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Bacterial resistance mechanisms for heavy metals of environmental concern

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SUMMARY

Bacterial species have genetically-determined systems for resistances to toxic heavy metals. Those for metals of environmental concern including mercury cadmium, arsenic and others are briefly summarized, considering the genes of the systems and the biochemical mechanisms by which the resistance proteins function.

INTRODUCTION

Bacteria can show remarkable resistances to essentially all toxic metal ions of environmental concern. Genes on bacterial plasmids have been identified that encode specific resistance systems for toxic heavy metal ions. These include Ag^+, AsO_2^-, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Sb^{3+}, TeO_3^{2-} and Zn^{2+}. There are reports (but less than satisfactory understanding) for resistances to $B_4O_7^{2-}$, Pb^{2+}, Sn^{2+} and organotin compounds. U(VI) reduction to U(IV) by bacteria has been recorded [46,47] and may constitute a resistance mechanism. However, it seems unlikely that U(VI) salts occur in bacterial growth environments at levels sufficient to have selected for uranium resistance in nature. Whether additional toxic inorganic compounds lack highly-specific biochemically-precise genetically-encoded resistance systems is not known; it may sometimes be only a matter of inadequate experimental efforts to date. That leaves the higher atomic number member of the Periodic Table (the lanthanides and actinides, including uranium and trans-uranium elements) and Groups Ia, IIa, as lacking genes for metal ion resistances. Group VIIa halides (Cl^-, Br^-, and I^-) also lack resistance genes, although this might indeed be expected from the environmental distribution of high levels of halides.

Our hypothesis has been that microbial resistance systems arose shortly after prokaryote life started (in an already metal-polluted world). Therefore, we expect that the resistance systems will be found in essentially all bacterial types. As with sugars and other carbon sources, whatever inorganic cations and anions were frequently found in the environment selected genes (and enzymes) required for their transport and metabolism. Unlike carbon compounds, however, some inorganic ions are required nutrients at low levels but toxic when present at higher levels (Cu^{2+}, Co^{2+} and Ni^{2+} are examples), whereas other inorganic ions (Hg^{2+} and AsO_2^- are examples) are always toxic and have no metabolic functions in bacteria. As with antibiotic resistances, the recent activities of humans create local environments of high selection, but there is nothing recent about toxic heavy metal resistance determinants and their mechanisms. In fact, resistances to the heavy metals arsenic and copper were frequent in bacteria isolated before the antibiotic era [29] and indeed were more frequent in such a collection than were resistances to antibiotics.

Since the discovery that mercury-resistant bacteria could volatilize mercury, as mono-atomic Hg^0, the subject of multigene bacterial plasmid-determined resistances to toxic inorganic cations and anions has burgeoned. The idea that mercury 'gas' was generated as a resistance mechanism seemed ludicrous, but is true [89]. Similarly our newer finding that less toxic arsenate is converted to more toxic arsenite as part of an oxyanion resistance system in both Gram-positive and Gram-negative bacteria [34,35] seemed implausible, but is clearly correct. To understand the environmental microbiology of toxic metals and to use microbial processes for bioremediation, we must first understand the basic biochemical activities and their genetic determinants. This review can only briefly cover the range and mechanism of bacterial toxic metal resistances. System-specific mini-reviews have appeared [16,69,70,84, 87,88], including six in a 1992 special issue of the journal *Plasmid* [5,8,37,52,56,97] and others are in this issue of the *Journal of Industrial Microbiology*.

In addition to mercury resistance determinants, we know of highly specific resistance systems for arsenic (and antimony), copper, cadmium (and zinc in Gram-positive bacteria, but a different system shared with cobalt, nickel and zinc in plasmids of Gram-negative bacteria), tellurite, silver and perhaps

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other toxic metal ions. Before going into specifics, three generalizations may be made: (i) The specificities of plasmid metal resistance systems are approximately the same as for antibiotic resistances, or sugar or amino acid metabolism (i.e. very specific). There are no general mechanisms of metal resistance. (ii) Metal ion resistance systems have been found on plasmids of every eubacterial group where they have been sought, from *Escherichia coli* to *Streptomyces*. Frequently, the genes (and mechanisms) initially found with plasmids (because of ease of experimental analysis) are subsequently found on the chromosomes of related bacteria. Insertion elements and transposons are associated and thought to be responsible for gene mobility. There are no published reports of biochemical mechanisms similar to those described here with Archaebacteria. Certainly Archaebacteria live in environments with high levels of toxic heavy metal ions and therefore are expected to have developed the same or alternative mechanisms of resistances. (iii) The third generalization is that mechanisms of resistance frequently involve enzymatic detoxification (initially a bizarre concept for a 'metal'), converting a more toxic to a less toxic or less available metal ion species (but familiar as intracellular oxidation and reduction chemistry), efflux 'pumping' out from the cells of toxic ions that slipped in via transport systems for nutrient cations or anions, and bioaccumulation (binding of toxic metal ions to bacterial proteins or polypeptides). Why it would not be easier to keep toxic ions out (by altering the specificity of membrane uptake pumps) rather than expend the metabolic energy bringing in the toxic ions and then more energy pumping them out requires an explanation. It seems that the metabolic penalty for having more specific uptake pumps is greater than the genetic cost of having genes on plasmids or transposons in the population that can spread and become induced (these systems are tightly regulated at the gene level) when needed. Efflux pumps indeed are the major currently-known group of such plasmid resistance systems. They can be either ATPases (as the Cd^{2+} ATPase of Gram positives and the arsenite ATPase of Gram negatives) or chemiosmotic (as the divalent cation efflux system of soil *Alcaligenes* and the arsenite efflux system of Gram-positive bacteria). It is thus clear that the mechanisms are not precisely the same in all bacterial types. While the mercury resistance systems are highly homologous in all bacteria studied, and the arsenic resistance systems are less homologous (energy-coupling differs between Gram positives and Gram negatives), and cadmium resistance uses an ATPase in Gram-positive bacteria (including *Staphylococcus*, *Listeria*, and *Bacillus*), but the energy for cadmium efflux in Gram-negative bacteria is chemiosmotic and the system appears to be of independent evolutionary origin. There is even a well-described bacterial metallothionein, found so far only with chromosomal genes of some cyanobacteria, and conferring resistances to Cd^{2+} and Zn^{2+} [24,28].

PLASMID MERCURY RESISTANCE

Highly homologous plasmid systems for resistances to inorganic and organic mercury have been found on plasmids of Gram-negative and Gram-positive bacteria. In a collection of some 800 plasmids that had been mobilized from various Gram-negative bacteria into *E. coli*, 25% expressed mercury resistance [77]. The mercury-resistance determinants from several different sources have been actively studied and more than ten *mer* operons have been sequenced either partially or in total (see refs. [52,82,83,96] for general reviews and specific citations). Mercury-resistance (*mer*) operons are divided into two groups: those that confer resistance to both organic and inorganic mercury (broad spectrum) and those that confer resistance only to the inorganic mercuric ion (narrow spectrum) [77]. In Gram-negative bacteria, the order and approximate functions of the *mer* genes are the same [82] (Fig. 1), with the exception of *mer* operon from *Thiobacillus ferrooxidans*.

These mercury-resistance systems start with a regulatory gene, *merR*, whose product is a unique positively-acting activator protein. The MerR protein contains a helix-turn-helix (HTH) DNA binding motif and binds as a homo-dimer to the operator DNA region, repressing the expression of its own as well as other *mer* genes. In the presence of mercuric ions, the MerR-Hg^{2+} complex (one Hg^{2+} per MerR dimer) twists open the operator DNA region allowing RNA polymerase to synthesize mRNA [62,88]. Ralston and O'Halloran [67] showed an unusual apparently 'cooperative' kinetics for Hg^{2+} induction of *mer* operon with an 'apparent Hill coefficient' of 2 to 3, although a single Hg^{2+} cation is known to bind cooperatively to two cysteines on one subunit and a third cysteine on the second subunit of the homo-dimer (as diagrammed in Fig. 1; with evidence summarized by O'Halloran [62] and Summers [88]). In the *mer* systems in Gram-negative bacteria, *merR* is transcribed separately and in the opposite direction from the remaining *mer* genes, allowing more complete repression of the *mer* operon in the absence of mercury than with Gram-positive bacteria, with which *merR* is the first gene on the multi-gene *mer* operon (Fig. 1), which therefore must function at a low level even in the absence of mercury.

