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Ion efflux systems involved in bacterial metal resistances

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SUMMARY

Studying metal ion resistances gives us important insights into environmental processes and provides an understanding of basic living processes. This review concentrates on bacterial efflux systems for inorganic metal cations and anions, which have generally been found as resistance systems from bacteria isolated from metal-polluted environments. The protein products of the genes involved are sometimes prototypes of new families of proteins or of important new branches of known families. Sometimes, a group of related proteins (and presumedly the underlying physiological function) has still to be defined. For example, the efflux of the inorganic metal anion arsenite is mediated by a membrane protein which functions alone in Gram-positive bacteria, but which requires an additional ATPase subunit in some Gram-negative bacteria. Resistance to Cd^{2+} and Zn^{2+} in Gram-positive bacteria is the result of a P-type efflux ATPase which is related to the copper transport P-type ATPases of bacteria and humans (defective in the human hereditary diseases Menkes' syndrome and Wilson's disease). In contrast, resistance to Zn^{2+}, Ni^{2+}, Co^{2+} and Cd^{2+} in Gram-negative bacteria is based on the action of proton–cation antiporters, members of a newly-recognized protein family that has been implicated in diverse functions such as metal **r**esistance/**n**odulation of legumes/cell **d**ivision (therefore, the family is called RND). Another new protein family, named CDF for 'cation **d**iffusion **f**acilitator' has as prototype the protein CzcD, which is a regulatory component of a cobalt-zinc-cadmium resistance determinant in the Gram-negative bacterium *Alcaligenes eutrophus*. A family for the ChrA chromate resistance system in Gram-negative bacteria has still to be defined.

INTRODUCTION

About 96% of the dry mass of a living cell is formed by the elements C, O, H, N, P and S. In addition to these non-metals, however, the cell requires metal ions. Metal ions are small, charged entities which are, to give a few examples, needed for redox processes (especially the ions of transition metals like Fe, Cu, and Ni), to stabilize other molecules by electrostatic forces (Mg^{2+} in MgATP, Zn^{2+} in various enzymes and DNA-binding regulatory proteins), to form complex compounds with diverse functions (Fe^{2+}-, Mg^{2+}-, Ni^{2+}- and Co^{2+}-haem, Fe-*S*-cluster), for the regulation of the intracellular osmotic pressure (K^{+}); and, simply but very important, to act as counter-ions for the non-metallic building blocks and metabolites of the cell, which are mostly negatively charged. Thus, a living cell cannot exist without metal ions.

Non-metallic compounds, mostly composed of C, O, H, and N, are synthesized by the cell when needed and degraded when they are not needed. In contrast, metal ions cannot be synthesized or degraded in accordance with current requirements of the cell. Therefore, the intracellular concentrations of essential metal ions may be regulated by sequestration or (with some metals) by changing the oxidation state of the metal. The main process, however, for regulating intracellular concentrations of inorganic cations and anions is by membrane transport systems.

The presence or absence of a metal ion transporter of particular specificity depends on the metal ion, the bacterial species and the physiological status of the cell. Under usual 'housekeeping' conditions, abundant intracellular ions are accumulated by high-rate, relatively nonspecific uptake systems that are synthesized constitutively. These systems are relatively cheap (from the point of view of energetic expenditure) and effective, since several cations or anions are transported by a single system [48,83]. Metal ion starvation induces high-affinity high specificity transporters which require extra energy for synthesis of the proteins, sometimes extra energy for functioning as well (compared to the less specific systems). Metal ion excess (in contrast to starvation) induces synthesis of metal ion efflux systems [48,83]. This review summarizes new understanding of the inorganic ion efflux systems involved in bacterial resistances to the cations and anions of As, Cd, Zn, Co, Ni and Cr.

A continuing theme in the review is that inorganic ion transport systems are providing the 'prototypes' for new 'families' of genes and their protein products. Thus, we cannot avoid a series of neologisms, as the proposed names for these families. Such neologisms are both a plague on unfamiliar readers and a benefit — when any one gains general currency. So this section introduces these exotic terms of membrane transport. Perhaps most familiar are **P-type ATPases** (ATPases forming a phosphorylated intermediate during their catalytic cycle), that were first known from the Ca^{2+} ATPase

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of animal muscles and the Na^+ K^+ ATPase, but are now being found in bacteria as well. The newly-coined family name **ABC transporters** (for ATP binding cassette) is a change from 'periplasmic protein-requiring' transport systems [17]. Other names (below) that may or may not catch on include **MFS** (for major facilitator superfamily) of chemiosmotic antiporters with 12 proposed trans-membrane segments [40], which include efflux systems for antibiotics and sugar-phosphate/inorganic phosphate antiporters. **MFP**, for membrane fusion proteins [72] is clearly going to get confused with **MFS**! The **RND** family [72] of proteins was named for resistances (as the CzcA protein for cadmium, zinc and cobalt efflux was the first member recognized), nodulation and cell division. All three processes appear to require chemiosmotic antiporters that contain more than one type of polypeptide. In this review the 'CDF' family for 'cation diffusion facilitator' is newly defined.

Resistance to arsenical compounds: permeases and missing ATPase proteins

Arsenic is not a metal, but a semi-metal; an element with both metallic and non-metallic properties. However, resistance to arsenical compounds is mediated by systems very similar to metal ion resistance systems; and resistance to inorganic oxyanions of arsenic is treated in this review. The uptake of phosphate (and arsenate) has become the paradigm for the uptake of nutrient and related toxic inorganic ions by bacterial cells. Arsenate uptake (Fig. 1) is always mediated by phosphate transporters [2,19,39,96]. Under abundant phosphate conditions, the high V_{max} but less specific Pit system [14] fulfills the phosphates need of the cell and leads also to arsenate accumulation. Under conditions of phosphate starvation, the more specific Pst system is induced [84]. Pst discriminates between phosphate and arsenate 100-fold better than Pit [62,67,68,95]. Thus, one way for the cell to adapt to arsenate stress is to inactivate the Pit system by a *pit* mutation, which leads to moderate arsenate tolerance due to the discrimination between arsenate and phosphate by the Pst system (Fig. 1).

While the Pit function is encoded by a single gene [64,86,93], Pst is composed of several components (Fig. 1). Phosphate-specificity is determined in the periplasmic space by the phosphate-binding protein, the *pstS* gene product [37,45]. Phosphate uptake by the PstABC complex requires ATP hydrolysis [83]. Thus, Pst is more energy expensive, compared to Pit. Therefore, arsenate resistance resulting from a *pit* mutation is frequently lost, when selection pressure by arsenate is relieved. A more specific way to deal with arsenate in the environment is acquisition of an arsenate efflux pump. Arsenate efflux resistance systems have been intensively studied in the Gram-negative bacterium *Escherichia coli* (Fig. 1) and in two species of the Gram-positive genus *Staphylococcus* (Fig. 2). Plasmid-harbored functions encoded by *ars* determinants in all three bacteria mediate efflux of arsenic in an energy-dependent process [44,78,80], driven in *E. coli* by ATP hydrolysis [65] and in *Staphylococcus* by the membrane potential [3]. The *ars* determinants also govern resistance to antimonite [55] (which appears to be an alternative substrate for the transport system) and to tellurite [89]. Figures 1 and 2 summarize our current knowledge about resistance to arsenate in *E. coli* and in *Staphylococcus*.

Because of the electrochemical gradient across the cytoplasmic membrane, export of any anion has a negative free energy for the bacterial cell. Thus, the cell needs only an arsenic-specific tunnel through the membrane to get rid of the toxic anion. These arsenic-specific tunnels are the products of the *arsB* genes in *E. coli* and *Staphylococcus* [9,22,71]. The two *Staphylococcus* ArsB proteins are nearly identical in amino acid sequence and the sequence similarity between the proteins from the Gram-negative and the Gram-positive bacteria is high (58% amino acid identities) [22,73]. the ArsB protein from *E. coli* (Fig. 1) has a size of 429 amino acids and is a membrane-integral protein with 12 transmembrane spans [100]. Interestingly, the ArsB protein is specific for the arsenite oxyanion [3,22] (and possibly antimonate); the arsenate anion is not a substrate, perhaps because an arsenate-specific tunnel would lead to a leakage of phosphate concentration, possibly Pst system induction, and a very energy-consuming futile cycle of phosphate uptake and efflux.

