the basic mechanism of resistance might be different in *E. coli* and in Pseudomonas (*3,5*). However, new DNA sequence analysis of the *E. coli* plasmid determinant has shown that the systems are basically equivalent and consist of four structural genes, now called *pcoA*, *pcoB*, *pcoC*, and *pcoD* in *E. coli*, and *copA*, *copB*, *copC*, and *copD* in Pseudomonas (*7*). The Pseudomonas and *E. coli* systems both have paired regulatory genes, with an apparently membrane-bound Cu^{2+} sensor (the *pcoS* or *copS* gene product) coupled with the *pcoR* or *copR* gene products that may be DNA-binding repressor proteins (*5,7*). The genes and their protein products are summarized in Figure 2, along with the percentages for

Plasmid Heavy Metal Resistance Systems and Mechanisms.

1. *mer.* Mercury and organomercurials are enzymatically detoxified.
2. *ars.* Arsenate is reduced to arsenite enzymatically. Arsenite is "pumped" from cells by a membrane protein or a two-component ATPase. Sb(III) is also a transport substrate.
3. *cadA.* Cadmium (and zinc) are "pumped" from the cells by a P-type ATPase.
4. *cop.* Copper resistance in *Pseudomonas* and *pco.* Copper resistance in *E. coli* results from bioaccumulation of copper from the medium and binding by surface proteins.
5. *czc.* Cadmium, zinc, and cobalt are "pumped" from the cells by a three-componenet membrance complex that appears NOT to be an ATPase. *cnr.* Cobalt and nickel are "pumped" from the cells by a related membrane complex that may (after mutation) also pump zinc.
6. *chr.* Chromate resistance results from reduced cellular uptake but an efflux pump has not been demonstrated.

Additional resistance systems await understanding: bismuth, boron, lead, silver, thalium, and tellurium.

Figure 1. Summary of systems and mechanisms for bacterial plasmid resistances to toxic inorganic cations and oxyanions.

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Recent work from other laboratories and close communications with NL Brown, DA Cooksey, P Corbisier, F Götz, BTO Lee, AL Linet, M Mergeay, AP Monaco, DH Nies, LT Phung, NJ Robinson, BP Rosen, H Schlegel, C Vulpe, and M Walderhaug are gratefully acknowledged. This report also summarizes work in our laboratory recently supported by grants from the National Science Foundation, the Department of Energy, and the Electric Power Research Institute.

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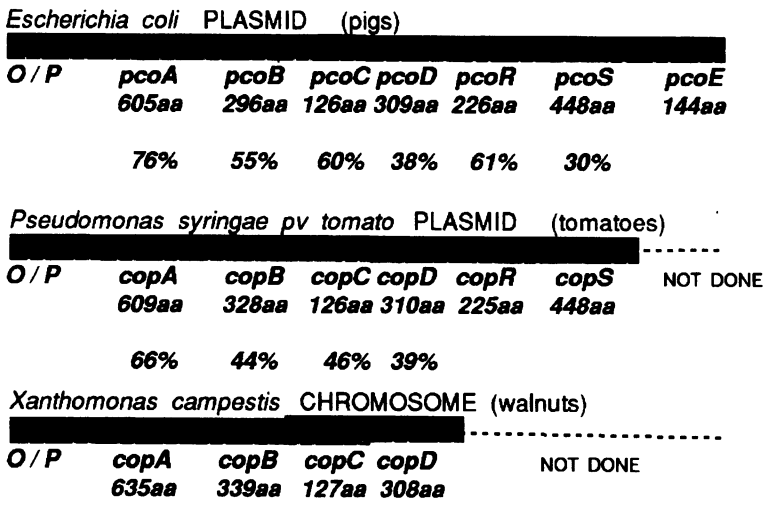

Figure 2. Comparison of the genes for copper resistance from an *E. coli* plasmid, an *P. syringae* pv *tomato* plasmid and the chromosomal determinant of *Xanthomonas campestris* pv *juglandis*. The *copABCDRS* gene products (*9,12*) and the *pcoR* gene product (*5*) have been published. The remainder of this analysis is based on preliminary sequences by NL Brown (personal communication) for the Pco sequence from *E. coli* and by Y-A Lee, M Hendson, and MN Schroth (personal communication) for the *Xanthomonas* sequence. The precise numbers may be subject to minor corrections and the "missing" genes and the nature of *pcoE* remain for additional work.

