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New developments in the understanding of the cation diffusion facilitator family

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Abstract Cation diffusion facilitator (CDF) proteins are a phylogenetically ubiquitous family of intermembrane transporters generally believed to play a role in the homeostasis of a wide range divalent metal cations. CDFs are found in a host of membranes, including the bacterial cell membrane, the vacuolar membrane of both plants and yeast, and the golgi apparatus of animals. As such, they are potentially useful in the engineering of hyperaccumulative phytoremediation systems. While not yet sufficient for reliable biotechnological manipulation, characterization of this family is proceeding briskly. Experimental data suggests that CDFs are generally homodimers that use proton antiport to drive substrate translocation across a membrane. This translocation of both substrate and protons is likely mediated by a combination of histidines, aspartates, and glutamates. Functional data has suggested that CDFs are not limited to metal homeostasis roles, as some appear to be determinants in the operation of high-volume metal resistance systems, and others may facilitate cation-donation as a means of signal transduction. This review seeks to give an overview of the data prompting these conclusions, while presenting additional data whose interpretation is still contentious.

Keywords Iron · Zinc · *Escherichia coli* · CDF-transporter

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Introduction

In addition to being important drug-resistance determinants in bacteria, transport proteins can also be used for bioremediative purposes. A common approach to metal phytoremediation is to isolate or engineer plants capable of accumulating a target metal, then disposing of both offsite. In order to accomplish this, it is necessary for these plants to not only take up metals, but store them as well. Two important ubiquitous plant transporter systems are the ZRT-like, IRT-like proteins (ZIP) and the cation diffusion facilitator (CDF) families [14, 47]. Members of these families are involved in metal hyperaccumulation and handle both essential and toxic metals. The ZIP family of proteins is responsible for uptake of metals such as iron and zinc, whereas CDF proteins are involved in vacuolar sequestration.

Whether natural or engineered, a commonly encountered problem with hyperaccumulators is that they yield a low biomass. In order obtain meaningful large-scale phytoremediation, higher biomass systems need to be developed. In theory, one way to achieve this would be to increase the number of uptake proteins for the metal of interest in a high-biomass-producing plant [49]. Alternatively, the specificity of uptake proteins could be manipulated to take up only the metal of interest, to the exclusion of otherwise competitive substrates, reducing the overall metal load.

Employing the latter method predictably requires a thorough knowledge of the residues involved in substrate-protein interaction. The CDF proteins are ideal for this type of analysis, as their ubiquity in all three domains of life allows inter-kingdom sequence alignments, making screens for conserved, potentially metal-binding, residues more comprehensive. Further, this similarity has allowed CDFs from less-characterized organisms to be reliably assayed in model systems [36] and even, in the case of Zrc1 from *Saccharomyces cerevisiae* as assayed in *Escherichia coli*, in models from separate kingdoms [31]. It is likely that substrate binding

and translocation schemes are all quite similar amongst CDF proteins. Knowledge gleaned from the more easily manipulated bacterial systems should be almost directly applicable to CDF proteins from plants or animals. However, because the identity and nature of CDF substrate binding sites is only now being closely examined, the validity of this prediction is still undetermined.

The CDF protein family's defining structural characteristics include six transmembrane domains (TMDs), a conserved signature sequence between TMDs I and II, histidine-rich cytosolic regions at both termini, and additionally between TMDs IV and V [47]. These general rules are often individually broken, as CDFs can vary considerably in sequence and size [23, 27]. So far, most CDFs appear capable of transporting Zn^{2+}, but some have also been able to transport divalent cations of Co, Mn, Fe, Cd, and Ni [3, 12, 17, 36, 48].

In most bacterial instances, CDFs appear to be involved in metal tolerance and/or homeostasis via the export of the cations from the cytoplasm to the periplasm or exterior of the cell [17, 19, 25]. A notable example is the second characterized CDF protein from *E. coli*, FieF (previously known as YiiP). This protein, as well as its identically named homolog in *Cupriavidus metallidurans* (formerly *Acaligenes eutrophus, Ralstonia/Wausteria metallidurans*), has been demonstrated to be responsible for decreased cellular iron accumulation, possibly making it the first iron-efflux system ever described [17, 36]. However, bacterial CDFs may not be limited to metal transport. The *fieF*-like *cepA* encodes a gene related to chlorhexidine resistance in *Klebsiella pneumoniae* [15].