The solution to the problem of arsenate efflux is the enzyme arsenate reductase, the product of the *arsC* gene [9,22,71]. Arsenate is reduced to arsenite and effluxed through the arsenite-specific ArsB protein. The ArsC reductase catalyzes a thioredoxin- or glutaredoxin-dependent [23,58] reduction of arsenate to arsenite (Figs 1 and 2). Phosphate is not reduced. There is no structurally-related analog of arsenite in the cell; and, therefore, arsenite can be pumped out of the cell easily by the ArsB protein. In *Staphylococcus*, arsenate and arsenite resistance requires only two proteins, ArsB and ArsC [22,71]. In *E. coli*, however, a third structural protein, the ArsA protein [9] is required (Fig. 1). ArsA is an arsenite- (or antimonite-) dependent ATPase [66].

In *E. coli*, the ArsB protein serves as membrane anchor for the ArsA ATPase [85]. The active form of ArsA is a dimer and arsenite (or antimonite) is required in addition to ATP for active dimer formation [20]. Two ATP-binding motifs were identified in the predicted amino acid sequence of the ArsA polypeptide [9] and both sites are able to bind ATP [26,27]. The ArsA dimer (Fig. 1) may have two catalytic units, each composed of the amino-terminal ATP-binding site of one monomer and the carboxyl-terminal ATP-binding of the other monomer [28,29].

Why does *E. coli* have ArsA and *Staphylococcus* not? There are two possibilities: with the membrane potential (external positive), anion export has always negative free energy, and an additional ATPase is not required but might be useful to increase the rate or affinity for arsenite efflux. Alternatively, in *Staphylococcus*, ArsA might be encoded by a chromosomal gene. Both hypotheses could be correct. Direct experiments indicate that efflux of arsenite via the staphylococcal ArsB can indeed be driven by the membrane potential [3]. Furthermore, when the *E. coli arsA* is expressed *in trans* to the staphylococcal *arsB* gene, increased resistance to arsenite is observed [3]. Thus, the ArsA protein in *E. coli* seems to increase the velocity of arsenite efflux compared to the situation in *Staphylococcus*. Finally, the recent release of 225 419 base pairs of the *E. coli* chromosome from minute 76 on the

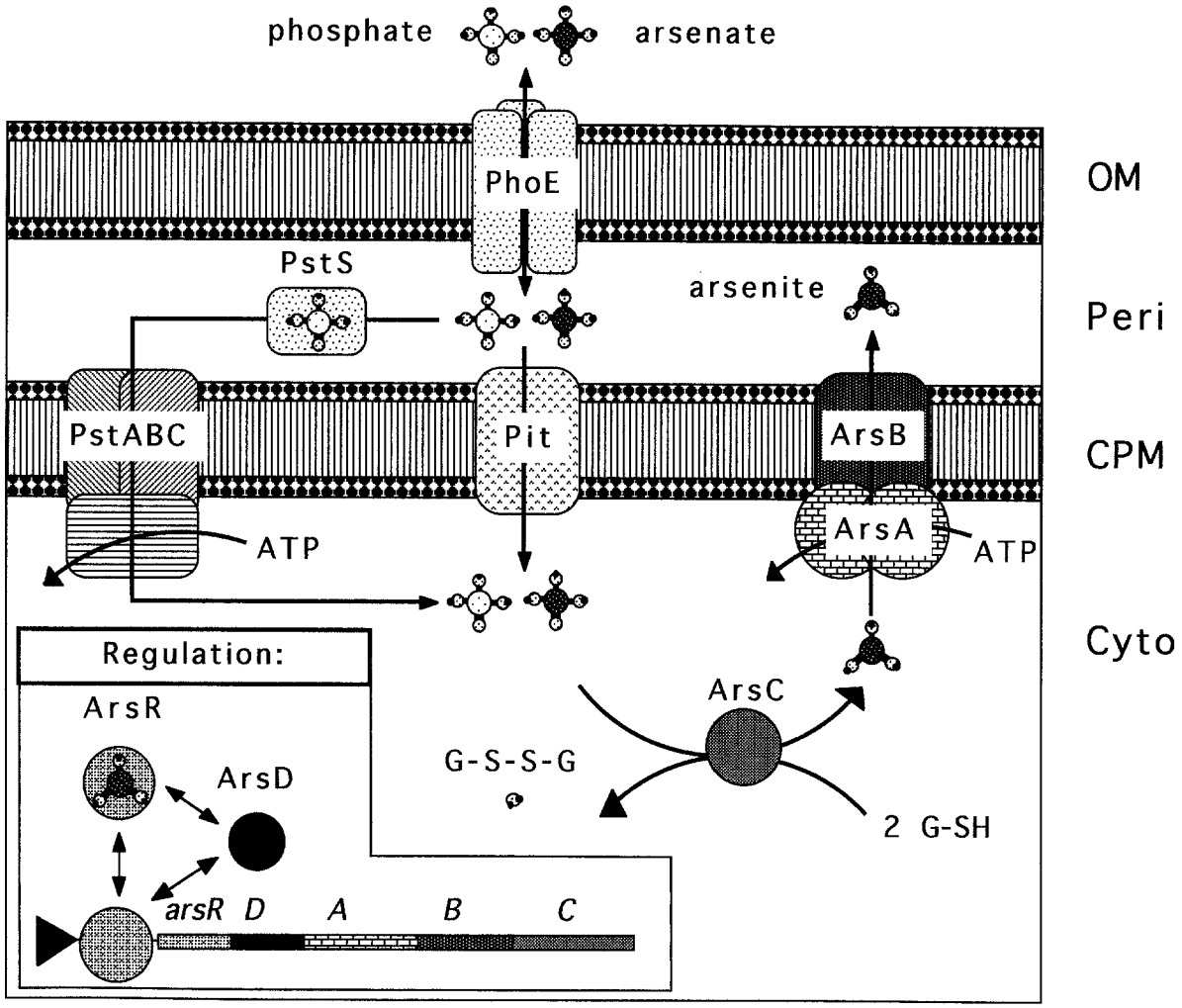

Fig. 1. Transport of and resistance to arsenate in *E. coli*. Arsenate and phosphate enter the periplasmic space through the outer membrane porin, the PhoE protein. Both anions are transported into the cytoplasm by the Pit protein or the Pst system (which is more specific for phosphate, as it uses the PstS phosphate-binding protein and the PstABC ATPase complex for inner membrane uptake). Within the cell, arsenate is reduced to arsenite by the ArsC protein (dependent on glutaredoxin and glutathione) and arsenite is pumped out of the cell by the ArsAB efflux ATPase. The *arsRDABC* operon is regulated by the ArsR repressor protein and the ArsD co-regulator protein. OM, outer membrane; Peri, periplasmic space; CPM, cytoplasmic membrane; Cyto, cytoplasmic space; G-SH, reduced glutathione; G-S-S-G, oxidized glutathione. Phosphate, arsenate and arsenite are shown as tetragonal or trigonal oxyanions.

genetic map (GenBank accession U00039; locus ECOUW77) includes three open reading frames, tentatively labelled *arsE*, *arsF* and *arsG* continuing after *arsD*. The predicted protein products of these open reading frames share 74%, 91% and 94% sequence similarity with ArsR, ArsB and ArsC, respectively, of the *E. coli* plasmid R773! The point here is that the *E. coli* chromosomal version of the *ars* operon is also missing the *arsA* gene. The interesting difference between the arsenic resistance systems with and without the ATPase subunit could mean that the Ars systems are in a sense an intermediate link between the ATP-driven transporters (for example ABC-type) and proton motive force driven transporters (for example the MFS class permeases [40].