amino acid identities between corresponding protein products. Southern blotting DNA/DNA hybridization fails to detect homologies between the *E. coli* and Pseudomonas genes (*8–10*). However, amino acid identities ranging from 38 to more than 75% allow little question but that the proteins will function in comparable roles. For the *E. coli* system, an additional reading frame called *pcoE* was identified in the DNA sequence; it appears to be transcribed in a Cu^{2+}-inducible manner. The function of this gene product is not known, and the comparable region for the Pseudomonas determinant has not been sequenced. Figure 2 also includes information from a chromosomal Xanthomonas copper-resistance determinant for which only the four structural genes have been sequenced (Y-A Lee, M Hendson, and MN Schroth, personal communication). It was earlier shown that Xanthomonas and Pseudomonas copper-resistance determinants do not detectably cross-hybridize (*10*). However, it is surprising that the copper-resistance systems from *P. syringae* and *E. coli* are more similar (Figure 2) than are the Pseudomonas and Xanthomonas determinants. The bacteria from which these copper-resistance systems have been isolated are all pathogens of agricultural interest, but are otherwise quite different in their sources, with the *E. coli* from pigs with diarrhea, and the Pseudomonas and Xanthomonas pathogens of tomato plants and walnut trees, respectively.

The Pseudomonas CopA, CopB and CopC proteins have been isolated (CopD remains to be identified). The CopA and CopC proteins are blue periplasmic proteins, containing 11 and 1 Cu^{2+} cation respectively by direct analysis (*11*). The CopB protein is an outer membrane protein and CopD is probably an inner membrane protein (Figure 3). How these four proteins function together to allow periplasmic "bioaccumulation" of copper by resistant cells is still unknown. However, colonies of the copper-resistant Pseudomonas turn bright blue, while the supporting agar becomes colorless, as the copper salts are taken up by the cells. The colonies of copper-resistant *E. coli* become brown, however, when grown on copper-containing agar. The nature of the brown pigment is not known.

Regulation of the copper resistance determinants is also under study. Thus far, the *pcoR* and *pcoS* [or *copR* and *copS* (*12*)] gene products are known only from DNA sequence analysis and the proteins have not be isolated. The sequences are typical of the 100 or so "two-component" regulatory systems that have been identified in bacteria (*13*), so there is little doubt of the overall mechanism (Figure 4). PcoS appears to be a membrane protein. It is homologous in sequence to other "sensor" proteins (kinases) (*13*) that respond to external stimuli by autophosphorylating (from ATP) a specific histidine residue (for PcoS, His_{257}) that is always conserved in this class of membrane sensors. The phosphorylated sensor protein then transfers its phosphate to a specific aspartate residue (Asp_{52} for PcoR) of the secondary transducer protein (*12,13*). In many cases, the transducer is activated by phosphorylation and binds to the appropriate DNA operator region, to initiate transcription of the operon, for example, here *pcoABCD*. However, there is a question as to whether PcoR might be a repressor protein that is inactivated (and released from the DNA) by phosphorylation. Furthermore, in the Pseudomonas *cop* system, there is evidence for a third regula-

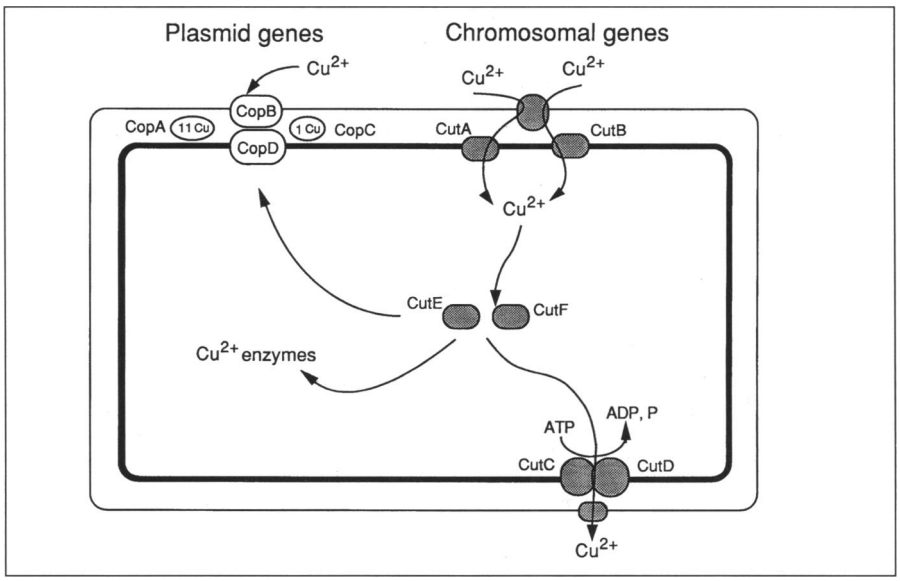

Figure 3. The proteins for copper transport and resistance in *Pseudomonas* and *E. coli* [modified and updated from Silver and Walderhaug (*3*), Brown et al. (*5*), and Silver et al. (*7*)]

tory component apparently encoded by a chromosomal gene.