Among the sequenced Gram-negative *mer* operons, only pDU1358 contains the gene that encodes organomercurial lyase, *merB*, that thus confers resistance to organomercurial compounds. The pDU1358 MerR is a 'broad spectrum' activator, as well, since it responds to inorganic Hg^{2+} and also to organomercurial compounds (such as phenylmercuric acetate) as inducers [61,106]. Amino acid sequences among known Gram-negative MerRs are quite similar with the exception that the pDU1358 MerR has C-terminal 10 amino acid residues that are completely different from those of others. Deletion mutation analysis revealed that these ten amino acids were necessary for pDU1358 MerR to respond to organomercurials but not to Hg^{2+} [61]. In vitro runoff transcription experiments showed that Hg^{2+} and phenylmercuric acetate function directly as inducers in the presence of purified pDU1358 MerR and *E. coli* RNA polymerase to activate the *mer* operon transcription [106].

In addition to the *merR* regulatory gene, in four of the Gram-negative *mer* operons (again the exception is the environmentally important *Thiobacillus ferrooxidans*), there is a second regulatory gene, *merD*. The predicted MerD amino acid sequences are highly homologous with those of MerR,

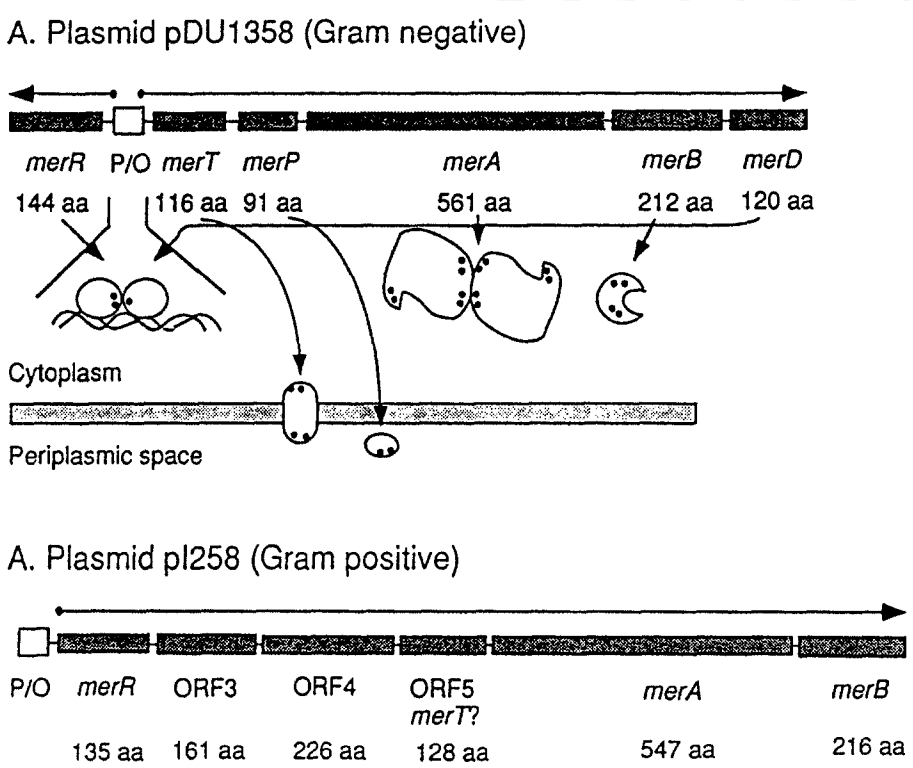

Fig. 1. Genes and polypeptides of mercury resistance systems of plasmid pDU1358 from Gram-negative bacterium *Serratia* spp. and plasmid pI258 from Gram-positive bacterium *S. aureus* [modified from refs 81, 82 and 83]. aa, length of gene products in amino acids. P/O, promoter/operator region. Dots (•) indicate positions of cysteine residues, except for the MerR homo-dimer, where the cooperative binding by two cysteines on one subunit with a third cysteine on the second subunit is indicated.

especially in the helix-turn-helix region. In vivo complementation analysis showed that MerD functioned as a trans-acting regulator [52,54] and in vitro experiments (gel retardation and footprinting) with purified pDU1358 MerD showed that it binds specifically to the same *mer* operator region and protects the same nucleotide residues as does MerR [54]. However, the merD protein binds to the *mer* operator with a much lower affinity than MerR [54], and only low levels of MerD proteins are synthesized.

The remaining genes of the Gram-negative *mer* operons are transcribed as a single mRNA in the opposite direction from *merR*. The first two genes, *merT* and *merP*, encode proteins involved in mercuric ion transport [25,49,52,82]. MerP is a Hg^{2+} binding protein (one Hg^{2+} per MerP monomer [72], utilizing a vicinal cysteine pair) located in the periplasmic space. MerP functions as a 'shuttle', delivering Hg^{2+} to the MerT inner membrane protein [52,82]. MerP mutants that have lost one or the other cysteine residue have reduced mercury binding [73]. Most interesting is the three-dimensional structure of MerP which has been determined by proton NMR [21]. This structure shows the small polypeptide consists of four β-sheet regions plus two α-helices. The oxidized cystine in the structure as determined shows that Cys_{14}-Ala-Ala-Cys_{17} occurs in a loop between the first β-sheet and the first α-helix. This structure becomes important (below) in consideration of comparable heavy metal-binding motifs in mercuric reductase and in the cadmium and copper translocating P-type ATPases.

MerT has two vicinal cysteine pairs. It has been postulated that one vicinal cysteine pair faces the periplasmic space and the other faces the cytoplasm. Hg^{2+} is released from MerP to the periplasmic cysteine pair of MerT and then transferred to the cytoplasmic pair, and (it is further hypothesized) handed subsequently to mercuric reductase in the cytoplasm (Fig. 1) [52,82].

The Gram-positive Hg^{2+} transport genes have not been as well defined. Three open reading frames in *S. aureus* plasmid pI258 *mer* operon between *merR* and *merA* (Fig. 1) appear to be involved in Hg^{2+} transport but the three gene products have not been directly identified [42,81]. Based on computer modeling analysis of the predicted amino acids sequences, these are likely to be membrane proteins. The predicted ORF3 product has a long hydrophobic region (a possible membrane leader sequence) followed by a more hydrophilic region containing three cysteines. The ORF4 product is very hydrophobic and has six possible transmembrane spans. The ORF5 product has two possible transmembrane domains and one vicinal cysteine pair on each side of the membrane. It has been proposed that ORF5 is the Gram-positive equivalent of MerT, based on a weak sequence homology between them [42,81].