The components encoded by the *ars* determinants were identified as prototypes of a new protein family [79]. While the topology of ArsB resembles the topology of the iron-hydroxamate permeases and major facilitator superfamily (MFS) that have 12 transmembrane spans, the ArsA protein is not similar to the ATP-binding domains of ABC-type transporters [17], except in the short ATP-binding motifs. However, the sequence similarity is too small to postulate any evolutionary relationship [71]. Therefore, for the moment, the ArsA ATPase lacks recognizable bacterial homologs. We predict that this situation will change over the next years, and significant homologs of ArsA were indeed found in the first region sequenced of the nematode *C. elegans* genome [discussed in ref. 79].

The *ars* mediated resistance to arsenicals is only expressed when the cell is exposed to a high concentration of these compounds [22,69,97]. The main regulator of the system is the ArsR repressor protein (Figs 1 and 2) with a binding-site just upstream of the *ars* determinant. Interestingly, arsenate is not an inducer of the system; arsenate must be reduced by ArsC to arsenite [18,21], the actual inducer [99]. ArsR has been

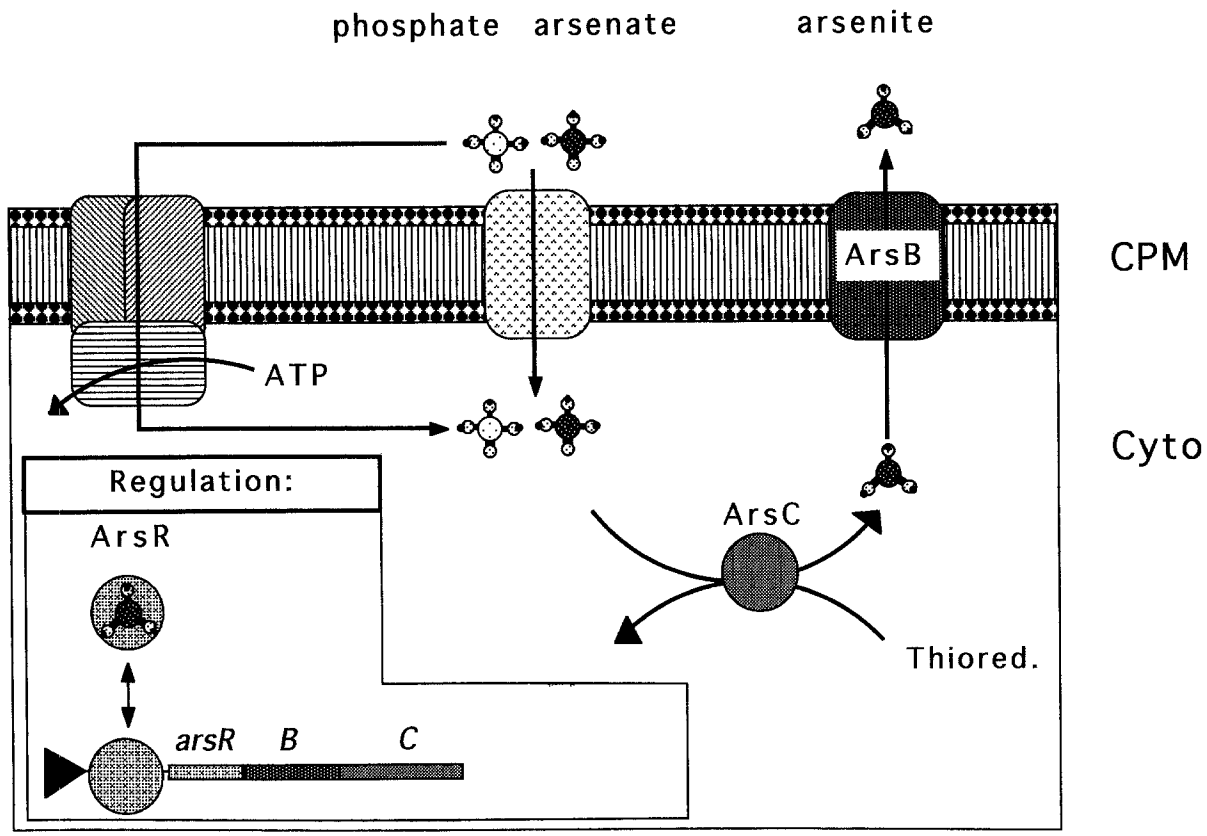

Fig. 2. Transport of and resistance to arsenate in *Staphylococcus*. As in Fig. 1, arsenate and phosphate are hypothesized to be transported into the cytoplasm by two membrane transporters [78]. Within the cell, arsenate is reduced to arsenite by the ArsC protein, coupled to thioredoxin, and the arsenite is pumped out of the cell by the ArsB protein alone. The *arsRBC* operon is regulated by the ArsR repressor protein alone. CPM, cytoplasmic membrane; Cyto, cytoplasmic space.

identified as a member of a new family of prokaryotic transcriptional regulators which have cysteine residues in their putative helix-turn-helix DNA-binding site [1]. It was proposed that binding of a metal(loid) ion to these predicted metal-binding sites could prevent the regulator from binding to the DNA and, thus, the regulator represses the respective operon only in the absence of the inducing metal ion [1].

Four *ars* determinants have been sequenced; however, only in the *ars* determinant of the *E. coli* plasmid R773 is a second regulator present which is encoded by the *arsD* gene. ArsD (Fig. 1) seems to function as a feed-back inhibitor on the level of transcription initiation and may prevent 'overshooting' of expression of the *ars* determinant [98].

The uptake of Cd^{2+} and Zn^{2+} by bacteria depends on the bacterial species

Cadmium is transported by the magnesium uptake system(s) in the Gram-negative *Alcaligenes eutrophus* [53] but by the manganese transport system in the Gram-positive *Staphylococcus* [61,90,94]. Not much is known about zinc uptake in bacteria: in *A. eutrophus*, it is accumulated by the magnesium transport system [53] and the same may be the case for enterobacteria [4].

P-type ATPases and heavy metal transport: from cadmium efflux to Menkes' disease

In Gram-positive bacteria, high concentrations of Cd^{2+} and Zn^{2+} lead to expression of the CadA resistance system, which is located on plasmid pI258 and related plasmids [56]. CadA mediates resistance by active ion efflux [91]. The CadA determinant is inducible [101] and the *cadC* gene product [102] is now known to be the trans-acting DNA-binding regulatory protein (Fig. 3) for this system (G. Endo and S. Silver, in preparation). This was predicted because of the amino acid sequence similarity to ArsR in the predicted cysteine-containing DNA-binding site helix-turn-helix region [1]. Resistance to cadmium and zinc in *S. aureus* is summarized in Fig. 3.

Cation efflux (Fig. 3) is catalyzed by the CadA protein [57,88], which is a P-type ATPase [81,82,87]. ATP is indeed required for CadA-catalyzed cadmium transport [88], but, surprisingly, dissipation of the electrochemical potential gradient also inhibited the CadA function [88], indicating a more complex action of CadA than a simple ATP-driven ion efflux. The CadA protein has been demonstrated to be phosphorylated in a Cd^{2+}-dependent reaction with properties [87] typical of those of P-type ATPases with a phospho-aspartate intermediate in the transport cycle.