The current (and tentative) picture for the *cut* (for Cu^{2+} transport) chromosomal gene products involved in uptake, intracellular movement, and efflux of Cu^{2+} (*3,5,7*) are also shown in Figure 3. In *E. coli*, these genes were initially tagged by mutations leading to copper sensitivity (*14*). Only one of the *cut* genes (*cutE*) has been cloned and sequenced to date (*15*) and its sequence does not immediately lead to an explanation of its intracellular role (Figure 3). Comparable *cut* chromosomal mutations to copper sensitivity have been obtained with Pseudomonas (DA Cooksey, personal communication). The recent identification of a previously sequenced gene for a P-type ATPase in Enterococcus as a *cut* gene (*16*) opens the search for comparable Cu^{2+} efflux ATPases in other bacteria. Furthermore, the human X-chromosome gene responsible for the lethal disease Menkes syndrome appears to determine a P-type ATPase (*17*). The discussion of the Menkes gene and bacterial P-type ATPases in the section on Cd^{2+} resistance below provides further information.

Metallothionein from the Cyanobacterium Synechococcus

A bacterial metallothionein from the cyanobacterium Synechococcus was purified and sequenced (*18*). The gene encoding metallothionein is called *smtA* (*19*). Bacterial metallothionein probably represents the independent evolutionary development of the same solution to heavy-metal binding obtained with animal and plant metallothioneins, since the sequence is shorter (only 56 amino acids) and contains fewer cysteine residues (9, compared with 20 out of 60 amino acids for animal metallothioneins, or 12 out of 75 amino acids for plant metallothionein). Unlike animal metallothioneins, the Synechococcus metallothionein contains two aromatic tyrosine residues in the intracysteine-rich region (*19*). Synechococcus metallothionein synthesis is regulated by heavy metals such as cadmium and zinc (*20*) by means of a repressor protein synthesized from a constitutively expressed divergently transcribed gene *smtB* (*20*), (Figure 5). In addition to rapid response to toxic metals by derepression (*20*), two long-term mechanisms of increasing *smtA* metallothionein gene expression have been found. Upon growth on high cadmium concentrations, amplification of the metallothionein gene region occurs (*21*) leading

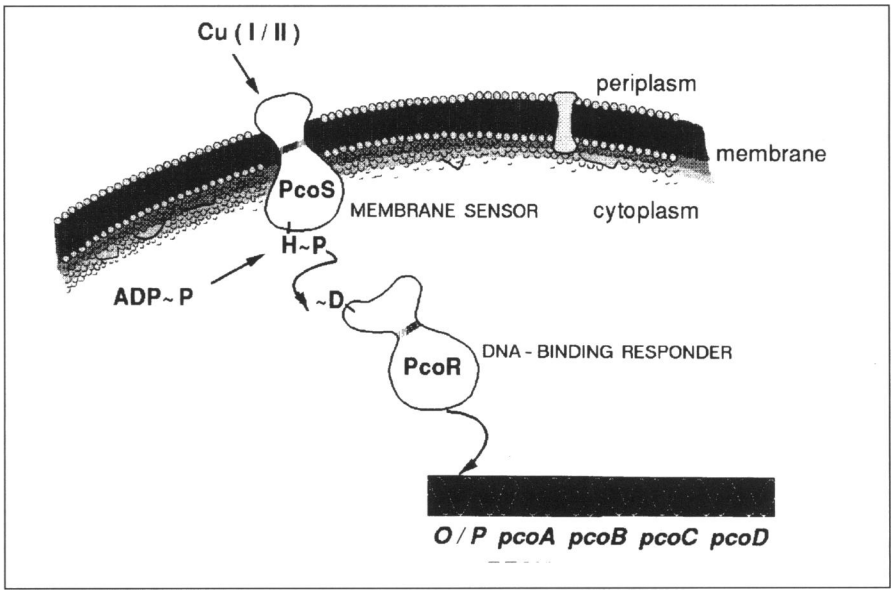

Figure 4. Regulation of the *E. coli* copper-resistance determinant [modified from a "general" signal transduction model of K.Volz (*7,12,13*)].