The *merA* gene encodes a NADPH-dependent FAD-containing mercuric reductase, reducing intracellular Hg^{2+} to Hg^0, which may freely diffuse out from the cells [52,82,96]. Mutagenesis analysis of MerA and both in vivo and in vitro biochemical studies of mutant proteins revealed the need for two vicinal cysteine pairs, at the redox active site (Cys_{135}–Cys_{140}) and at the carboxyl end (Cys_{558}–Cys_{559}) [19]. These

four cysteines are conserved in all known MerA sequences [see refs. 52 and 79 for citations]. Mercuric reductase from a Gram-positive *Bacillus merA* gene has been crystallized and the structure was solved [75]. The structure is remarkably similar to that of human glutathione reductase, except for the N-terminal 160 amino acid residues, which do not occur in glutathione reductase. These 160 amino acids lack a fixed position in the crystal [75] and have been postulated [e.g. 82,83] to function much like a baseball mit, taking Hg^{2+} from MerT and passing it on to the vicinal cysteine pair at the carboxyl end of the reductase polypeptide. This amino-terminal region is homologous with MerP and contains a third vicinal cysteine pair [52,82,83]. The MerA protein functions as a dimer containing one NADPH and one FAD in each subunit. Hg^{2+} binds to the MerA dimer using four cysteine sulfurs (Cys_{135}– Cys_{140} from one subunit and Cys_{558}–Cys_{559} from the other) [19,52,75,83].

The three sequenced *mer* operons from Gram-positive bacteria and one from a Gram-negative (plasmid pDU1358) have *merB* genes determining the enzyme organomercurial lyase (that cleaves the C–Hg bond to release Hg^{2+} which is subsequently reduced by the mercuric reductase) following *merA*. A reaction mechanism has been proposed [79,96] based on mutagenesis and biochemical studies of the organomercurial lyase by Begley et al. [2] and Walts and Walsh [100]. In brief, one cysteine is initially used for organomercurial substrate binding. Following a S_E2 reaction, the C–Hg bond is broken and a C–H bond is formed. Hg^{2+} binds to a second cysteine residue, and is then released from the enzyme to a still-unidentified thiol compound (generally glutathione in vitro but perhaps the mercuric reductase enzyme itself in vivo).

Not all known *mer* operons have exactly the same gene number and organization. Some Gram-negative *mer* systems have an additional gene, *merC*, which is involved in membrane transport of mercury [39]. And among the ten complete *mer* operons sequenced so far, the one from the *T. ferrooxidans* chromosome is an overall exception. Instead of being organized contiguously, like the other *mer* operons, the *Thiobacillus* system has the *merA* gene at one chromosomal locus, contiguous with one *merC* gene [30], and then in a different chromosomal locus, there are two *merR* genes, each with additional (and functional) *merC* genes, together with partial (non-functional) *merA* genes. Both *Thiobacillus merR*s are functional and control the expression of the *merC* and the partial *merA* as well as the contiguous *merC merA* region [30].

ARSENIC RESISTANCE

Plasmid-mediated arsenic-resistant bacteria have been widely found in various sources [9]. Four of these systems have been cloned and sequenced to date. Closely related arsenic resistance systems are found on plasmids and the chromosome of *E. coli* and plasmids of *Staphylococcus* [69–71,84]. However, the number of genes is different (Fig. 2). The *ars* operon from *E. coli* plasmid R773 consists of total five genes, *arsR*, *arsD*, *arsA*, *arsB* and *arsC* in order [13,74,103], but only three genes (*arsR*, *arsB* and *arsC*) were found from the *ars* operons from *S. aureus* plasmid pI258, *S. xylosus* plasmid pSX267 [33,71], and the *E. coli* chromosome (NCBI accession #U00039). The *E. coli* chromosomal system appears to be functional, since insertions in the *arsB* gene led to increased sensitivity to arsenic (M.S. Dubow, personal communication). Furthermore, disruption of the *arsR* gene upstream from an *arsB-lux* fusion resulted in constitutive light emission, rather than *lux* expression inducible by arsenic and antimony salts (M.S. Dubow, J. Bacteriol., in press). This latter result again demonstrates that ArsR is a repressor of transcription. Several summaries of understanding of plasmid-mediated arsenic resistance have appeared recently [9,37,68–70,80,84]. A diagram of the relationship between the *E. coli* plasmid and chromosomal systems is shown for the first time in Fig. 2.

The *ars* operons are transcribed as a single polycistronic mRNA [33,37], regulated by the *arsR* gene which encodes a trans-acting repressor protein, and can be induced by arsenate, arsenite, antimonite and bismuth in vivo [33,71,102; Chen, Y. and S. Silver, unpublished]. In vitro gel retardation and footprinting analyses show that the *E. coli* ArsR protein forms a dimer and binds to a short DNA region of imperfect dyad symmetry just upstream of the *ars* mRNA initiation site [102]. The ArsR proteins belong to a newly recognized family of metal-responsive regulatory proteins [1,70], which is rather different from other DNA binding proteins. Bairoch [1] proposed a helix-turn-helix for ArsR, although the usual modelling algorithms did not recognize this. The hypothesized helix-turn-helix region contains two cysteine residues. Recent site-directed mutagenesis analysis of the *E. coli* ArsR revealed that both cysteines were necessary for inducer binding, but not for DNA binding [78].

In addition to *arsR*, the plasmid R773 *ars* operon has a second regulatory gene, *arsD*, that is missing in *S. aureus*. ArsD has not yet been shown to bind to the promoter region, and the protein contains no recognized DNA binding motif. However, in vivo genetic analysis showed that the ArsD protein is an inducer-independent trans-acting regulatory protein which controls the highest level of expression of the *ars* genes, rather than being needed for regulating low level *ars* gene expression [103].

Following *arsD*, there is the *arsA* gene in the plasmid R773 *ars* operon, but missing from both staphylococcal plasmids [33,71] and the *E. coli* chromosomal system. The *arsA* gene encodes an ATPase subunit, as inferred initially from sequence homologies within the ATP-binding regions with other ATP-binding proteins [13]. ArsA contains two ATP-binding sites and it appears to have evolved by duplication and fusion of 'half-genes' with single ATP-binding motifs. Both sites are necessary for resistance and ATPase activity [38]. The purified ArsA protein forms a dimer (presumed to be the active form) and was shown to be an arsenite- or antimonite-stimulated ATPase, tightly bound to the ArsB membrane protein [37,69,70,84].

The *Staphylococcus* and *E. coli* plasmid *arsB* genes both encode 429 amino acids long integral membrane proteins. The *E. coli* plasmid R773 ArsB has 12 transmembrane spans [104] and is the membrane anchor for the ArsA ATPase subunits [18]. The ArsB proteins of *E. coli* and *S. aureus* plasmids have 58% identical amino acid residues, and the homology is uni-

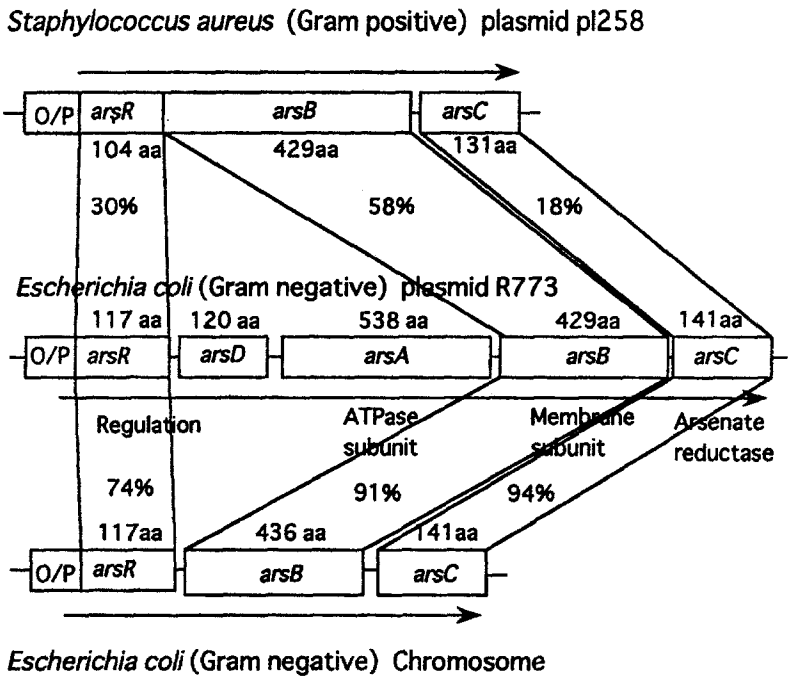

Fig. 2. Genes and products for arsenic resistance in *S. aureus* and *E. coli*. Alignment and functions (below) of arsenic resistance genes (boxes) with lengths of gene products in amino acids (aa) (above and below genes) and percent identities of amino acid products (between) (redrawn from ref. 84, with addition of the chromosomal *E. coli* system).