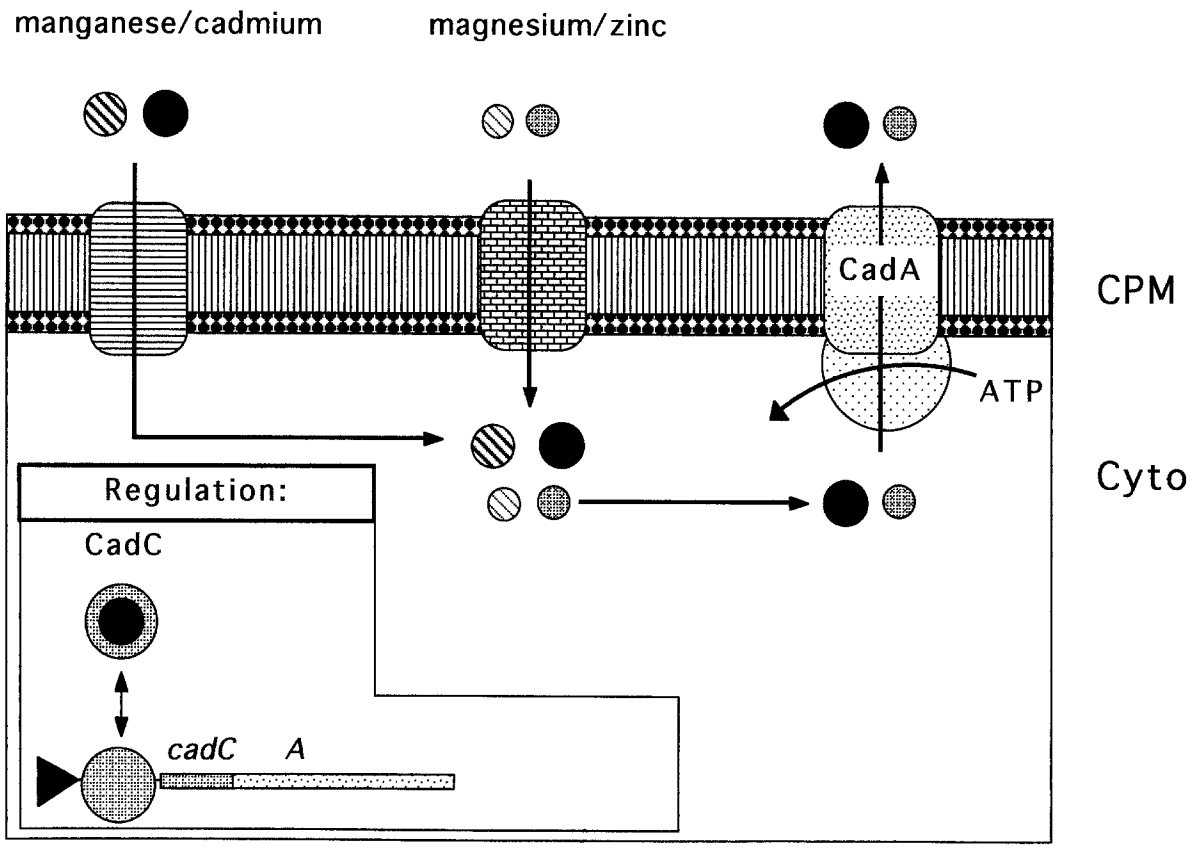

Fig. 3. Transport of and resistance to cadmium and zinc in *S. aureus*. Cadmium is transported into the cytoplasm by the manganese transporter and zinc probably by the magnesium transporter. At high concentrations, both cations are pumped out by the single polypeptide CadA P-type ATPase. The *cadCA* operon is regulated by the *cadC* repressor protein [12,102]. CPM, cytoplasmic membrane; Cyto, cytoplasmic space.

P-type ATPases are a large family of ATPases found in plants, animals, eukaryotic microbes and bacteria, all of which are cation pumps. Different P-type ATPases transport different cations and can function in either direction, inward or outward. All P-type ATPases have a conserved aspartate residue in the 'kinase' domain of their amino acid sequence. This aspartate residue is phosphorylated by ATP during the ATP hydrolysis cycle, and dephosphorylated again during the transport cycle. More than 70 P-type ATPases are known [71] including 16 in prokaryotes. They were grouped into four clusters [16] according to substrate specificity. However, these clusters do not mirror the evolution of the eukaryotes or bacteria. One of the four clusters has been grouped around the CadA protein [16,82].

This CadA-cluster contains besides the initial (and only well-studied) CadA ATPase of plasmid pI258 [57,87,88] the following P-type ATPases: (i) three additional Cd^{2+} CadA ATPases from *Bacillus*, from *Listeria* [33,34] and from the chromosomal determinant of methicillin resistance in *S. aureus*; (ii) two closely homologous P-ATPases from sequences of *Helicobacter* (P. Clancy, personal communication) and the chromosome of *E. coli* (new Genbank accession U00039) for which there are no transport or genetic data to suggest which (probably divalent) cation is transported; (iii) four probably copper transporting bacterial P-type ATPases, two from *Enterococcus hirae* [59,82] and two from the cyanobacteria *Synechocystis* and *Synechococcus* [82]; and (iv) remarkably three sequences from mammals that are thought to encode copper translocating P-type ATPases [5,6,41,42,82]. There is genetic analysis but no transport data supporting the idea that the CopA and CopB ATPases of the Gram-positive bacterium *E. hirae* are involved in copper transport [59]. The *copAB* operon from *Enterococcus hirae* potentially encodes two P-type ATPases that both contain putative heavy metal ion binding motifs in their N-terminal regions. However, these motifs are unrelated and that in CopA resembles the cadmium-binding motifs of cadmium ATPases (with a Cys-Ala-Asn-Cys tetrapeptide) while the CopB sequence contains three closely spaced repeated motifs Met-X_4-Met-X_2-Met related to presumed copper-binding determinants of the quite unrelated *Pseudomonas* CopA and CopB proteins involved in copper resistance [59]. Expression of the *E. hirae copAB* operon is induced by both very low and very high copper concentrations. Disruption of the *copA* gene renders the cells copper-dependent (suggesting that it determines an uptake ATPase), whereas disruption of *copB* results in a copper-sensitive phenotype (therefore suggesting that it may determine a copper-efflux ATPase) [59]. CopA exhibits only 32% sequence identity to CopB, and both proteins are indeed slightly more related to CadA than to each other [59,82].

Three mammalian P-type ATPase sequences have been published recently that show high sequence similarity to the bacterial CadA and CopA P-type ATPases. These are the can-

didate gene products for the human X-chromosome lethal disease Menkes' syndrome [8,41,92] (and its mouse equivalent that is called 'mottled' [42]) and the autosomal (lethal unless treated) Wilson's disease [5,6]. Both Menkes' and Wilson's were known as diseases of copper transport, with the primary defect in Menkes' being an inability to transport copper across the intestinal mucosa (leading to copper starvation for the body organs and failure to produce functional copper-containing enzymes needed for proper connective tissue formation) and the defect in Wilson's disease apparently involving a failure of copper transport in the liver, leading to over-accumulation and necrotic collapse. Now both diseases can be understood as due to failure to produce closely related essential copper P-type ATPases. The candidate gene product for humen Menkes' syndrome is a 1500-amino acid protein, which would be very similar to the CadA ATPase except that instead of having one single or two cadmium-binding motifs near its N-terminus, the presumed Menkes' gene product has six such motifs, which require the first half of the total polypeptide length [8,41,92]. Several mutants alleles were found of the mouse equivalent of the presumed Menkes' gene, two of which result in no stable mRNA (and fetal death; 'dappled' allele) or mRNA of altered size ('blotchy' allele; viable but with disease similar to human Menkes). This mouse gene produces a protein that is 90% identical over its 1492-amino acid length to the product of the human Menkes' gene [42]. Menkes' gene (and its mouse equivalent) is expressed in most mammalian tissues, with the exception of liver. The new Wilson's disease gene [5,6] produces a closely related polypeptide that is 47% identical to the Menkes' protein in the first half that contains the six metal-binding motifs and 67% identical in the second (and P-type ATPase region) half molecule. The Wilson's gene mRNA is expressed in liver but not in other tissues [5,6].

Most of the proteins in the CadA subfamily of cadmium- and copper-translocating P-type ATPases (and their three mammalian subfamily members) are known only as DNA sequences. Transport measurements were done only with CadA [88] and protein phosphorylation only with CadA and *E. hirae* CopB. However, the high sequence similarity of the proteins grouped in this subfamily leads to the conclusion that all these proteins have to be divalent cation transport ATPases. Therefore, studies with the bacterial cadmium resistance system will continue to help understand the fatal human copper storage diseases Menkes' and Wilson's.

The RND family: bacterial metal ion transporters of a new family

The best-studied metal cation efflux system in Gram-negative bacteria is the Czc system (determining resistances to cadmium, zinc and cobalt) of *A. eutrophus* CH34 [43]. This bacterial strain has two 'megaplasmids' (totalling more than 400 kb DNA) and at least seven metal resistance determinants, including three mercury resistance operons [13], a system that confers **n**ickel and **c**obalt **r**esistances (*cnr* determinant), and additional systems for resistances to chromate (*chr*) and to copper (*cop*) [13,54].