In the yeast *S. cerevisiae*, Zrc1 and Cot1 perform the same tolerance function; however, they translocate metals from the cytoplasm into the vacuole [11, 31]. Further, a third CDF from *S. cerevisiae*, Msc2, appears to regulate Zn^{2+} homeostatic export specifically from the nucleus into the cytoplasm [27]. The best-studied plant example is AtZat (previously known as ZAT1p) from *Arabidopsis thaliana* [7]. As with Zrc1 in yeast, this transporter translocates Zn^{2+} from the cytosol into the vacuole.

In mammals, all characterized CDFs transport Zn^{2+}, and so these proteins are named zinc transporter X (ZnTX), where X is a number corresponding to the order in which the protein were discovered. These proteins seem less involved in survival-dependent metal homeostasis than they are in shuttling Zn^{2+} into intracellular and secretory vesicles, which could have a homeostatic component [21, 29]. Some notable examples include ZnT5, which is localized to the secretory vesicles of pancreatic β cells, and may be responsible for providing the Zn^{2+} needed to form insulin crystals [23]. In mice, ZnT4 likely delivers the micronutrient zinc from the tissue of mammary glands into milk, as its absence in mice leads to a lethal milk syndrome where sucklings perish from an insufficient zinc supply [21]. However, the ortholog of ZnT4 in humans (Slc30A4) does not appear to be a factor in acrodermatitis enteropathica, the phenotypically equivalent disease in humans [33]. ZnT1 is located in the cytoplasmic membrane and appears to act as an exporter of Zn^{2+} from the cells of a host of tissues [46]. Interestingly, a protein closely related to ZnT1, CDF1 from *Caenorhabditis elegans*, may have a regulatory role in the *Ras* oncogene signaling pathway [8, 22].

CDF structure

Hydrophobicity plots and experimental data suggest that CDFs generally contain six individual TMDs [3] (D. Fu, personal communication). Using LacZ/PhoA translational fusions at various points along the protein sequence of CzcD from *C. metallidurans* CH34, the presence of the first four amino terminal membrane spanners has been confirmed [3]. Data from a similar experiment, using cysteine-specific biotinylation and labeling of the inter-TMD coils of FieF from *E. coli*, suggested six membrane spanners (D. Fu, personal communication). Further, circular dichroism spectroscopy of FieF and CzcD indicated a molar α-helical content of over 50%, supporting the hypothesis that the TMDs of CDF proteins are helices (D. Fu, personal communication; D. Nies and A. Anton, personal communication).

While tertiary structure remains a point of speculation, there are some recent instances of quaternary structural data. FieF from *E. coli* appears to assume a homodimeric structure [60]. This analysis was based on a technique involving both size-exclusion HPLC as well as light scattering/absorption, which together helped negate the effects of the size and makeup of the micelles in which the protein was necessarily solubilized. A series of detergent types was used to create the micelles but, statistically, all yielded the same protein mass of 68 kDa, twice the predicted mass of the *fieF* gene product [60]. Additionally, the Zn^{2+}-transporting CDF PtdMTP, from the *Populus trichocarpa* X *P. deltoids* poplar hybrid, forms homooligomers in vitro, which can be disrupted using dithiothreitol (DTT), suggesting that disulfide bridges may play a role in their formation [6]. Indeed, FieF dimers can be substantially stabilized through Hg^{2+}-mediated cysteine crosslinking at C127. However, there is currently no data to suggest that these bridges occur in vivo (D. Fu, personal communication). The possibility of dimer formation has also been demonstrated for AtZAT from *A. thaliana* [7].

Substrate transport

Transport proteins require a source of energy, because they are generally pumping a substrate against an electrochemical gradient. Perhaps the most intuitive source is ATP hydrolysis. While ATPase transporters are quite effective, their function is also energy-intensive, a liability for a homeostatic protein. Alternatively, some transporters couple the movement of substrate up its electrochemical gradient to the movement of a co-sub-

strate down its own gradient. The two substrates can be translocated in the same direction across the membrane (symport) or in opposite directions (antiport) depending on the relative concentrations of the substrate on either side of the membrane. In *E. coli*, the proton motive force, generated by the extrusion of protons into the periplasm via the electron transport chain, is coupled to several transport processes. In the case of uptake proteins like LacY, proton transport down their gradient is coupled in a symport arrangement to lactose uptake from the periplasm (as reviewed by Abramson [1]). Efflux antiport proteins can use the same proton gradient to exude substrate from the cell, often against its gradient [56]. So far, all CDFs fall into the latter category. They use the energetic capital from the entropically favorable diffusion of one cation to facilitate the less favorable diffusion of another. This is an important distinction as, out of context, ''CDF'' could be taken to simply mean ''cation pore''.