to higher levels of metallothionein synthesis. In addition, continued exposure to high cadmium concentrations results in deletion (*22*), (Figure 5) of most of the *smtB* gene, at a site between two (of seven) highly iterated palindromic octanucleotide sequences (HIP1; 5'GCGATCGC3') that occur in the metallothionein gene region (Figure 5). This HIP1 sequence occurs elsewhere in known Synechococcus DNA sequences at a frequency of approximately every 650 nucleotides (*22*), whereas the HIP1 frequency is lower in bacteria other than cyanobacteria and is virtually unknown in *E. coli*. In summary of Figure 5, three levels of gene regulation occur: rapidly by derepression of the SmtB repressor, and more slowly in response to continued toxic metal stress by amplification of the gene copy number and by deletion of the repres-

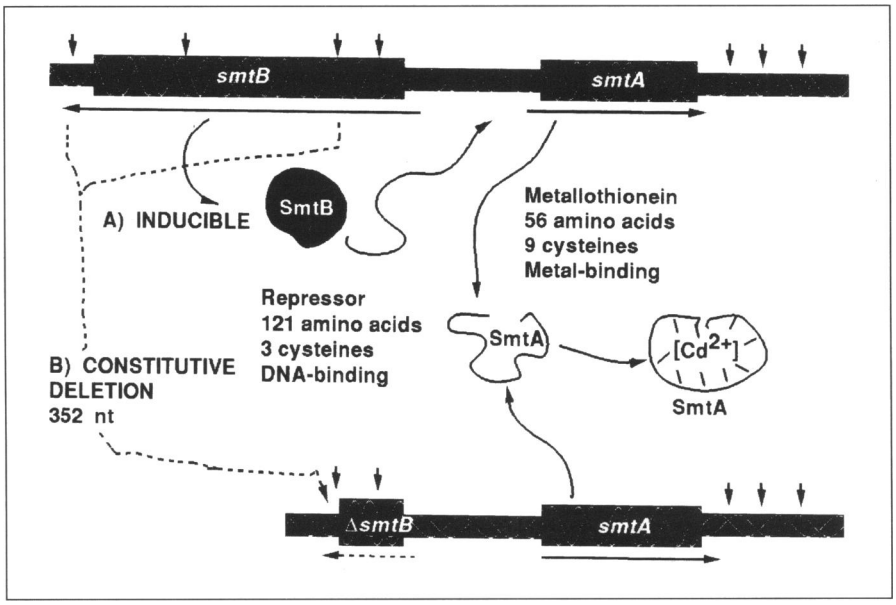

Figure 5. The metallothionein genetic determinant of Synechococcus. (A) Wild-type, repressible system with divergently transcribed *smtA* (metallothionein) and *smtB* (regulatory) genes. Arrows at top, locations of seven HIP1 (highly iterated palindromic sequences 5'GCGATCGC3'). The repressor protein SmtB and bacterial metallothionein SmtA (hypothesized as gaining a more rigid structure with bound Cd^{2+}). (B) Genetic determinant (constitutive metallothionein synthesis) after deletion of 352 nucleotides from the first to the third HIP1, from the left as shown). Summarizes results by NJ Robinson and colleagues (*19–22*).

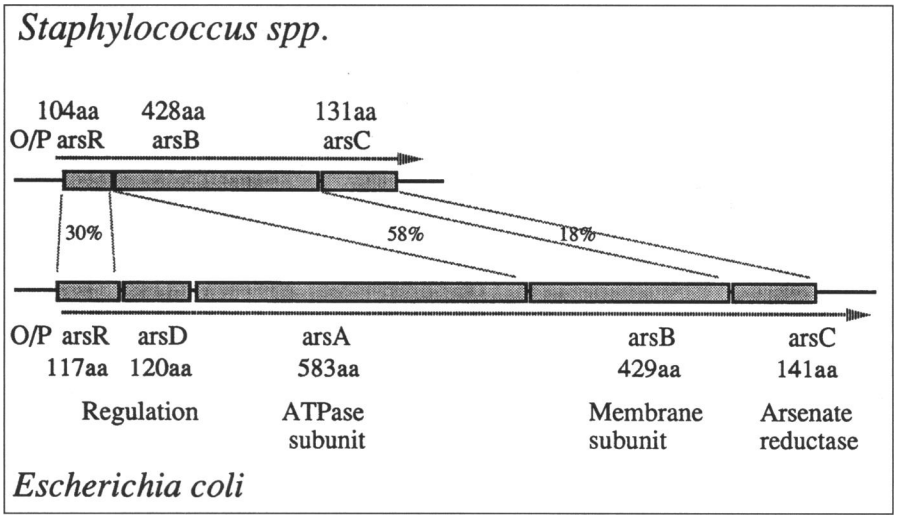

Figure 6. Alignment and functions of arsenic resistance genes from *E. coli* and *Staphylococcus* [Silver and Walderhaug (*3*), *Silver et al. (27)*, with permission].

sor gene between fixed chromosomal points (Figure 5).