The *czc* determinant was cloned and sequenced [51,52]. The determinant consists of the three 'structural' genes *czcC, B* and *A* plus two regulatory genes *czcR* and *czcD*. More recently, the two additional systems *cnr* (for **c**obalt and **n**ickel **r**esistances) [36,77] and *ncc* (for **n**ickel, **c**admium and **c**obalt resistances) [74] also have been cloned and sequenced from *Alcaligenes*. These systems contain structural genes closely homologous to *czcC* (*cnrC* and *nccC*), to *czcB* (*cnrB* and *nccB*) and to *czcA* (*cnrA* and *nccA*).

The difference in the substrate specificity between CzcABC and CnrABC is interesting, and the NccABC system is even a kind of missing link between *cnr* and *czc* because it mediates resistance to nickel, cobalt and cadmium [74]. Furthermore, *cnr* may mediate zinc resistance when the expression of the CnrABC complex is altered by a mutation in one of the *cnr* regulatory genes [10] and *czc* may catalyze nickel resistance after mutation (Nies, unpublished). The A-subunits of all three cation transporters share the putative charge-relay proton transport motif (Fig. 4) and are probably the actual cation-proton antiporter subunits. The B- and C-subunits are required to increase the efficiency of cobalt transport and to include nickel (*cnr*), nickel and cadmium (*ncc*), or zinc and cadmium (*czc*) as substrates.

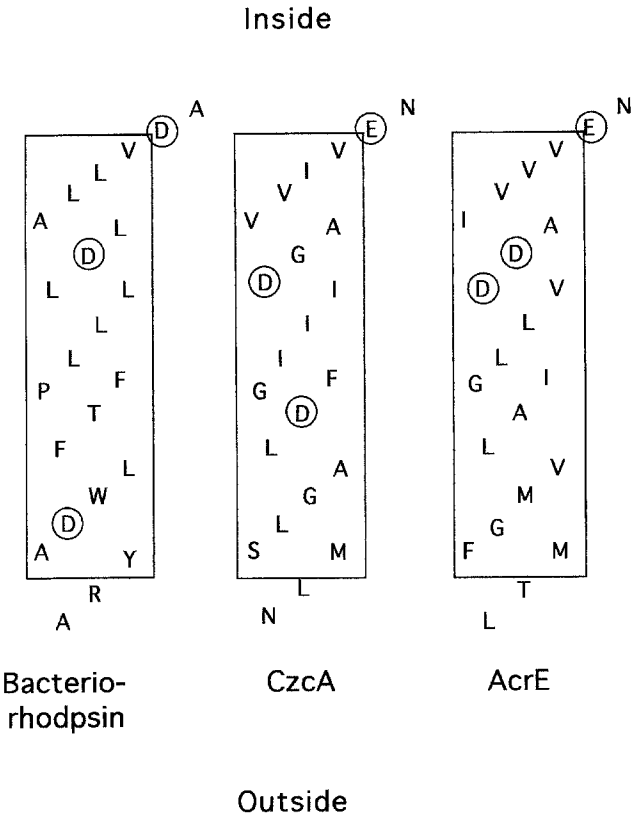

Fig. 4. Transmembrane α-helices that might form change-relay systems for proton transport. The boxed region corresponds to predicted transmembrane α-helices of bacteriorhodopsin, CzcA and AcrE, which are further hypothesized to form a charge-relay system for proton transport. Acidic amino acids (D, E) are shown in a circle. In bacteriorhodopsin, the two aspartic acid residues are spaced further apart compared to CzcA, and the proton is not transferred directly from one aspartate residue to the other, but via a retinal molecule which is covalently bound to a lysine residue in another transmembrane span [32].

All four cations, Cd^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} enter the cell via magnesium transport system(s) [53], and apparently the resistance (efflux) systems have a basic common mechanism but also differences that account for the varying cation specificities for efflux.

Cadmium, zinc and cobalt efflux in *A. eutrophus* is mediated by a cation-proton antiporter unlike efflux in Gram-positive bacteria which is based on an ATPase-driven mechanism. The antiport process is catalyzed by the CzcABC protein complex which is composed of the three subunits CzcA, CzcB and CzcC in a ratio of 1:1:1 (D.H. Nies, unpublished). The proton transport activity of the CzcABC complex has been measured in inside-out membrane vesicles by fluorescence quenching assays and the divalent cation transport with radioactive cations (D.H. Nies, submitted). The stoichiometry of the antiport is discussed to be two protons per zinc ion (D.H. Nies, submitted). Zn^{2+} may be bound at two cooperative binding sites, both with high affinity (K_m = 22 μM Zn^{2+}); Cd^{2+} binds to one site (K_m = 140 μM Cd^{2+}) with high affinity; and Co^{2+} to one site with low affinity (K_m = 10 mM Co^{2+}) (D.H. Nies, submitted). The maximal rate of transport of all three cations is more or less the same (D.H. Nies, submitted). Therefore, it is probably not cation binding but transport through the cytoplasmic membrane that is the rate-limiting step of the efflux process (D.H. Nies, submitted).

It is important to understand how *Alcaligenes* solved the problem of transporting toxic levels of the three different divalent cations with a common system without causing starvation for the two, Co^{2+} and Zn^{2+}, that are essential trace nutrients. Regulation must occur not only at the level of transcription but also at the level of cation pump function. The kinetic properties of the CzcABC complex assure that the toxic cation cadmium is always pumped out of the cell. Zinc, in contrast, is exported slowly at low concentrations and rapidly at high concentrations (D.H. Nies, submitted). Cobalt provides a problem with the *czc* system, since it is a poor inducer of *czc* and is also bound and transported with low affinity. One solution for problems with cobalt efflux for *A. eutrophus* was to evolve related cobalt efflux systems which are not induced by zinc, such as the *cnr* resistance determinant.

The three subunits of the CzcABC protein complex differ in structure and function, as first demonstrated by analysis of *czc* deletion mutants [52]. The complete CzcABC increases the MIC to Zn^{2+} and Co^{2+} 100-fold and the MIC to Cd^{2+} 10-fold. When *czcC* is deleted, and CzcAB are produced alone, the MIC to Zn^{2+} increases 20- to 50-fold, but the MIC to cobalt increases only 2-fold (and resistance to Cd^{2+} is lost) [52]. Therefore, CzcA and CzcB alone are able to catalyze a highly efficient zinc efflux. On the other hand, CzcC is needed to modify the substrate specificity of the complex to include cadmium and for practical purposes cobalt. The CzcC subunit does not contain apparent candidate motifs for a metal-binding site. Therefore, CzcC may function by altering the conformation of the CzcAB complex. After expression in *E. coli*, CzcC was shown to be located in the membrane fraction, independent of the presence of CzcA or CzcB (Nies, unpublished). Thus, CzcC is membrane-bound by itself and it does not need CzcA or CzcB as an anchor (as the ArsA requires the ArsB membrane protein). The amino acid sequence of CzcC, however, is highly hydrophilic and shows no signs of transmembrane segments. The question of whether CzcC is located on the periplasmic or the cytoplasmic face of the membrane cannot be solved at the moment, since data obtained with *phoA*- and *lacZ*-reporter gene fusions are ambiguous (Nies, unpublished). However, cation specificity for an efflux carrier like CzcABC should occur on the cytoplasmic membrane surface.

Although the CzcAB complex catalyzes zinc efflux highly efficiently, if *czcB* is deleted, this zinc efflux is lost [52]. Therefore, CzcA alone catalyzes cobalt efflux with low efficiency but CzcB is needed to add the substrate specificity for Zn^{2+}. The predicted amino acid sequence of CzcB [52] starts with a highly hydrophobic amino terminus followed by two small histidine-rich segments which are homologous to each other. These two motifs are very good candidates for the required zinc-binding sites, since they are absent in the otherwise related CnrB protein that does not recognize Zn^{2+}. In the field of metal resistances, it has been shown until now only with the MerP mercury-binding protein, that a proposed metal-binding domain is indeed involved in metal binding [70]. Moreover, the structure of this protein is known in some detail [15]. Comparable data are not yet available for CzcB, however, the number (two) of potential binding sites fits with the two zinc-binding sites concluded from the vesicle transport experiments [50]. CzcB is a membrane-bound protein which is located on the cytoplasmic face of the membrane (D.H. Nies, unpublished).