At this point, all available data suggest that CDF proteins function as proton antiporters. In the yeast *S. cerevisiae*, isolated wild type vacuole vesicles accumulate Zn^{2+} in an ATP-dependent manner, an effect that can be largely abolished through mutation of the vacuolar CDF, Zrc1 [31]. Mutations in the vacuole acidifying V-type ATPase also render cells hypersensitive to Zn^{2+}, probably due to the lack of vacuolar protons to use as counterions in sequestering zinc into the vacuole via Zrc1 [13, 51]. Treatment of ATP-energized wild type vesicles with the ionophore valinomycin, which allows K^+ to freely cross the vacuolar membrane, had no negative effect, excluding a K^+ gradient as the driving force in this case [31]; some early complementation data had suggested K^+ as the counterion for metal transport in CzcD from *Bacillus subtilis* and ZitB from *E. coli* [19, 25]. More recently, in reconstituted proteoliposomes, ZitB has been shown to transport Cd^{2+} in a proton-dependent manner, while K^+ had no effect [9]. Interestingly, the same study indicated the stoichiometry for this process to be 1.23:1 Cd^{2+}/H^+, generating a positive charge trans to Cd^{2+} uptake [9]. The imposition of an artificial electrochemical gradient ($\Delta\Psi$) across the membrane of ZitB proteoliposomes facilitated transport of Cd^{2+} down the electrochemical gradient, and up the Cd^{2+} concentration gradient [9]. While this does not preclude H^+ antiport, it does suggest that CDFs may have more than one power source, which may partially explain the K^+ data. Proton antiport is also supported in studies using everted membrane vesicles (EMVs) overexpressing ZitB and FieF (both from *E. coli*), as these vesicles accumulate Zn^{2+} only in the presence of NADH, ATP having no effect [4, 17].

The kinetics of these processes are only beginning to be explored. Recent proteoliposome studies indicated the K_m for ZitB Zn^{2+} transport to be 105 μM [9]. On the other hand, experiments using EMVs suggested this K_m value to be closer to 1 μM [4]. By comparison, the high volume, ATP-driven Zn^{2+} efflux pump in *E. coli*, ZntA, has a K_m in EMVs of 9 μM [53]. Because ZitB is thought to be primarily a homeostatic protein, and the concentration of free Zn^{2+} within the bacterial cell is thought to be in the femtomolar range [44], all of these values would seem to render these proteins incapable of performing homeostatic maintenance roles. However, neither technique claims to faithfully reproduce the physiological conditions under which the proteins are meant to function. Additionally, neither technique accounts for the binding of Zn^{2+} or Cd^{2+} to embedded metal-binding proteins and/or the membrane itself. Given the fact that virtually none of the intracellular zinc, which is in the millimolar range [44], is present as free ions, it is reasonable to predict that much of the zinc used in the transport assays was unavailable for transport due to the high metal-binding capacity of the systems themselves. This would inflate the K_m values because a higher dose of substrate is needed to overcome this binding potential before the zinc is even available to the transporters. The detection methods themselves may also have inflated these values. The proteoliposome study, for instance, used a fluorescent zinc probe with a Kd^{Zn} of 8 μmol [9] (D. Fu, personal communication).

The apparent discrepancy between the two ZitB K_m values may also be due to as yet unknown cofactors involved in the transport process. The production of proteoliposomes involves the dissolution of the native membrane, and purification of the protein of interest, which could also remove any cofactors. EMV production utilizes the native membrane and involves no protein purification steps, and so may help with the retention of protein cofactors associated with the membrane and/or the transporter itself.