Bacterial Plasmid Arsenic Resistance

Bacterial plasmid arsenic resistance results from the rapid energy-dependent efflux of oxyanions added as either arsenate [As(V)], arsenite [As(III)], or antimonite [Sb(III)]. The same basic mechanism (and genes) exists in both gram-negative and gram-positive bacteria (Figure 6). The mechanism of bacterial arsenic resistance has been repeatedly reviewed (*3,4,23,24*), but there are several recent and surprising findings. After an operator/promoter regulatory region, both the gram-positive and the gram-negative versions of the ars operon start with the *arsR* regulatory gene (Figure 6) that encodes a transacting repressor protein (*25,26*). ArsR of the gram-negative plasmid R773 and the gram-positive version from two plasmids (*26,27*) are identical in 30% of their amino acid positions. The ArsR proteins are not very similar to other known bacterial regulatory proteins, but ArsR is weakly similar to the SmtB regulatory protein for cyanobacterial metallothionein regulation (above) and to the small CadC protein in the cadmium resistance system (below). The ArsR structure and means of binding to the DNA operator region (*25,27*) are not known. Since the regulatory protein is made as part of the same transcriptional unit as the other arsenic resistance genes, the system cannot be turned "tightly off": low levels of ArsR are always needed.

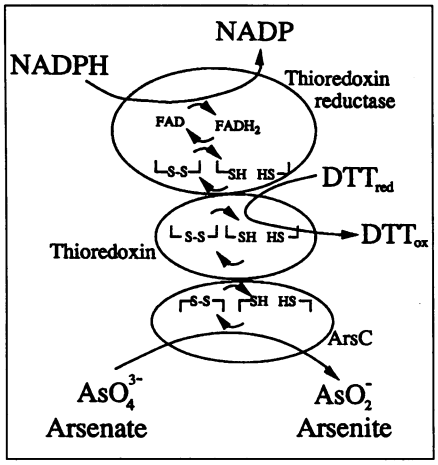

Figure 7. The function of the ArsC arsenate reductase [Silver et al. (*24*), with permission].

The arsenic resistance operons of gram-positive and negative bacteria diverge after *arsR*. The plasmid R773 system has two genes, *arsD* and *arsA*, that are missing from the staphylococcal arsenic resistance systems (*26,27*). ArsD appears to be a minor regulatory protein that "puts a cap" on the amount of transcription that occurs in derepressed cells. ArsA is the ATPase subunit, binding to the ArsB membrane protein and forming the arsenite efflux ATPase (*3,4,23,24*). ArsA from plasmid R773 has been studied extensively *in vitro*. It is an oxyanion-stimulated soluble ATPase protein (*4,23*). How can one have an ATPase efflux pump in gram-positive bacteria without the ATP-binding protein? This major question is not resolved, but there is preliminary evidence that ArsB alone functions in Staphylococcus as a chemiosmotic secondary transport system (*24,28*). It was hypothesized (*23*) that an ancestral electrogenic oxyanion system (ArsB) may have acquired through evolution a second ATPase protein component, converting it into a more effective ATPase primary pump. The basic similarity of membrane regions from differing primary and secondary membrane transporters (*29,30*) is consistent with this idea. In summary, there are still major unresolved questions concerning the mechanism of arsenic resistance.

The next gene common to all three sequenced arsenic resistance determinants is *arsB* (Figure 6), the determinant of the membrane protein that is needed for arsenic efflux. The ArsB proteins from gram-negative and gram-positive bacteria are 58% identical in amino acids. They are so similar in overall sequence that chimeric ArsB proteins with the first third of ArsB from *E. coli* and two-thirds of ArsB from *Staphylococcus* (and the reverse) have been made by swapping gene segments (S Dey, D Dou, BP Rosen, personal communication). These chimeric ArsB proteins give partial resistances to arsenate and arsenite, but they are not equivalent to the native gene products. Higher levels of resistances occur with the ArsA protein present in addition to ArsB. The details of how the regions of the chimeric ArsB proteins function remain to be deduced.