The large (more than 1000 amino acids in length) CzcA protein is the functional center of the Czc (and related) efflux complex. CzcA alone may function as a cation-proton antiporter, albeit with low efficiency. From hydropathy analysis of the predicted sequence, four domains are predicted [48], two hydrophobic regions composing the transport 'tunnel' and two cytoplasmic hydrophilic domains. These regions are arranged in the following order: hydrophobic amino-terminus, hydrophilic domain I, tunnel region I, hydrophilic domain II, tunnel region II, and hydrophilic carboxyl-terminus [48,52]. This structure appears to be the result of an ancient gene duplication event shared by a family of proteins for which CzcA is the prototype [71].

One predicted transmembrane α-helix in tunnel region I (Fig. 4) is highly conserved between the CzcA and the CnrA and NccA proteins. This segment includes two negatively-charged aspartate residues, which are predicted by our computer-based model to be within the membrane. This unusual placement of aspartates resembles an α-helix in bacteriorhodopsin, where two comparably-placed aspartates (Fig. 4) are essential for proton transport [32]. The aspartate residues may form a charge-relay system [24]. Therefore, tunnel region I of CzcA and CnrA may be part of the proton-specific transmembrane pathway, while tunnel region II, which does not contain charged amino acid residues in predicted membrane segments, may form the divalent cation pathway. We recognize that this model is highly speculative, but it provides a basis for direct mutagenesis experimentation. This segregation of proton translocation from divalent cation translocation

might have arisen at the time of gene duplication and fusion: since proton transport uses a charge-relay system, negatively charged amino acid residues are needed; however, divalent cations travel as cations. Thus, different 'tunnel pathways' are required for proton transport and cation transport, respectively. To couple proton influx with divalent cation efflux, a 'universal' six α-helix membrane-spanning 'tunnel domain' was duplicated, and one copy subsequently evolved into a proton pathway while the second evolved into a divalent cation pathway, whose specificity was later modified by addition of ancilliary (separate) polypeptides (CzcB and CzcC). The respective hydrophilic domains of each half-protein may serve in gating functions and to couple proton and divalent cation translocation. Fig. 5 gives a diagrammatic presentation of this working hypothesis for the function(s) of the CzcABC protein complex.

The CzcA protein may prove to be the prototype of a large family of transporters which couple transport of two chemically unrelated substrates. This activity may include symporters and exporters as well as antiporters such as CzcA. The family of proteins homologous to CzcA has recently been named 'RND' (for metal **r**esistance/**n**odulation of legumes/and cell **d**ivision) [72], an indication of the wide range of cellular functions that may require coupled transport functions. Other than CzcA, CnrA and NccA, RND-related polypeptides have not been shown to be involved in cation transport or even proton transport. They have functions in cell division (EnvE) and nodulation (NolGHI) for which transport involvement has not been shown, or resistance to toxic xenobiotic organic molecules such as acriflavine and ethidium bromide (AvrA) [38,71,72] for which proton/xenobiotic antiporter activity is known.

In contrast to the CzcA transport protein, other RND transporters appear to function with protein members of still another 'family', the MFP **m**embrane **f**usion-forming **p**roteins (EnvD, NolF, and AvrE) [72], that may form a membrane fusion pore between the cytoplasmic and the outer membrane of Gram-negative bacteria [35]. We have found no evidence based on amino acid sequence comparisons to support the notion that either CzcB or CzcC belong to the MFB family. AvrE has been shown with *phoA* protein fusion techniques to be a periplasmic protein [38]. The inner/outer membrane fusion may be required for the transport of molecules that would otherwise be unable to leave the periplasmic space through outer membrane porins. Thus, the RND xenobiotic transporters together with the MFP protein may transport their substrates directly across the two membranes.

MFP proteins associated with the RND xenobiotic transporters are composed of a short N-terminal hydrophobic domain followed by a large C-terminal part. Thus, the hydrophobicity profiles of the MFP proteins resemble those second subunits of the RND metal transporters. A weak sequence similarity between CzcB and members of the MFP family has indeed been observed [63], but the sequence similarity is too

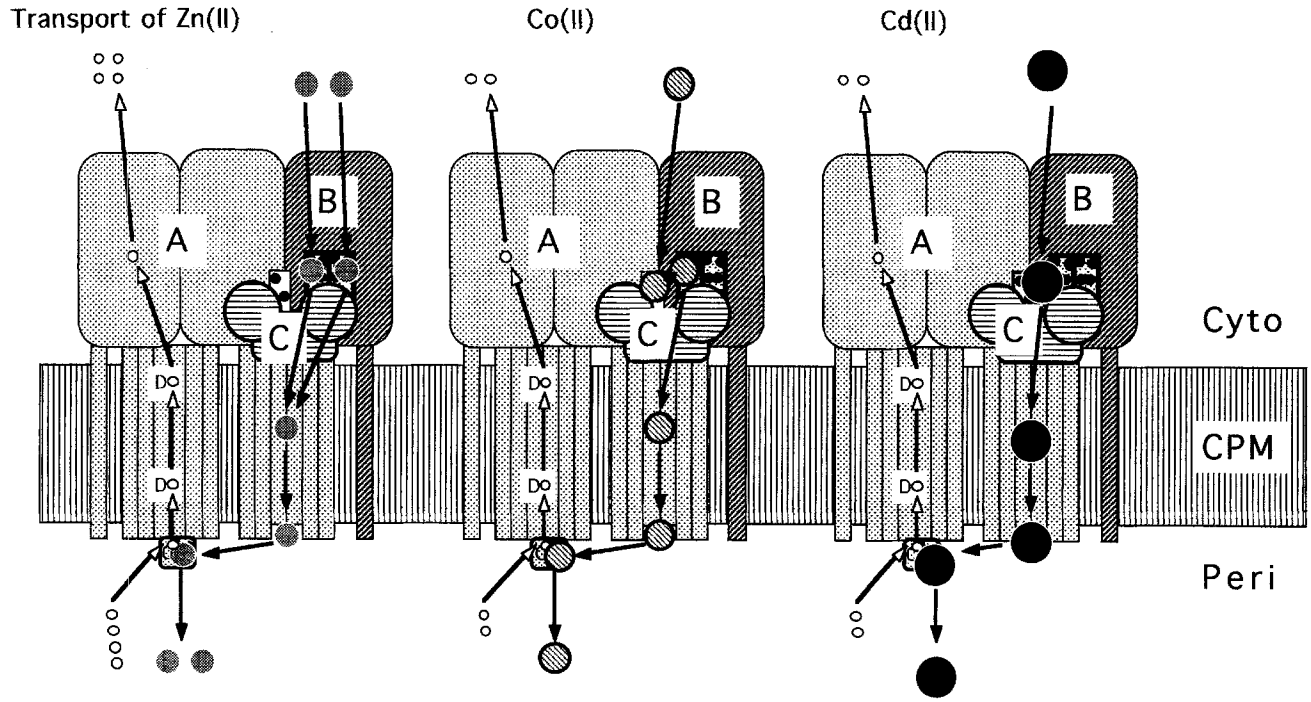

Fig. 5. Model for the function of the CzcABC cation-proton antiporter. Zn^{2+} is bound to either of two metal-binding sites of the CzcB subunit and then released into the cation tunnel. Per zinc cation exported, two protons are imported by the proton tunnel via the two aspartic acid residues. A potential metal-binding site in the periplasmic space may have a function in the coordination of proton- and cation-transport. Co^{2+} is bound to a binding site of the CzcA subunit and transported into the cation tunnel via the zinc-binding site(s) of CzcB. The larger Cd^{2+} cation is bound between the cobalt- and the zinc-binding sites and released into the tunnel. Peri, periplasmic space; CPM, cytoplasmic membrane; Cyto, cytoplasmic space. A, B and C indicate the CzcA, CzcB and CzcC subunits of the efflux protein complex, respectively. D; asparaginic acid residues in a transmembrane span of the CzcA-protein.

small to speculate about similar functions in the transport process. It might be, however, that the MFB proteins and the CzcB-like proteins have a common ancestor.