form throughout the entire protein length. In fact chimeric proteins that contain partial *E. coli* and partial *S. aureus* ArsB sequences are functional and confer resistance, although there are small differences as to which combinations provide stronger resistance [20]. The *S. aureus* ArsB alone is responsible for arsenite resistance and efflux with membrane potential as energy source [3]. Coexpression of the *E. coli* ArsA with the *S. aureus* ArsB dramatically increased the level of arsenite resistance, suggesting a direct physical interaction between these two proteins [3], one from a Gram-negative bacterium and the other from a Gram-positive bacterium. In contrast, the *E. coli* plasmid R773 ArsB functions with obligatory ATP-coupling in the presence of the ArsA protein, but as a membrane potential-driven secondary pump in the absence of ArsA, as with the *S. aureus* ArsB [70].

The last gene in all four operons (*arsC*) encodes an arsenate reductase enzyme (131 amino acid residues for *S. aureus* and 141 for *E. coli*), that reduces intracellular arsenate to arsenite, which is extruded from the cells by the pump [34]. Although the ArsC proteins of Gram-positive and Gram-negative bacteria have only about 18% identical amino acids, the plasmid R773 and pI258 proteins both were shown to reduce arsenate to arsenite in vivo [34], and in vitro. Purified ArsC enzymes show no activity by themselves, but need coupling proteins to carry out the reduction reaction. A striking difference between ArsC proteins is in the energy-coupling systems. For the *S. aureus* enzyme, coupling is with thioredoxin (the small intracellular protein that functions as a general disulfide-reducing agent) both in vivo and in vitro [34,35]. In contrast, the *E. coli* plasmid R773 enzyme coupled with glutaredoxin (a protein similar to thioredoxin, but with sometimes different substrate specificity; for some reductases, these two coupling proteins are exchangeable) [22].

A tentative model (Fig. 3) for the reaction pathway of AsO_4^{3-} reduction to AsO_2^- by arsenate reductase is presented here, based on theoretical arguments and new unpublished experiments (E. Garber and S. Silver, unpublished). Two of the four cysteines of the pI258 polypeptide sequence are postulated to be involved (Cys_{10} and Cys_{82}). The model starts with an 'open' form of the arsenate reductase monomer. Upon interacting with the tetragonal oxyanion arsenate, arsenate reductase undergoes a conformational change to a 'closed' form. A change in CD spectrum on binding oxyanion has been measured (E. Garber, unpublished). The bound arsenate (possibly via hydrogen bonds analogous to those used in phosphate binding by the phosphate-binding protein PstS [48]) is hypothesized to undergo an S_N2 displacement reaction in which a cysteine thiol displaces a water molecule (Fig. 3(B, C)). This would result in an arsenic thiol ether linkage. The second cysteine would form a disulfide with the first, concurrent with the two-electron reduction of tetragonal arsenic(V) (arsenate) to trigonal arsenic(III) (arsenite) (Fig. 3(C, D)). The formation of the cystine disulfide protects the protein from attack by reactive arsenite. After release of arsenite (Fig. 3(D, E)), reduced thioredoxin converts the cystine to cysteines (Fig. 3(E, F)). This model is tentative, and represents a current interpretation of the data. It should be viewed as a basis for further experiments.

It was reported that the *E. coli* R773 *ars* operon also confers resistance to tellurite [95]. The three structural genes (*arsABC*) are required for tellurite resistance and efflux. However, no tellurite resistance was detected with *S. aureus* cells

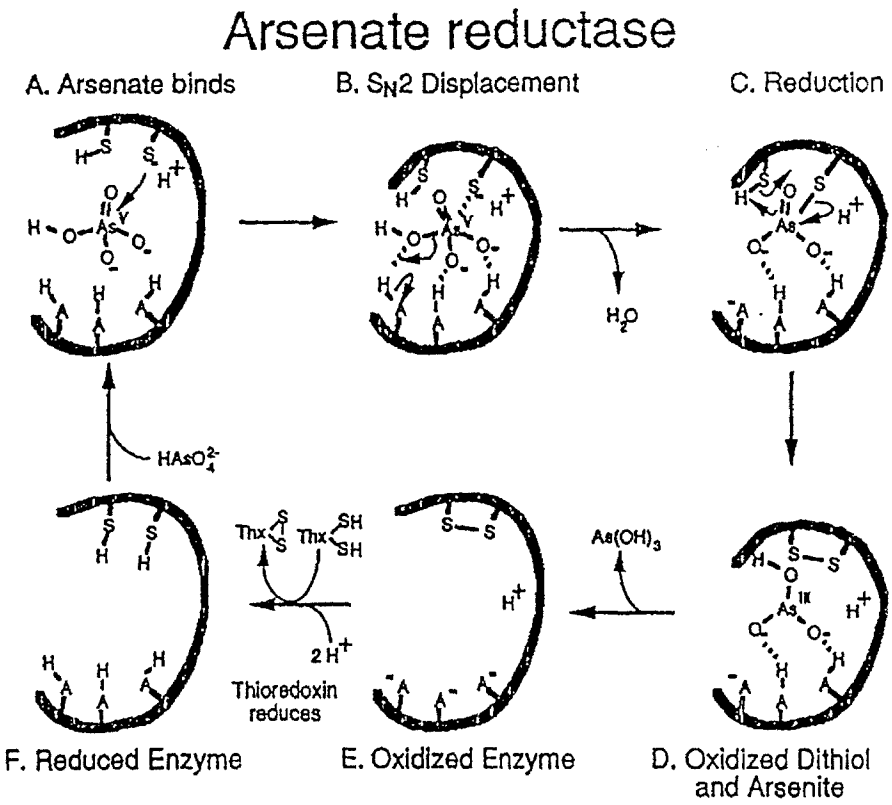

Fig. 3. Proposed reaction mechanism for arsenate reductase. (A) Resting enzyme, with two active site cysteines, three hydrogen-bond donors (analogous to those for phosphate binding of the phosphate binding protein), and tetragonal $HAsO_4^{2-}$. (B) Hydrogen binding of arsenate oxyanion and formation of 'donor' thio–ether bond. (C) Two electron transfer from one cysteine to arsenate, forming (D) trigonal arsenite and oxidized dithiol. From step B to C a water is released; from step D to E, arsenite is released. Trx, thioredoxin. (E. Garber and S. Silver, unpublished).

containing the pI258 *ars* operon (Ji and Silver, unpublished results).

COPPER RESISTANCE IN GRAM-NEGATIVE BACTERIA

To date, strong copper resistance has been described only with plasmids in *Pseudomonas* and *E. coli* and on the chromosome in *Xanthomonas*. The systems are homologous and contain basically the same genes [4,5,16,51,82,85] (Fig. 4).