The final gene common to all three arsenic resistance operons is *arsC*, but the protein products are only 18% identical in amino acid sequences. Nevertheless, the function of *arsC* is the same: to provide arsenate resistance to a system limited to arsenite and antimonite resistances in the absence of *arsC*. Recent experiments have shown that the *arsC* protein is an enzyme, a reductase that converts AsO_4^{3-} [As(V)] to AsO_2^- [As(III)] (*31*). The gram-positive version of this small, soluble protein couples *in vitro* to thioredoxin (*31*), which is a general intracellular redox protein. *In vitro* ArsC reductase activity can be driven by thioredoxin and an artificial reduced thiol compound, dithiothreitol (DTT: Figure 7), or by the larger protein thioredoxin reductase and NADPH (Figure 7). Since thioredoxin reductase catalyzes an oxidized to reduced dithiol cycle in thioredoxin, it is expected that thioredoxin similarly carries out reduction of an oxidized dithiol [two of the four cysteine residues in staphylococcal ArsC (*26,27*); however, there are only two cysteines in the *E. coli* ArsC] and that ArsC protein is oxidized as it reduces arsenate to arsenite (Figure 7). The picture of the arsenic resistance system given here shows major changes from that reported in 1992 (*3,4*).

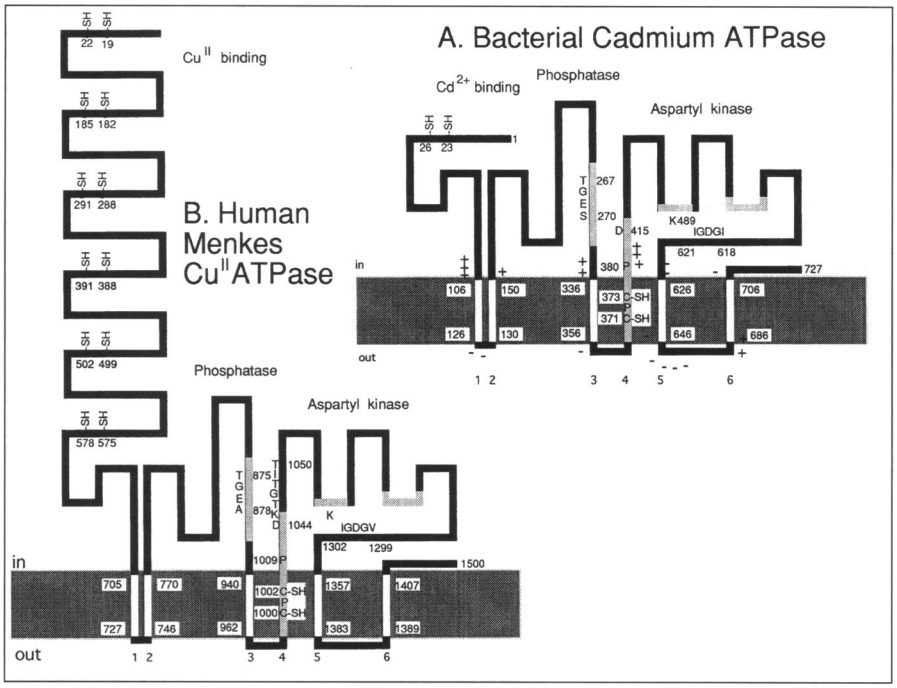

Figure 8. Structure and functions of (*A*) the CadA Cd^{2+} efflux ATPase of gram-positive bacteria [Silver and Walderhaug, (*3*), with permission] and (*B*) the candidate human Menkes disease copper ATPase (*17*).

CadA Cd^{2+} Efflux ATPase

The CadA Cd^{2+} (and perhaps Zn^{2+}) efflux ATPase is a P-type ATPase (*32–34*) found in gram-positive bacteria. Three versions of this genetic determinant have now been sequenced, including one from *S. aureus* plasmid pI258 (*32*). A closely related system was found in a alkalophilic soil bacterium *Bacillus firmus* (*35*) and still another has been sequenced from the chromosomal cadmium-resistance determinant of a methicillin-resistant *S. aureus* isolate (DT Dubin, personal communication). These are three of the ten currently sequenced P-type ATPases from bacteria, half of which are yet to be published. There are more than 30 P-type ATPase enzymes known from animal sources (*3,33,34*, and references therein). Whereas the P-type ATPases were once thought to be exclusively animal in origin (and they include the Ca^{2+} ATPase of the muscle sarcoplasmic reticulum and the numerous known Na^+/K^+ ATPases), additional P-class ATPases have been identified in plants and lower eukaryotes as well (*34*).