Substrate binding

While many of the substrates transported by CDF proteins have been identified, there is currently little data as to how these proteins acquire their substrates. Although it seems logistically simple, direct uptake of free substrate may be complicated by the extremely low concentration of free substrate ions such as Zn^{2+} within cells. Many CDF substrate ions are bound either to proteins that use the ions in their catalytic sites, or to storage molecules such as ferritin or bacterioferritin, in the case of iron [2]. These storage partners could interact with CDF proteins to donate substrate for transport and thus refresh their ability to bind any free substrate ions. Although there are presently no data to support it, such an arrangement coincides well with both the hypothesized homeostatic function of many CDF proteins and the low free-ion concentrations of their substrates. Another interesting possibility, as discussed below, is that CDF proteins may be involved in the activation/deactivation of proteins whose function is dependent upon the presence/absence of bound substrate.

Data for a CDF-chaperone interaction involving two homologous eukaryotic CDF proteins have recently

been made available. Raf1 is a protein kinase involved in cell differentiation and proliferation pathways in many eukaryotes, and as such is also a potential cancer determinant [35]. Indirect evidence has suggested that this kinase is activated, at least partially, by the action of the CDF ZnT1 from humans, and its homolog CDF1 from *C. elegans*. Mutation of the Zn^{2+}-binding cysteine-rich domain of Raf1, as well as UV-induced cross-linking of this domain, results in an increase in Raf1 kinase activity, suggesting the importance of zinc in keeping the kinase in the inactive conformation [20, 34]. New data has shown that both ZnT1 and CDF1 directly interact with Raf1 via the CDF C-terminus and the N-terminal half of Raf1, which is involved in regulation of kinase activity [22]. These data support the hypothesis that these two CDFs receive their substrate directly from Raf1, as a component of the latter's protein activation pathway. The same study supported this further by showing reduced Raf1-ZnT1 interaction in the presence of 4 mM Zn^{2+} in vitro, as well as reduced Raf1 activation in the presence of 0.1 mM extracellular Zn^{2+} in *Xenopus* oocytes injected with mRNA for both Raf1 and ZnT1 [22]. Assuming that the typical intracellular free zinc concentration in eukaryotic systems is in the femtomolar range, similar to that of *E. coli* [44], the ability to remove substrate directly from zinc-binding proteins seems a logical route for CDF substrate acquisition. However it is not conclusive that a chaperone is needed.

Data for Zn^{2+} and Cd^{2+} transport rates upon initial exposure to substrate suggest a 1.23:1 stoichiometry for Me^{2+} :ZitB [9]. Interestingly, isothermal titration calorimetry (ITC) analysis of FieF indicated separate binding sites for Zn^{2+}, Cd^{2+}, and Hg^{2+} [10]. One of these sites was mutually competitive between all three metals, while the others excluded one metal or another. The universally competitive site preferred Zn > Cd≫Hg, according to the binding enthalpy (ΔH) [10]. It should be noted that most known CDFs transport Zn^{2+} and Cd^{2+}, usually in that order of preference, while none are known to transport Hg^{2+}. Zinc appears to bind another site with little competition from either of the other two cations.The binding of Zn^{2+} and Cd^{2+} to this site was inhibited by low pH, suggesting that metal binding is linked to deprotonation [10]. Zinc also competed for the binding sites of Cd^{2+} and Hg^{2+}, even though they exclude each other [10]. It is not yet clear what, if any, relationship these selectively competitive sites have with one another.

One of the Cd^{2+}/Zn^{2+} binding site in FieF has been mapped to C287, where cysteine-specific labeling was blocked by Cd^{2+} (D. Fu, personal communication). Because of its position within the presumably cytoplasmic C-terminus (cis to metal transport), C287 may be directly involved in substrate binding. The equivalent cysteine in CzcD (*C. metallidurans*) at position C290, appeared necessary for function, while C294 in ZitB did not, although an additional cysteine at C299 may have compensated for the C294S mutation [4, 25]. Additionally, C127 of FieF has been shown to possess the ability to bind Hg^{2+}, creating an Hg^{2+}-mediated disulfide bridge, a phenomenon uninhibited by excesses of Zn^{2+} or Cd^{2+} (D. Fu, personal communication). The broad implication of these findings is that CDFs may bind metals in more than one way, and for more than just translocation. Other potential uses for these sites include regulation, catalysis, and sequestration.

Structure-mechanism relationships

If CDF proteins are ever to be manipulated to a biotechnological end, the relationship between the structural components and substrate translocation must be elucidated. This includes determination of which structures are involved in substrate binding and which are involved in the transport process itself.