There are four structural genes in both the *cop* operon in *Pseudomonas* and *pco* in *E. coli*. Three of the *cop* gene products have been isolated. CopA and CopC are periplasmic copper-containing proteins that are abundantly synthesized in Cu^{2+}-induced resistant cells [11]. They contain 11 Cu^{2+} per CopA polypeptide and 1 Cu^{2+} per CopC, respectively. Unlike some transition-cation binding proteins (such as MerP and MerA of the mercury resistance systems, the CadA Cd^{2+} efflux ATPase and the human Menkes and Wilson disease ATPases (see below), all of which have cation binding motifs (Cys-Xaa-Xaa-Cys)), the CopA protein has four direct repeats (and once in CopC) with a consensus sequence Asp-His-Ser/Gly-Xaa-Met-Xaa-Gly-Met and one cysteine containing motif at its C-terminus. These have been proposed as the Cu^{2+}-binding motifs. Other types of presumed Cu^{2+}-binding motifs were also identified in CopA [4,16]. Related consensus sequences were found in the *E. coli* PcoA and PcoC amino acid sequences. CopB (and PcoB) is a transmembrane protein located in the outer membrane and is also a Cu^{2+}-binding protein [11]. CopB has five potential Cu^{2+}-binding motifs with a consensus sequence similar to that of CopA (and PcoA). However the Cu^{2+}-binding capacity of CopB is not known. CopD (and PcoD) has been hypothesized to be an inner membrane protein. Mutagenesis analysis showed that deletion of *copA* resulted in hypersensitivity to Cu^{2+}, suggesting that CopD together with CopC is involved in Cu^{2+} transport, as with the MerP/MerT Hg^{2+} transport system [12].

The *cop* operon-mediated Cu^{2+} resistance results from high Cu^{2+} accumulation in the periplasmic space between inner and outer cell membranes [4,11,16]. *Pseudomonas* cells carrying *cop* operon form blue colonies when grown on Cu^{2+}-containing agar plates [16]. It has been proposed that when excess Cu^{2+} ions are present in the medium, copper is bound and transported into the periplasm by CopB protein, and trapped there by binding to CopA proteins, CopC and CopD may be involved in Cu^{2+} transport from the periplasmic space into the cytoplasm. Why this plasmid-mediated transport system is needed is not known. The *pco*-determined Cu^{2+} resistance mechanism in *E. coli* is not clear. Pco has been reported to result in lowered rather than increased cellular copper content [4,5]. It seems likely but difficult at this stage to understand how both systems function in a similar matter. Furthermore, when *E. coli* cells containing the *pco* operon grow on Cu^{2+}-containing plates, a brown precipitate is formed around the

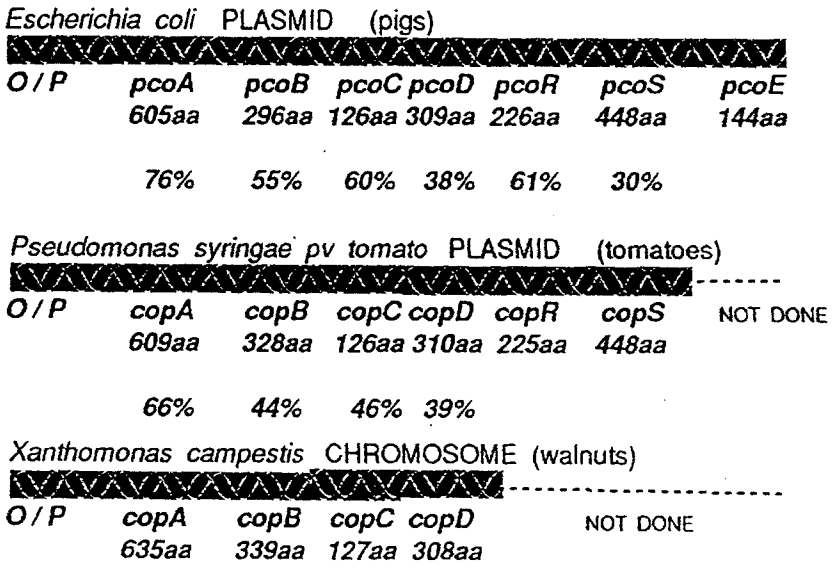

Fig. 4. Genes and products of the plasmid copper resistance system of *Pseudomonas syringae* (isolated from copper-treated tomatoes), compared with that of *E. coli* (from piglets) and *Xanthomonas campestis* (from walnut trees). The numbers are percentages amino acid identities between gene products (from ref. 85, with permission).

bacterial colonies instead of blue colonies that resistant *Pseudomonas* cells form [4,16]. The chemical nature of this brown precipitate is not known.

The two remaining genes in both operons, *copS* and *copR* (or *pcoS* and *pcoR*), are involved in regulation. The gene products (from their predicted amino acid sequences) probably comprise a two-component regulatory system with CopS (PcoS) acting as a membrane-bound cation-sensor and CopR (PcoR) functioning as a DNA-binding transcriptional regulatory protein [4,85], as is the case with well-studied two component regulatory systems. In addition to the genes described above, there is an additional gene (*pcoE*) downstream of *pcoS* in the *E. coli* system. Transcription of *pcoE* is controlled by Cu^{2+}, but there is no evidence concerning what the PcoE function might be.

CADMIUM RESISTANCE IN GRAM-POSITIVE BACTERIA RESULTS FROM A P-TYPE ATPase

The 727-amino acid long Cd^{2+} efflux P-type ATPase (CadA) from staphylococcal plasmid pI258 (Fig. 5) was the first example sequenced of a system now known to occur widely in Gram-positive bacteria, including *Bacillus*, *Staphylococcus* and *Listeria* [32,40,41,60,82,86,87]. The ATPase protein is inducibly synthesized when resistant cells are exposed to Cd^{2+}; it extrudes intracellular Cd^{2+} out of the cells using ATP as energy source [93,105]. The predicted CadA amino acid sequence contains several domains which are homologous with other P-type ATPases. This enzyme has been discussed in several reviews recently [82,85,86].

A basic model of CadA was proposed [82] (Fig. 5). In this model six transmembrane α-helical regions were hypothesized, including two (from amino acid position 336 to 356 and from 464 to 484 as indicated in Fig. 5 as segments 3 and 4) that may be part of a Cd^{2+} transport channel. In segment 4, there is a proline residue (position 372) which is conserved in all P-type ATPases. Pro_{372} is flanked by two cysteines (Cys_{371} and Cys_{373}). There are three large cytoplasmic domains. The first one (position 1 to 105) may be involved in Cd^{2+} binding, since it is this region that is homologous with MerP and MerA of the mercury resistance determinants. This region contains a Cys-Xaa-Xaa-Cys consensus sequence, which has been proposed to be a metal-binding motif [83,86,87]. An unpublished *S. aureus* chromosomal CadA has two such Cd^{2+}-binding motifs (as an N-terminal repeat of 70 amino acids) (Dubin, D.T., personal communication). The second cytoplasmic domain (Fig. 5) consists of approximately 190 amino acids and may function both as a 'funnel' to move Cd^{2+} from the initial binding site to the membrane surface and as a phosphatase to remove phosphate from Asp_{415}-P_i. The third cytoplasmic domain contains an ATP-binding motif (Lys_{489}-Gly-Ile-Val_{492}) and a heptapeptide aspartyl kinase (Asp_{415}-Lys-Thr-Gly-Thr-Leu-Thr_{421}) that is highly conserved in all P-type ATPases. The aspartate residue is absolutely conserved in this class of proteins and is phosphorylated by ATP to form a phosphorylated intermediate Asp_{415}-P. The phosphorylated CadA intermediate has been directly demonstrated [92].