The basic properties of P-type ATPases are demonstrated in the model of the CadA membrane polypeptide in Figure 8. First, the protein is embedded in the membrane by six hydrophobic sections that presumedly form transmembrane α-helices. These regions are thought to form the transmembrane cation channel. All P-type ATPases are cation-translocating (*34*); some function for uptake (of K^+ and Mg^{2+}) whereas others function for efflux (Cd^{2+} and Ca^{2+}). In each case, the P-type ATPases are thought to be about 75% intracellular, about 20% in the membrane, and with very little extracellular sequence (*8*). Positively charged amino acids reside more frequently on the inner membrane surface, whereas negatively charged amino acids are found more often on the outer membrane surface.

There are a number of key conserved positions in P-type ATPases, and in particular in the CadA sequence and candidate human Menkes sequence in Figure 8. Starting from the amino terminus, the CadA protein has a hypothesized Cd^{2+}-binding motif of perhaps 30 amino acids. This motif occurs also in the Cd^{2+} ATPase of Bacillus (*35*), twice (back to back) in an unpublished chromosomal Cd^{2+} resistance determinant in a methicillin-resistant *S. aureus* (DT Dubin, personal communication), and six times over a 600 amino acid region at the beginning of the gene product for human Menkes disease (*17,36*). This is a striking example of where studies on bacterial model systems have facilitated understanding of human disease (*17,36*) (Figure 8).

Menkes syndrome is a human disease of copper deficiency (*37*) and the characteristics of the defective cells are consistent with the new hypothesis that Menkes disease patients will be defective in synthesis of a P-type ATPase that is a Cu^{2+} efflux enzyme, more similar to the bacterial Cd^{2+} ATPases in sequence than it is to known eukaryotic P-type ATPases (*17*).

After the first membrane hairpin, all P-type ATPases have a region of 150 to 300 amino acids that includes a "phosphatase motif" Thr-Gly-Glu-Ser in CadA or Thr-Gly-Glu-Ala in the Menkes sequence (Figure 8). This region has been assigned the role of "transducing" the intracellular protein-bound cation to the membrane channel region. The second membrane hairpin appears to be involved in transmembrane cation movement and includes a conserved proline residue in all P-type

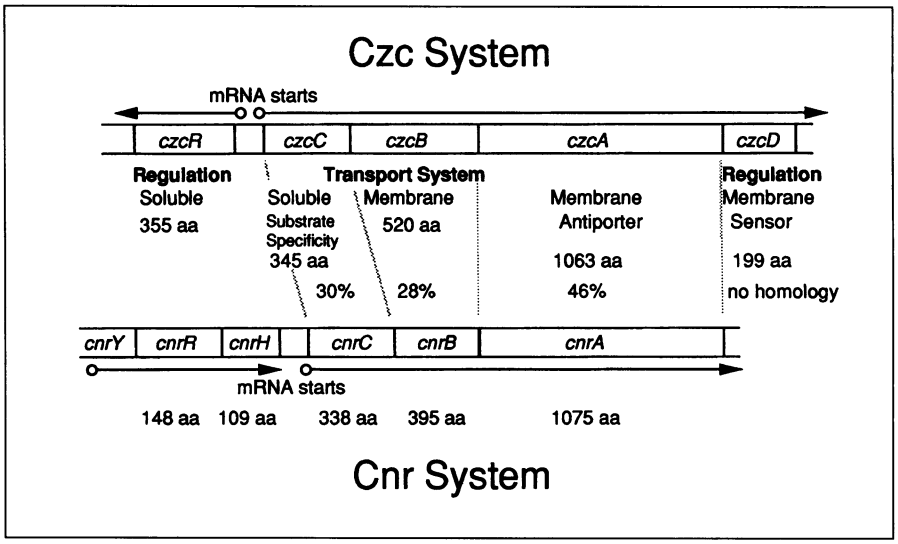

Figure 9. Tentative model for arrangement (and roles) of the genes in the Czc (Cd^{2+}, Zn^{2+} and Co^{2+} efflux) and Cnr (Co^{2+} and Ni^{2+} resistance) systems from *A. eutrophus* strain CH34 (*42–45*).

ATPases. For CadA and Menkes, this appears in a conserved Cys-Pro-Cys sequence and along with the metal-binding motif cysteines. In addition to these two cysteines in the membrane region, only the metal-binding motif cysteines occur in the entire 727 amino acid CadA polypeptide or 1500 amino acid Menkes sequence (*17*). The Cys-Pro-Cys sequence is found in this position in six of the ten known prokaryote P-type ATPases (all except in KdpB, MgtA, MgtB, and the Enterococcus possibly-Cu^{2+} ATPase (*16*). Following the conserved proline is a second proline residue, eight positions further along in CadA, in the Menkes ATPase, and in the six closest prokaryotic P-type ATPase sequences. Following the second hairpin is an ATP-kinase domain of approximately 300 amino acids in P-type ATPases. This domain includes the aspartate-415 in CadA, which is in the Asp-Lys-Thr-Gly-Thr-Leu (or Ile)-Thr sequence conserved in all P-type ATPases. The aspartate residue is phosphorylated by ATP during the transport cycle. There follows a series of conserved residues involved in ATP binding, and the "kinase" region ends with a highly conserved "hinge motif" that is thought to be involved in allosteric movement of the kinase region so as to modify the cation binding affinity. After a third membrane hairpin, the CadA structure in Figure 8 ends at position Lys_{727}. The Menkes ATPase sequence continues further and ends at Leu_{1500}.