A reasonable initial hypothesis is to identify the cytoplasmic (cis to substrate) histidine-rich regions as metal binding sites. However, deletion of either the four N-terminal (ΔM1–H11), or five C-terminal (ΔC294–H313 histidines from ZitB did not render cells of the zinc-sensitive *E. coli* strain GG48, which were overexpressing the mutant proteins, any more sensitive to Zn^{2+} than those expressing wild type ZitB [25]. This suggests a non-essential role for the individual histidine-rich regions, as only one of the two is needed for function. Additionally, there are several CDF proteins lacking these histidine-rich regions altogether. These belong to the FieF-like group 3, whose substrate is thought to be Fe^{2+} [39]. A representative of this group, MamB, is found in the magnetosome of several magnetotactic bacteria, and may be responsible for magnetite accumulation [18]. Despite this, FieF from *E. coli* has been shown to transport Zn^{2+} in EMVs [17]. In the case of the CDF1/ZnT1 data, it is possible that one or both of these regions are involved in chaperone docking [22]. Alternatively, perhaps the histidines do bind metal, but not necessarily as part of the transport process per se. Histidine residues could bind metal in a regulatory capacity, leading to a shift in redox potential at critical residues, and/or a conformational change that exposes the substrate-binding site.

Histidine-bound substrate in close proximity to transporter could also increase the local effective concentration of the substrate at the cis face of the protein. This makes sense in the case of ZitB, which is thought to be a homeostatic transporter of Zn^{2+}. With intracellular free zinc concentrations normally in the femtomolar range [44], a homeostatic transporter would need an affinity in the same range. A collection of histidines could "funnel" substrate to the transporter by keeping its equilibrium concentration higher at the cis face of the transporter, in comparison to the actual cytoplasmic concentration, making the transporter more sensitive than the near-femtomolar specificity that would otherwise be required. A similar idea was recently suggested by Banerjee et al. [5] in their structural study of the ATP-binding-cassette (ABC)-transporter periplasmic subunit ZnuA from *Synechocystis 6803*. Residues 138–173 of this zinc uptake protein contain 12 acidic residues and 8

histidines. This highly charged, randomly coiled region protrudes into the periplasm, cis to metal uptake, where its histidines and negatively charged groups may have a role in sequestering Zn^{2+} near the metal binding site via weak interactions [5]. Interestingly, this region appears to be conserved only in ABC proteins known to transport zinc, presumably to compensate for low periplasmic free zinc concentrations [5].

A lack of detailed structural data precludes any predictions as to whether or not the C or N-terminal regions of ZitB are similar to ZnuA in their flexibility or what, if any, secondary structure they may assume. In the light of hydrophobicity plots it is reasonable to predict that the highly charged C-terminus of ZitB and other CDFs resides in the cytoplasm, cis to uptake [3]. Mutational analysis of this region has shown at least three mutations that affected the phenotypic activity of the transporter: G248T and W245L, which partially reduced the zinc resistance of zinc-sensitive *E. coli* GG48 cells expressing the mutated ZitB on a plasmid, and E214A, which reduced zinc resistance to a level near that of non-complemented GG48 cells [25]. The latter result seems contrary to the "funneling" hypothesis since, if E214 were merely part of the funnel, the mutation would not be expected to completely abolish the ability of ZitB to protect the cells from the relatively high Zn^{2+} concentrations used [25]. On the other hand, this residue is only 15 residues away from the C-terminal end of TMD VI, and so it could conceivably be part of the cation-binding site proper, a hypothesis supported by its high level of conservation. Further evidence against the idea of a ZnuA-like flexible length of random coil in ZitB is the G248T mutation. Unless it is a part of an important secondary structure, there is nothing to suggest that the mutation of the residue would result in any phenotypic change, as glycine is far better known for its utility in β turns and helix-helix contact points than its ability to interact with ions. Indeed, a cursory secondary structure prediction of the C-terminus of ZitB suggests that this glycine lies in the turn between two β-strands (data not shown). This does not preclude the C-terminus from sharing its ZnuA counterpart's function, just its coiled structure.