It is interesting to note that the newly cloned and sequenced human Menkes [6,87] and Wilson's [6,7] disease 'candidate genes' show amino acid sequences predicted from the cDNA sequences more closely homologous to the bacterial P-type cadmium ATPases than to other P-type ATPases of eukaryotes [see refs. 6, 83, and 87 for literature citations]. Both Menkes and Wilson's disease result from defects in human Cu^{2+} metabolism, but the diseases result from defects in different human tissues [17]. The expression of the newly-identified genes also differ (with the 'Menkes gene' expressed in most body tissues, except in the liver, and the 'Wilson's gene' expressed primarily in the liver) in a pattern consistent with Menkes being a disease of initial copper uptake and distri-

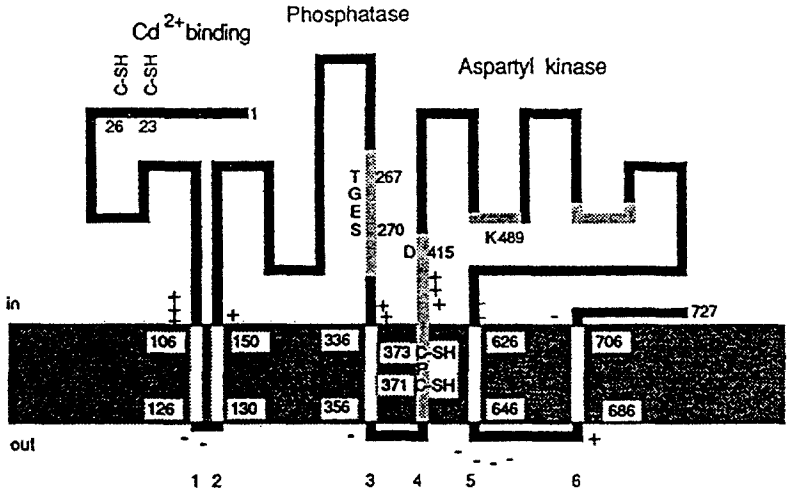

Fig. 5. The cadmium-resistance ATPase of *S. aureus*. The predicted motifs (Cd^{2+}-binding, phosphatase, membrane channel, and aspartyl kinase) regions are shown with key predicted amino acids (from ref. 82, with permission).

bution, and Wilson's being a disease resulting from an inability of the liver to process copper normally. Both proteins are P-type ATPases and they share about 70% identical amino acid residues. The Menkes and Wilson's proteins are probably Cu^{2+} transport ATPases. Like the CadA, they have a conserved sequence (Cys-Pro-Cys) in the equivalent transmembrane segment which may form the Cu^{2+} transport pathway. Instead of one metal binding motif (Cys-Xaa-Xaa-Cys) as in the pI258 CadA Cd^{2+} efflux ATPase, the predicted Menkes and Wilson's ATPases have six repeats of a homologous 30 amino acid segment within the first 600 amino acids of the proteins. These 'motifs' are postulated to be involved in the initial Cu^{2+} binding. It is apparent that studies of bacterial heavy metal resistance mechanisms will provide clues useful for understanding of human diseases.

CADMIUM RESISTANCE IN GRAM-NEGATIVE SOIL BACTERIA RESULTS FROM A MULTI-PROTEIN NON-ATPase EFFLUX SYSTEM

The large plasmids of the soil chemilithotroph *Alcaligenes eutrophus* have numerous heavy metal resistance determinants [in strain CH34, there are two plasmids with three determinants for mercury resistance that are related to those described above, and others for chromate resistance (see below) and for resistances to divalent cations (two systems) called Czc (for Cd^{2+}, Zn^{2+} and Co^{2+} resistances) and Cnr (for Co^{2+} and Ni^{2+} resistances)]. More recently, a third *Alcaligenes* divalent cation resistance system, called *ncc* for nickel, cadmium and cobalt resistances, became available [76]. This sequence is most closely similar to that of *cnr* (Fig. 6). And finally, a sequence referred to as *helABC*, for *h*emolysin *e*fflux from *Legionella* [50] was determined. The HelA, B and C protein sequences are closer to those of CzcA, B and C than to those of Cnr (Fig. 6). Why a determinant of virulence in the pathogen *Legionella pneumonia* would be related to heavy metal efflux systems in soil microbes is not clear. Mutations in *cnrY* result in constitutive expression of the *cnr* operon, and also in additional Zn^{2+} resistance [15], again showing that the Czc and Cnr systems are basically very similar.

The Czc system, which confers resistance to Cd^{2+}, Zn^{2+} and Co^{2+}, has been studied in most detail and functions as a cation/proton antiporter (and not an ATPase), effluxing cations from the cells [56,58,59]. The *czc* operon of *A. eutrophus* plasmid pMOL30 consists of three structural genes: *czcA*, *czcB* and *czcC* whose products form a complex membrane cation efflux pump. Additional genes (*czcR* (or *czcN* and *czcI*) and *czcD*) are involved in *czc* operon expression (Fig. 6) [56–59].There is not current agreement between workers on the number and nature of the regulatory genes (see reports in this issue by Nies and by Mergeay for more specifics). Both *czcA* and *czcB* genes encode membrane proteins. The CzcA protein is considered to be the inner membrane anti-porter cation carrier, while CzcB is considered to have an ancillary role in transport. Deletion mutations in *czcA* result in loss of resistances (and efflux) to all three cations, whereas deletion of *czcB* results in complete loss of resistances to Cd^{2+} and Zn^{2+}, and only partial loss of Co^{2+} resistance. When the *czcC* gene was eliminated, the cells retained full resistance to Zn^{2+} and partial resistance to Co^{2+}, but were sensitive to Cd^{2+} [59]. The CzcC protein is now thought likely to be an outer membrane protein (Fig. 6) based on analysis by Dong and Mergeay (see their paper in this volume). The evidence for this requires using a potential ATG start codon that would add 73 amino acids to the sequence proposed by Nies et al. [59]. This would add a candidate leader sequence and make CzcC similar to the sequences proposed for CnrC, NccC and HelC (Fig. 6). For HelC, the existence of a HelC-β-lactamase fusion that gives ampicillin resistance indicates that HelC must lie outside of the cytoplasm [50]. In summary, CzcA is likely to be the primary pump protein inefficiently transporting Co^{2+}; both CzcB and CzcC proteins function as substrate range modulators. CzcB may funnel cations across the periplasmic space (Dong and Mergeay, personal communication) so that free periplasmic cation is not released. The addition of CzcC to the CzcAB complex extends the substrate range to include Cd^{2+}