After the sequence of CadA was available (*32,35*), it was found that the second gene transcribed with *cadA* (called *cadC*) was necessary for full resistance (*38*) and that the *cadA* system is tightly repressed but inducible by toxic cations (*39,40*). It is still unclear whether the *cadC* gene is directly involved in the resistance mechanism (*38*) or is involved in regulation (*39*). The CadC amino acid sequence is somewhat related to the ArsR arsenic regulatory protein sequence (see above). Direct measurements of uptake of radioactive Cd^{2+} by inside-out membrane vesicles from cells containing CadA (*38*) added direct biochemical support for the deductions from sequence homologies. CadA-dependent Cd^{2+} uptake requires ATP (*38*), and the CadA protein can be labeled with ^{32}P from ATP, with the labeled protein showing exchange and alkali-sensitivity characteristics similar to other P-type ATPases (*41*). The CadA P-type ATPase, for which work is only beginning, may become increasingly valuable as a model P-type ATPase for detailed molecular genetic and biochemical studies.

Gram Negative Soil Bacterium *Alcaligenes Eutrophus*

The gram-negative soil bacterium *Alcaligenes eutrophus* harbors two divalent cation efflux systems on its large megaplasmids (each more than 150 kilobases in length): the Cd^{2+}, Zn^{2+}, and Co^{2+} resistance system called Czc and the Co^{2+} and Ni^{2+} resistance system called Cnr. Cnr has only recently been shown to be related to Czc at the level of genes and their protein products (*42,43*). For both systems, energy-dependent efflux of divalent cations has been demonstrated to be the resistance mechanism (*6,42*). The predicted gene products from DNA sequences show no sign of ATPase motifs (*42,43*). There are preliminary suggestions that the mechanism of energy-coupling may be a chemiosmotic divalent cation/proton antiporter (*6,44*). The primary differences between the two systems appeared to be cation specificity and the nature of gene regulation. New mutations of the *cnr* system add zinc resistance (and presumedly efflux) (*43,45*) to cobalt and nickel resistances; and *cnr* and *czc* may be less different than initially thought.

As cloned and sequenced, the *czc* system was found to consist of four genes (Figure 9). The first is *czcA*, the determinant of a large membrane protein that is thought to be the principal component of the transport system (*6*), since deletions involving *czcA* lost all resistances (*42*). The smaller membrane (CzcB) and soluble (CzcC) proteins appear to play secondary roles in determining specificity (*6,42*). The fourth gene product, CzcD, is thought to affect regulation of the system, but not resistance directly (*6,42,44*). More recent sequencing of the "upstream" region of *czc* and czc-β-galactosidase fusion experiments (*44*) indicate that the divergently transcribed *czcR* gene and *czcD* cooperate in gene regulation. CzcR may be an activator protein rather than a repressor (*44*). Although the paired regulatory proteins CzcD and CzcR are thought to be respectively membrane sensor and DNA-binding responder (*6,44*), these proteins do not fit into the familiar two-component class of kinase-"sensor" plus transphosphorylated "responder" as do PcoR and PcoS of the copper resistance system (above).

The new sequence of the *cnr* system (*43*) has yielded a few surprises. First, although the two genetic systems are sufficiently different that their DNAs do not hybridize on Southern blots, the gene products of CzcA (and CnrA), CzcB (and CnrB), and CzcC (and CnrC) are significantly homologous (Figure 9). Therefore, it is likely that the two systems will function in a fundamentally similar manner. However, the regulatory regions appear at the moment to be unrelated (Figure 9). The *cnr* lacks both *cnrR* and *cnrD* genes, but has instead three unrelated upstream genes *cnrY*, *cnrR*, and *cnrH* (Figure 9) that appear to be involved in gene regulation (*43,45*). It is the *cnrY* region that contains the mutations to constitutive function that also extend the system to zinc resistance (*43,45*). We can look forward to more understanding and possibilities with regard to toxic divalent cation efflux.

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