The core of investigations into transport protein function is to determine how the proteins get their substrate(s) across the membrane. Carboxylate groups have been implicated as key players in transporting protons across membranes, and they are also significant zinc ligands [59, 61]. Bacteriorhodopsin, a well-studied example, seems to pass protons along a sequence of glutamates and aspartates, from one side of the membrane to the other (as reviewed by Lanyi [24]). Changes in side-chain p K_a are caused by conformational shifts, and subsequent electrostatic interactions, as a result of the protonation of a preceding residue in the proton-transfer sequence. Since CDF metal-substrate transport is coupled to proton antiport, one could predict that CDFs employ this process in some manner. Results of mutagenesis of the glutamate and four aspartates within the TMDs of ZitB, and their CzcD counterparts, are in agreement with this idea, as alanine mutation of all such residues had a detrimental effect on phenotype [4, 25]. Further, in the case of D186, an alanine mutation abolished zinc resistance in the host, while a glutamate mutation rendered it only about half as resistant as wild type [4, 25]. A similar result is seen with glutamate and alanine D50 mutants; however, the alanine-substituted protein appears to be somewhat toxic or to cause hyperaccumulation, as zinc resistance is below that of the negative control, which carries no ZitB at all [4]. These acidic residues are also well conserved throughout the CDF family (Fig. 1).

Another conserved acidic residue, D163/D158 from the fifth TMD of ZitB and CzcD, respectively, is functionally intolerant to even glutamate substitution [4, 25]. Analysis of D157, the equivalent residue from FieF (*E. coli*), has shown it to be functionally tolerant of D157C mutation (Fig. 2) (D. Fu, personal communication). Despite being embedded in the membrane, D157C was susceptible to cysteine-labelling from both the periplasm and the cytosol (D. Fu, personal communication). Interestingly, this labeling was blocked by pre-incubation with Zn^{2+} or Cd^{2+}, in a concentration-dependent manner, with complete blockage occurring between 32 and 64 μM, respectively (divalent cations of Hg, Mg, Ca, Mn, Fe, and Co had no effect up to 200 μM; D. Fu, personal communication). Taken together, the high conservation of this residue, homologous mutational analysis, its availability to both sides of the membrane, and indirect data of its ability to bind demonstrated FieF substrates, suggest that D157 is directly involved in metal-substrate binding and/or transport.

Histidines, of which there are two within the TMDs of ZitB, might also act as zinc-binding residues. Upon mutation, H53R results in loss of a zinc-resistant phenotype in cells overexpressing the mutant protein [25]. Sequence alignment of a range of CDFs indicates high conservation of this residue. The second, H159, produces the same result when mutated to either arginine or alanine [4, 25]. While this residue is conserved only in 8/12 randomly selected, inter-kingdom CDF sequences, it is interesting to note that the other 5 are substituted with glutamate, another potential zinc ligand (Fig. 1). Together, these data suggest that ZitB, and likely other CDFs, use a combination of histidine, glutamate, and/or aspartate in moving zinc across the membrane, with the latter two residues probably also involved in proton translocation. The specific ion involved with each residue is not known, nor can it be assumed that some of the residues are not involved with both (or neither) of the ions.

The role of CDF proteins and other transporters of the cytoplasmic membrane in Gram-negative metal homeostasis

Genomic sequencing of *C. metallidurans* CH34 (http://genome.jgi-psf.org/draft_microbes/ralme/ralme.home.