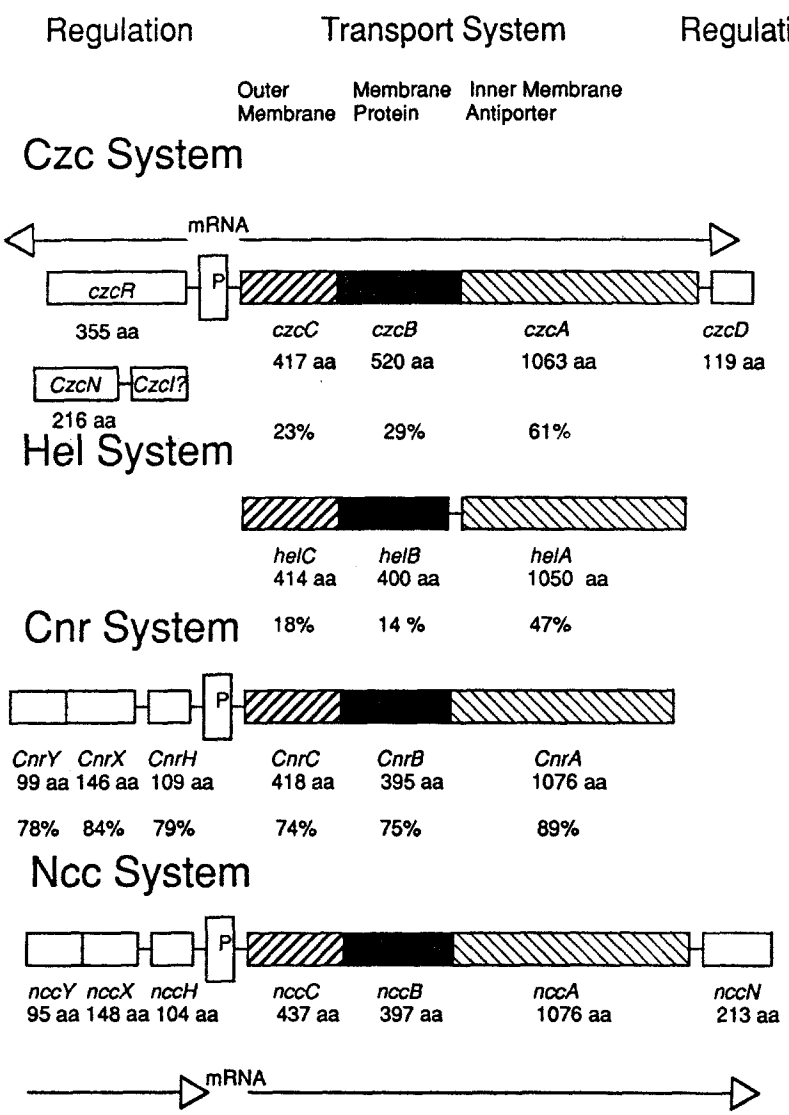

Fig. 6. The Czc [cadmium, zinc and cobalt; 57,59], Ncc [nickel, cadmium and cobalt; 76; NCBI accession number L31363] and Cnr [cobalt and nickel resistance; 43; NCBI accession number M91650] systems of *Alcaligenes* plasmids and the new HelA [*he*molysin release from *Legionella*; GenBank accession U11704; 50] systems. Arrangement, percent identities of predicted amino acid products and predicted functions of the gene products are given. Whereas the three structural genes (*A*, *B* and *C*) are closely homologous in all four systems, the number and existence of regulatory genes is currently less clear.

and leads to efficient transport across the outer membrane [56,59,82]. The assignment of cellular locations and functions to the CzcB and CzcC families of proteins is very new and heavily dependent upon sequence homologies rather than direct experimentation. Thus, these ideas must be considered tentative.

The expression of *czc*, *cnr* and *ncc* operons is regulated, but the full number of regulatory genes involved is unclear and available evidence confusing. The first regulatory gene identified was *czcD* [56,59] (Fig. 6), which is thought to encode a membrane protein that is a cation sensor. Nies [57] then sequenced the region upstream of *czcCBAD* and proposed an additional regulatory gene, called *czcR* (Fig. 6), which might function as a transcriptional activator. However, Mergeay and coworkers (Mergeay, M., personal communication) have analyzed the same region and found two potential reading frames upstream of *czcC* (*czcN* and *czcI* in Fig. 6) that may be involved in operon regulation. CzcN may be a transmembrane protein that could play a sensing role in regulatory response to Cd^{2+} and Co^{2+} similar to that proposed for CzcD. The new Ncc system contains an open reading frame closely homologous to CzcN, but at the other end of the determinant and with the opposite orientation (Fig. 6). Further complicating our current understanding, the Cnr and Ncc systems have unrelated (to Czc) regulatory genes called *cnrY*, *cnrX* and *cnrH* (or *nccY*, *nccX* and *nccH*) [43,76]. The *cnrH* gene is considered to produce an activator protein, that from sequence homologies may be a member of a new class of sigma factor components of RNA polymerase that regulate a range of extracellular functions [76]. However, the data for this hypothesis are only starting to be available. How these closely related systems function with fundamentally different regulatory genes is not resolved.

The *cnr* operon from *A. eutrophus* plasmid pMOL30 also functions by energy-dependent cation efflux [56]. Three structural gene products predicted from DNA sequence (CnrCBA) are homologous with those of CzcCBA (Fig. 6), suggesting both systems function fundamentally in a similar manner. Recently, still another related operon (*ncc*) has been cloned and sequenced from an *A. xylooxidans* plasmid [76]. This *ncc* operon confers resistance to Ni^{2+}, Cd^{2+}, and Co^{2+}, and consists of seven genes (Fig. 6). Six gene products that are predicted from the DNA sequence show high homologies with *cnr* gene products and the last gene (*nccN*) product appears closely homologous to the newly proposed [76; Dong and Mergeay, personal communication] *czcN* gene product (Fig. 6).

CHROMATE RESISTANCE AND CHROMATE REDUCTION IN GRAM-NEGATIVE BACTERIA

Both resistance and reduction occur, but resistance to chromate governed by plasmids of Gram-negative bacteria appears to have nothing to do with chromate reduction. Furthermore, it is not clear whether the chromate reduction ability found with several bacterial isolates confers resistance to CrO_4^{2-} or not [63]. Plasmid-mediated chromate resistance is due to reduced cellular accumulation of chromate [55,64]. Whether the reduced accumulation results from accelerated efflux is unknown.

The two sequenced chromate resistance determinants (*chr* operons) from plasmids of *P. aeruginosa* [10] and *A. eutrophus* [55] are quite similar. Both *chr* operons contain the long *chrA* gene. The two predicted ChrA proteins are highly hydrophobic and have 29% identical amino acid residues [10,55]. ChrA was identified as a membrane protein in *E. coli*, although the cloned *chr* operon does not confer chromate resistance in *E. coli* [55]. The actual role of ChrA in chromate transport and the energy coupling remain unknown. In both *chr* operons, an additional open reading frame (intact in *Pseudomonas* but partial in the cloned fragment from *Alcaligenes*) was found. Its possible function or role in chromate resistance is unknown. For the *Alcaligenes chr* operon, there is an additional gene, *chrB*, which is likely to be a regulatory gene involved in *chr* operon expression [8,82].

Bacterial reduction of chromate [Cr(VI)] to Cr(III) has been observed with numerous Gram-positive and Gram-negative bacteria, under both aerobic and anaerobic conditions. However, the enzymes carrying out chromate reduction have not been characterized in much detail [8,63]. Chromate reduction activity can be associated with either the cytoplasmic fraction [31,90] or cell membranes [101]. Ishibashi et al. [31] preliminarily characterized a soluble chromate reduction activity from a *P. putida* strain and found the reduction activity was NAD(P)H-dependent. A cell-free extract from *Streptomyces* also reduces Cr(VI) to Cr(III) in the presence of NAD(P)H. The chromate reductase from *Enterobacter cloacae* strain HO1 has been studied extensively [63]. However, the reductase enzyme has not been purified and the basic reduction mechanism is still unclear [8,63,101]. This *Enterobacter* strain confers resistance to chromate under both aerobic and anaerobic conditions, but chromate reduction occurs only anaerobically [65,66] and the reductase is probably membrane bound [101]. It is believed that *E. cloacae* strain HO1 utilizes Cr(VI) anaerobically as a terminal electron acceptor [63,65,66]. The chromate reductase from *Pseudomonas ambigua* strain G1 was purified and partially characterized [90]. This soluble enzyme has a molecular weight of 25 kDa on SDS-PAGE gels but probably is a dimer or trimer with a molecular weight of 65 kDa on a gel filtration column. The purified *Pseudomonas* enzyme reduces Cr(VI) to Cr(III) in vitro using NAD(P)H as electron donor with Cr(V) as a possible intermediate [90].

TELLURITE RESISTANCE AND TELLURITE REDUCTION IN GRAM-NEGATIVE BACTERIA

Several independent plasmid-governed tellurite resistance determinants have been studied and their DNA sequences have been obtained. Nevertheless, we do not understand the mechanism of tellurite resistance for any of these systems and resistance appears not to involve in tellurite efflux or reduction [44,45,97–99]. Sequencing of the tellurite resistance operons has so far revealed a surprising diversity of gene structures and organization. The first sequenced Te^R operon (*ter*) from *Alcaligenes* plasmid pMER610 consists of five genes, *terA* to *terE* [36]. All five genes are transcribed and translated. Northern blot analysis with different probes showed that the *ter* operon was transcribed into five mRNAs with three possible promoters (two located upstream of *terA* and one before *terD*) [27]. Computer analysis of the TerC amino acid sequence suggests that it is probably a membrane protein. TerD and TerE are related (66% identical amino acids) and likely to have arisen by gene duplication. Mutational deletion of both *terD* and *terE* eliminates tellurite resistance, but a mutation in either single gene results in retained partial resistance, suggesting TerD and TerE are functionally exchangeable [27]. The inducibility of the *ter* operon by tellurite is unclear. The operon appears to be transcribed constitutively but tellurite resistance seems to be inducible.