The CzcD-protein: a new family of transport regulators

Although the *czc, cnr* and *ncc* cation resistance determinants are all regulated by added toxic excess cations [54,75,76], the genes responsible for transcriptional regulation of *czc* and *cnr* are quite different. The reasons for these differences are not understood and currently in a state of flux. Therefore, conclusions about regulation of divalent cation resistance in *Alcaligenes* should be considered tentative. Induction of *czc* requires a novel two-component regulatory system which is composed of the membrane-bound sensor CzcD and a soluble regulatory protein CzcR. These proteins are unrelated to members of the large family of two-component trans-phosphorylating sensor/transducer regulatory proteins [49], and therefore the biochemical mechanism of sensing and regulating may be quite different. The CzcR protein is also not sequence homologous to the new SmtB/CadC/ArsR family of prokaryotic cation transcriptional repressors (see above). CzcR contains at its N-terminal end a potential helix-turn-helix DNA-binding motif with a paired cysteine residue [49]. Thus, CzcR may be a functional analog to the CadC family of metal-dependent repressors. The proposed regulatory proteins of *cnr* and *ncc* are unrelated to CzcD and CzcR [36,74], and we are left with the current conundrum of how basically homologous cation efflux systems became associated with basically unrelated gene regulation.

Northern blot mRNA/DNA hydridization experiments (Schwidetzky and Nies, unpublished) showed that zinc is the best inducer of *czc* transcription. Cadmium was an inducer of medium impact and cobalt a poor inducer. However, all three metal cations (and a variety of other cations as 'gratuitous inducers') induce the system for resistance to all three substrate metal cations. Thus, the substrate specificity of the efflux complex is not the same as that of transcriptional control.

CzcD, the novel sensor required for regulation of the *czc* determinant [49], is homologous to four predicted proteins and one fragment of an open reading frame in bacteria and in yeast. The products with unknown function are an open reading frame ORF2 in *Bacillus stearothermophilus* [31] and an incomplete open reading frame *B. caldolyticus* [30]. Those open reading frames were found when genes encoding glycogen branching enzymes from the two *Bacillus* species were cloned. ZRC1 [25] and COT1 [11] are putative proteins from *Saccharomyces cerevisiae*. The ZRC1 protein is required in yeast for a zince–cadmium resistance based on an unknown mechanism. While multiple copies of the ZRC1 gene enable yeast cells to grow in the presence of high concentrations of zinc, disruption of the chromosomal ZRC1 locus renders the respective mutant strain more sensitive to Zn^{2+} [25].

The COT1 protein has a similar function as ZRC1 with respect to the cobalt cation. Overexpression of COT1 increases cobalt tolerance while mutation of the COT1 gene makes the cells more sensitive to Co^{2+}. The COT1 protein is located in the mitochondrial membrane fraction and is involved in the uptake of Co^{2+} by mitochondria [11].

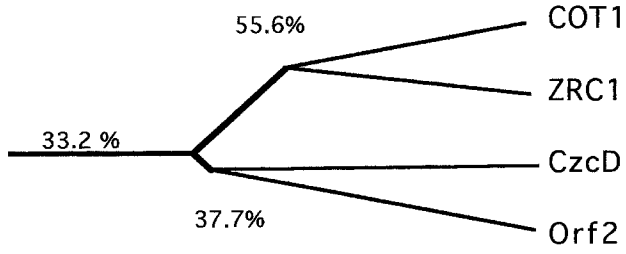

Fig. 6. Phylogenetic tree of the four CDF (**c**ation **d**iffusion **f**acilitator) proteins. The numbers give the percentage of identical amino acids between COT1/ZRC1 (55.6%), CzcD/Orf2 (37.7%) and ZRC1/CzcD (33.2%). All other pair-wise comparisons gave smaller values.

All four putative proteins CzcD, Orf2, ZRC1 and COT1 are related (Fig. 6) and form a protein family which is referred to as the CDF family for '**c**ation **d**iffusion **f**acilitator'. The sequence similarity is strongest in the hydrophobic parts of the proteins: all four proteins probably have six transmembrane α-helices. The differences between the proteins are in the hydrophilic N-terminus in addition to the six transmembrane α-helices. Although the sequence similarity between the two bacterial CDF proteins CzcD and ORF2 is higher compared to the yeast proteins, Orf2, ZRC1 and COT1 share a common motif, a C-terminal hydrophilic domain with potential metal-binding sites. The two yeast proteins ZRC1 and COT1 are 55.6% identical. Both proteins contain a second hydrophilic domain with potential metal-binding motifs in their middle part (Fig. 7)

The sequence similarity of the four CDF proteins is so high that it allows some working hypothesis about the function of these proteins: the CDF proteins are membrane-bound proteins involved in zinc, cobalt and cadmium transport. That would lead to the assumption that CzcD functions as a sensor by a slow uptake of the zinc, cobalt and cadmium cations. This completes the model for the function of the Czc metal resistance system (Fig. 8).

Chromate resistance: does ChrA protein start another new family?

Chromate resistance (Fig. 9) in *A. eutrophus* is mediated by the *chr* determinant located on plasmid pMOL28 [46]. This

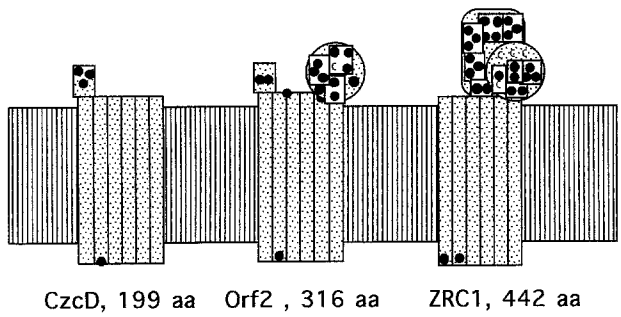

Fig. 7. Predicted secondary structure for the three CDF proteins. The boxed regions of all three proteins correspond to the six predicted transmembrane spans of each protein. Dots mark the histidine residues and 'C' cysteine residues. Boxed white regions confer to potential metal-binding sites.