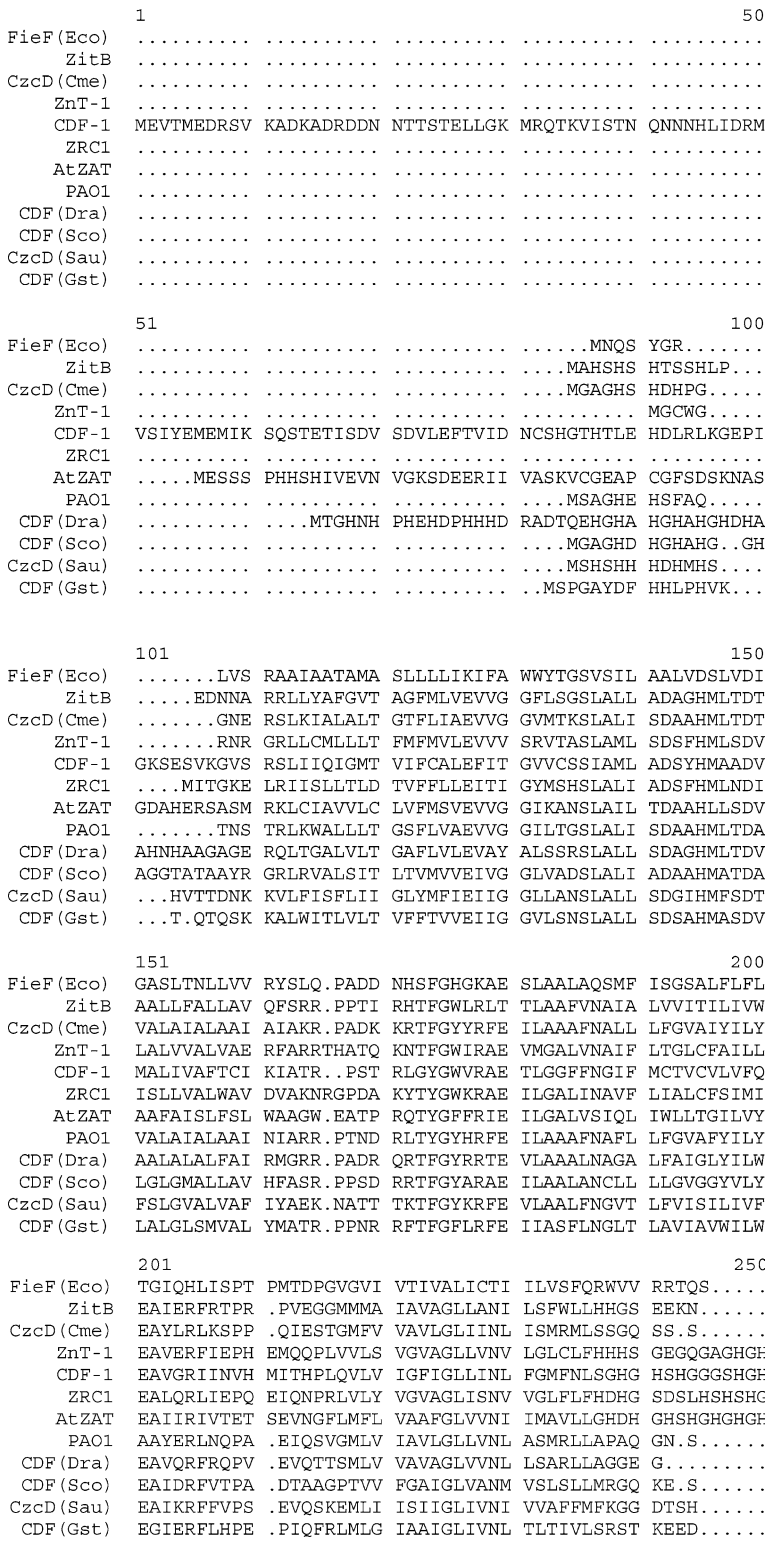
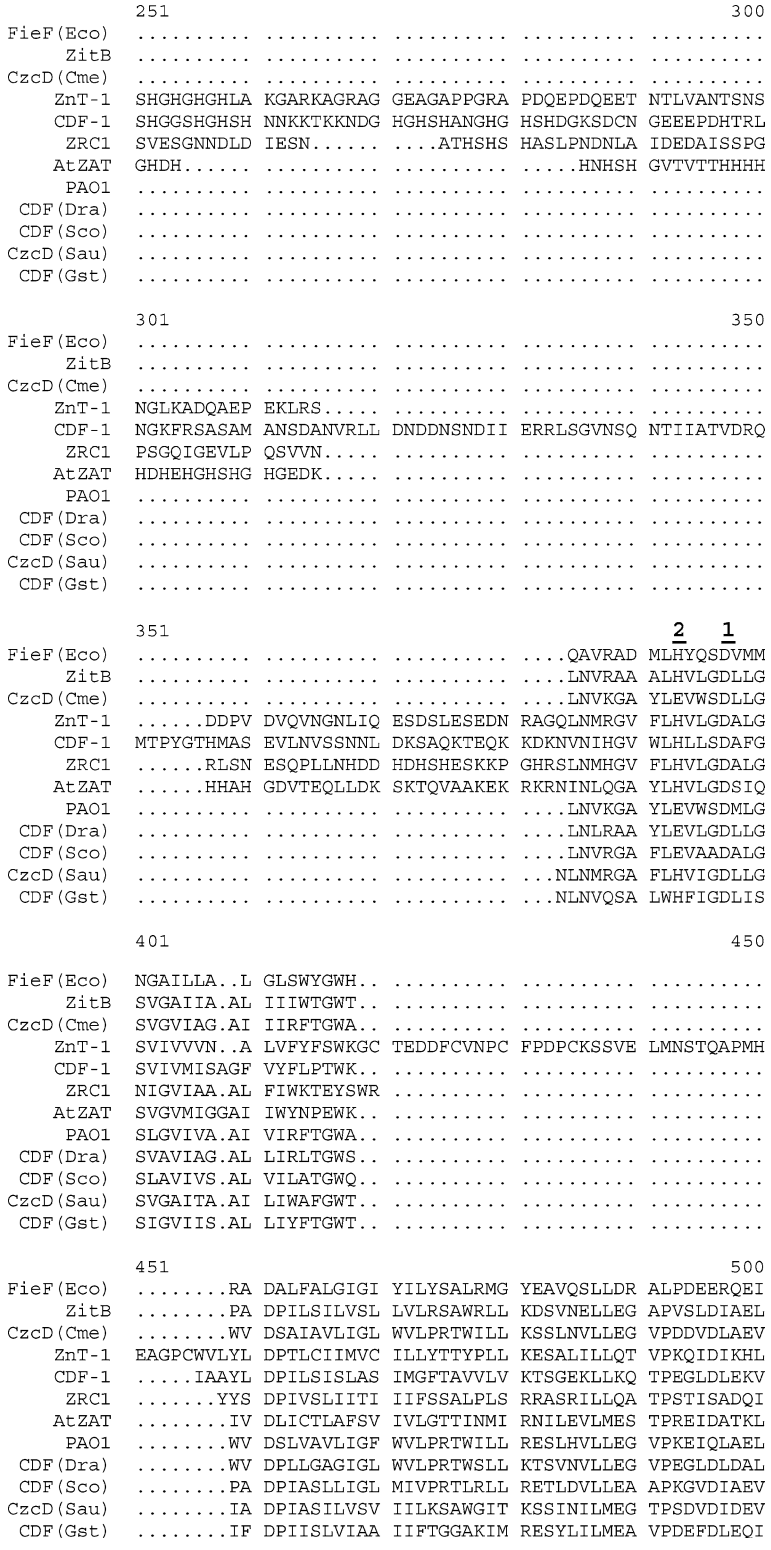