The second sequenced Te^R determinant (*teh* operon) from the *E. coli* K-12 chromosome (originally reported by error as from *Klebsiella* plasmid pHH1508a) has only two genes (*tehA* and *tehB*) [91,98,99]. TehB is highly hydrophobic and therefore likely to be a membrane protein. The *teh* operon is widely spread in additional wild-type *E. coli* strains [91]. Cells confer resistance to tellurite only when *teh* operon is expressed at high level (as when cloned into high copy number plasmids) [97]. The third Te^R determinant that was cloned and sequenced comes from the IncPα plasmid RK2 [97,98]. Three genes were found, named as *kilA*, *telA* and *telB*. The mnemonic *kilA* refers to a 'kill gene' function where this gene is also involved in maintaining plasmid stability by killing cells that have lost the plasmid; the relationship between this 'kill' function and tellurite resistance is not understood. It is likely that TelA is a cytoplasmic protein and TelB is a membrane protein. All three genes are needed for resistance [97]. Surprisingly, the only difference between Te^R and Te^S variants of plasmid RK2 is a single amino acid change at position 125 of the TelB protein (Ser_{125} results in Te^S and Cys_{125} Te^R) due to a single-nucleotide mutation [97,98]. There are two cysteines in TelB

and both have been hypothesized to lie on the cytoplasmic surface [98]. Transition of Te^S to Te^R with one of the cysteine residues lost indicates an important functional role for this cysteine, but how it is involved is not known.

Many bacteria (including both Gram-positive and Gram-negative species) reduce tellurite to metallic tellurium [97]. Bacteria grown on media containing potassium tellurite form black colonies in which crystals of Te^0 are deposited just inside the cellular inner membrane [97]. Reduction activities of cell-free extracts from several bacteria were observed but not well characterized [97]. A tellurite reductase was purified from a Gram-negative bacterium *Thermus thermophilus* [14]. The purified protein has a molecular weight of approximately 53 kDa and reduces tellurite to tellurium in the presence of NAD(P)H. This protein also reduces selenite and sulfite. Tellurite reduction to tellurium has been proposed as a possible resistance mechanism [44,97]. However, there is no evidence showing the involvement of Te^R gene products in this reduction reaction.

BACTERIAL METALLOTHIONEIN IN CYANOBACTERIA: A NEW SYSTEM

Bacterial metallothioneins, analogous to the small (approximately 60 amino acids long), thiol-rich (perhaps 20 of those 60 amino acids are cysteines) metal-binding proteins of animal systems have not been widely found, although some effort has been made by ourselves as well as others. Metallothioneins of apparently several separate evolutionary origins have been found in many animals, in higher plants, and in lower eukaryotes including yeast and *Neurospora*. In spite of promising reports of metallothionein in *Pseudomonas* [26] (but the protein could not be obtained reproducibly in the original or other laboratories), at this time the only prokaryotic cells with well-studied metallothionein are a few cyanobacterial strains in the genus *Synechococcus* [23,24,28]. This small 58-amino acid-long polypeptide product of the *smtA* gene contains fewer (only nine) cysteine residues than animal metallothionein; the cysteines are clustered in groups of 4 and 5, respectively, as the cysteines in animal metallothioneins are clustered in amino-half and carboxyl-half domains that appear to bind divalent cations independently. Deletion of the *smtA* gene from the chromosome results in hypersensitivity to Zn^{2+} and Cd^{2+} by the *Synechococcus* cells. Animal metallothioneins may play an important role as an intracellular Zn^{2+} homeostasis, and it is possible that cyanobacterial SmtA has a similar role [94]. The details of SmtA-mediated cation resistance are not known, but it is possible that this bacterial metallothionein serves as an intracellular 'sink' for toxic excess cations.

The synthesis of the SmtA metallothionein can be induced by addition of high levels of inducing cation (Zn^{2+}, Cu^{2+} and Cd^{2+}) and is regulated at three levels (Fig. 7): (a) Repression of transcription by the SmtB regulatory protein occurs. *smtB* is constitutively expressed in the opposite direction of *smtA* and the SmtB protein functions as a trans-acting transcriptional repressor to limit expression of the *smtA* gene [53]. Addition

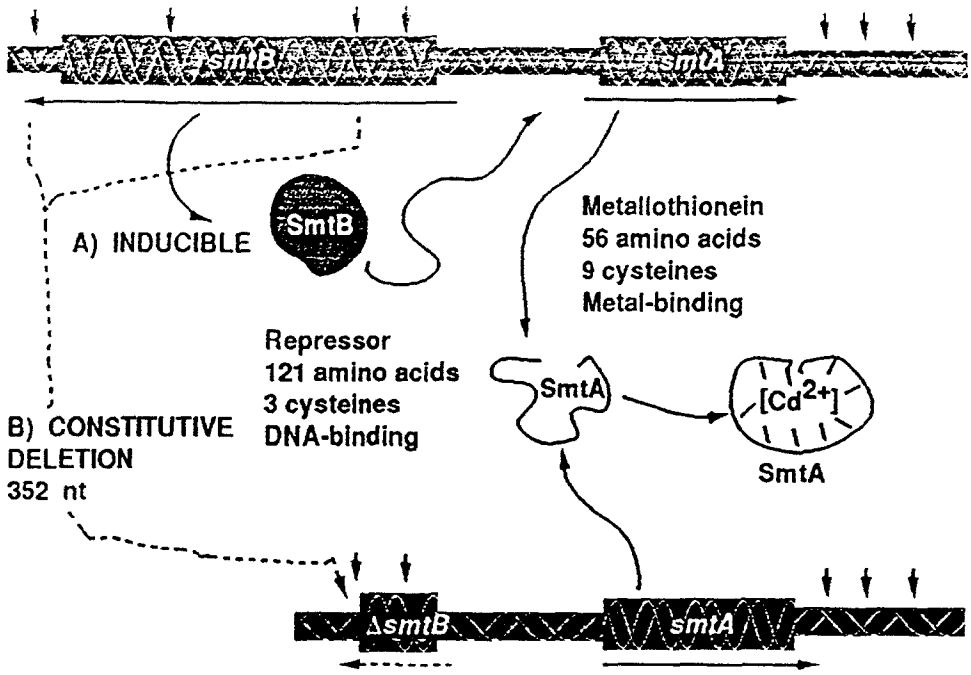

Fig. 7. The cadmium-resistance metallothionein system of *Synechococcus*. (A) Normal, inducible system with divergently transcribed *smtA* (metallothionein) and *smtB* (regulatory) genes. (B) Constitutive metallothionein synthesis after deletion of 352 nucleotides from the first to the third palindrome, from the left as shown). Vertical arrows show the locations of seven 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+}) are shown. This figure summarizes results from N.J. Robinson and colleagues [23,24,28,53]; from [80] with permission.

of high cations functionally inactivates the SmtB protein. (b) Amplification of *smtA* gene copy number happens when the cyanobacteria are grown in high Cd^{2+} concentrations. Transcription from the amplified *smtA* genes results in high cell levels of metallothionein [24]. Finally, (c) deletion of the *smtB* repressor gene upon continuous exposure to high Cd^{2+} levels results in fully constitutive synthesis. Deletion of the *smtB* gene was demonstrated by Gupta et al. [23] and this occurs by removal of 352 nucleotides between the second and the third of the highly iterated palindromic octanucleotide sequence 5′-GCGATCGC-3′ in the *smt* region (Fig. 7). There are seven of these sequences found within or near the *smt* locus (Fig. 7), including three within *smtB*, one upstream of *smtB* and three downstream of *smtA*.

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