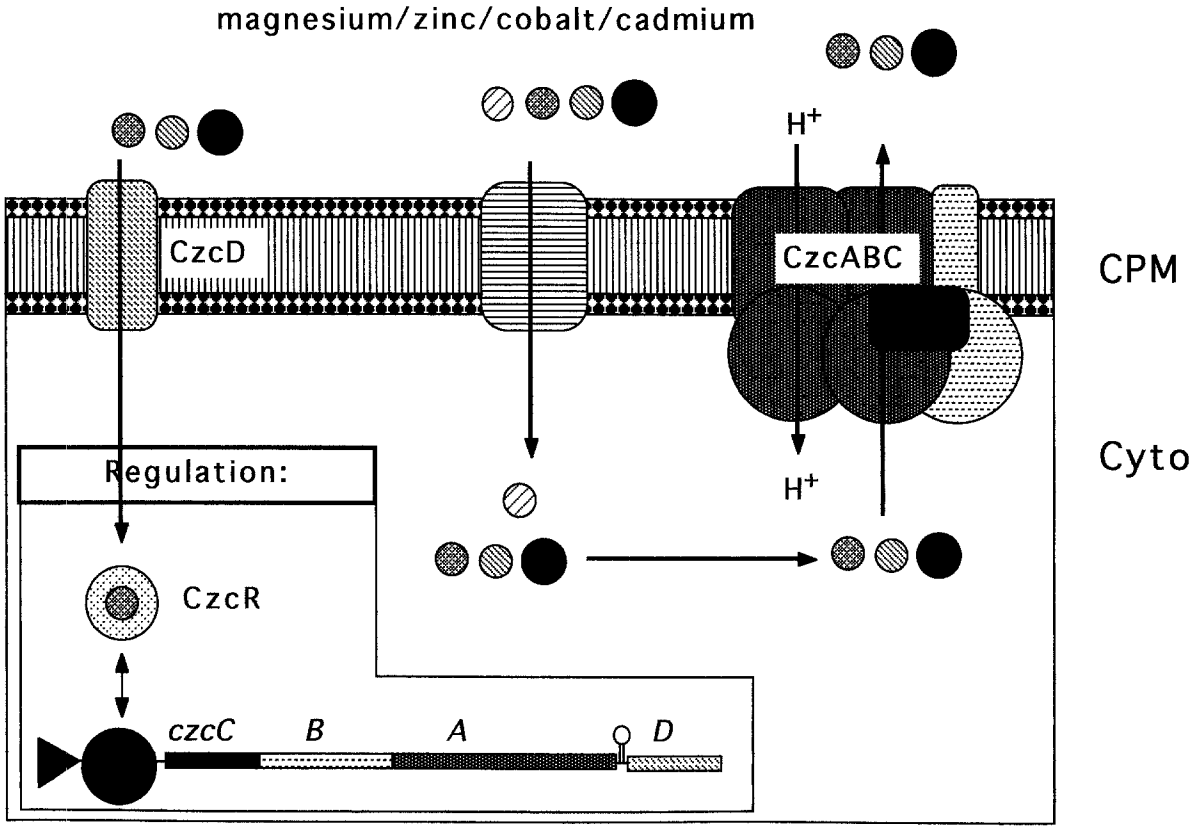

Fig. 8. Transport of and resistance to cobalt, zinc and cadmium in *A. eutrophus*. The three cations are transported into the cytoplasm by (the) magnesium transporter(s). At high concentrations, the cations are pumped out by the CzcABC cation-proton-antiporter. The *czcCBAD* operon is regulated by the CzcR regulator and the CzcD sensor. CPM, cytoplasmic membrane; Cyto, cytoplasmic space.

determinant encodes two proteins, ChrB and ChrA, with the *chrB* gene preceding the *chrA* gene in the same transcriptional direction [47]. Between these genes is an unusually long intergenic region (of 420 bp) that is thought to be involved in regulation of *chr* (Nies, unpublished). The ChrA protein of *A. eutrophus* is homologous to the ChrA protein from a *Pseudomonas* plasmid [7] with 29% identical amino acids. Protein databases lack other proteins with significant sequence similarities to ChrA. However, both ChrA proteins resemble in their hydropathy profile many large chemiosmotic transport proteins such as ArsB and LacY that are thought to pass across the membrane in 12 α-helical segments [48]. Since there are no orphan proteins, perhaps future research will show that the ChrA proteins are indeed patriarchs of still another new family of structurally and functionally homologous membrane transport proteins.

No *chrB* gene was found in the cloned *chr* determinant of *Pseudomonas*, but this may be because the initial fragment cloned and sequenced ended in the mid-intragenic regions [7]. Resistance with this cloned determinant is not inducible [7], which might suggest that ChrB is involved in regulation of *chr*. However, deletion of *chrB* leads to hyperaccumulation of chromate in *A. eutrophus* [47]. Downstream from the *chrA* gene in *Pseudomonas* is an additional open reading frame potentially encoding a polypeptide 87 amino acids long, unrelated to proteins currently available in protein sequence databases. A closely homologous open reading frame was disrupted in the chromate-resistance fragment from *Alcaligenes*, so this open reading frame is not essential for chromate resistance, a situation similar to the lack of essentialness of the *merD* and *arsD* gene products from the mercury and arsenic resistance operons respectively. Other than the requirement for the *chrA* gene and protein, it is clear that more effort is needed to define both the upstream and the downstream ends of the chromate resistance determinant.

Chromate is transported into *A. eutrophus* (and other bacteria [83]) via the sulfate uptake system(s) [53]. Chromate resistance results from reduced accumulation of chromate in both *Alcaligenes* and *Pseudomonas* [54,60]. However, chromate efflux has not been demonstrated. Therefore, we can not be certain whether the ChrA protein is involved in oxyanion efflux, as in other efflux resistance systems or might alternatively (but less likely) be the first example of a direct block on initial oxyanion uptake.

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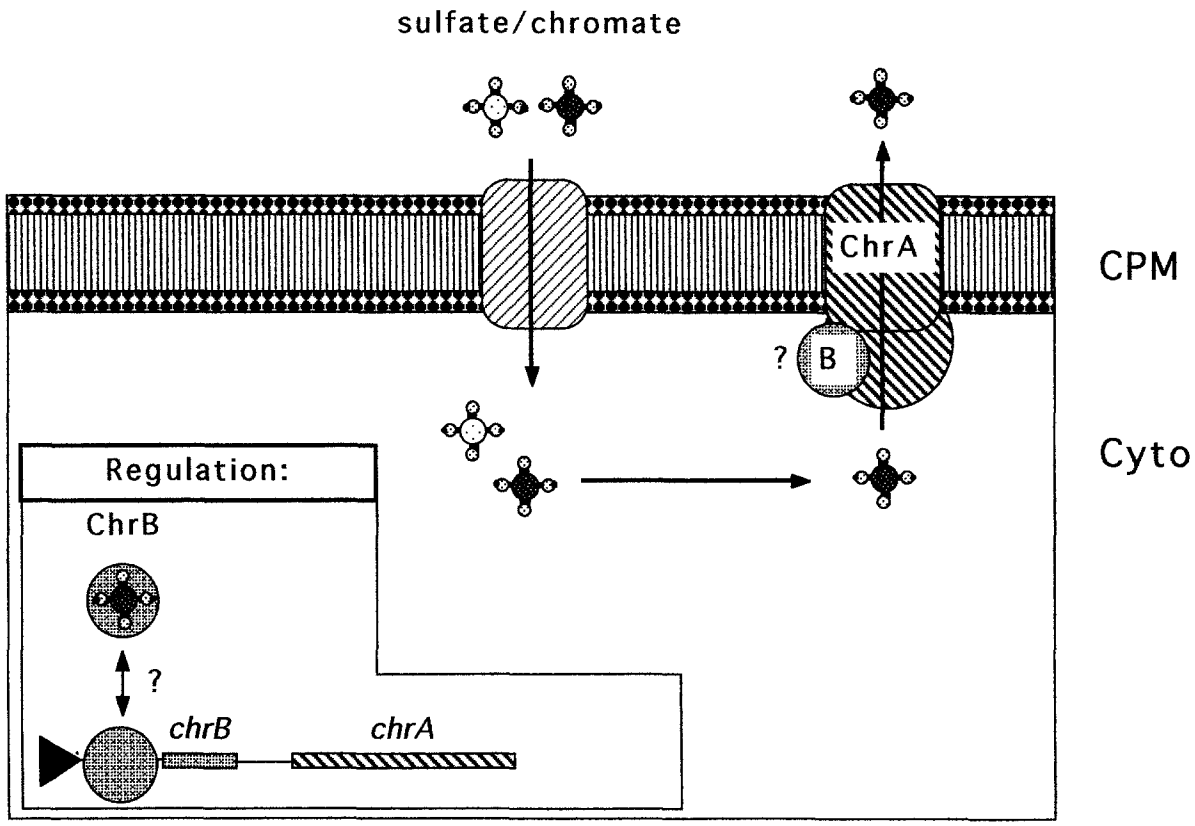

Fig. 9. Transport of and resistance to chromate in *A. eutrophus*. Chromate is transported into the cytoplasm by the sulfate transporter(s). At high concentrations, chromate is pumped out by the ChrA protein (possibly with ChrB associated). The *chrBA* operon may be regulated by the ChrB protein. CPM, cytoplasmic membrane; Cyto, cytoplasmic space.

Fonds der Chemischen Industrie and by Forschungsmittel des Landes Sachsen-Anhalt. D.H.N. dedicates this review to Anke, who did much early molecular genetics on *czc*, *cnr* and *chr* and who is waiting in Berlin with Ayla and Erin while he is already in Halle.

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