Fig. 1 Inter-kingdom CDF protein sequence alignment. Accession numbers (characterized proteins in bold): ***FieF*** (*Escherichia coli*) P32159, ***ZitB*** (*E. coli*) P75757, ***CzcD*** (*Cupriavidus metallidurans*) P13512, ***ZnT-1*** (*Rattus norvegicus*) Q62720, ***CDF-1*** (*Caenorhabditis elegans*) NP509096, ***ZRC1*** (*Saccharomyces cerevisiae*) P20107, ***PAO1*** (*Pseudomonas aeruginosa*) AAG03786, ***AtZAT*** (*Arabidopsis thaliana*) NP850459.1, *CDF(Dra)* CDF from *Deinococcus radiodurans* A75437, *CDF(Sco)* CDF from *Streptomyces coelicolor* T35276, *CDF(Sau)* CDF from *Staphylococcus aureus* BAA36686, *CDF(Gst)* CDF from *Geobacillus stearothermophilus* BAA19587. *1* Location of FieF D157, *2* location of ZitB H159

html) [32, 57] identified putative genes for two additional CDF genes besides the known CDF-protein CzcD. One was named FieF due to its similarity to FieF from *E. coli*, the second, divalent metal efflux (DmeF) [36]. Expression of *fieF* from *C. metallidurans* led to decreased iron-accumulation and zinc-accumulation in *E. coli*. In *C. metallidurans*, a *fieF*-disruption resulted in a small decrease in the resistance against Co(II), Ni(II) und Cd(II). The DmeF protein showed a similar substrate spectrum as FieF from *C. metallidurans* but it was of central importance for Co(II)-homeostasis: a disruption of *dmeF* caused functional inactivation of high-level

Fig. 1 (Contd.)



resistance systems such as Czc (Co/Zn/Cd) or Cnr (Co/Ni) [36].

Czc [40] and Cnr [28] are resistance nodulation division (RND) systems of the heavy metal efflux (HME) family, also known as transenvelope transport-exporters. These transporters are homotrimers with 12 TMDs and huge periplasmic portions necessary for substrate recognition and interaction with auxiliary proteins [55]. Significant progress has recently been made in solving the three-dimensional structure of components of the tripartite RND-type transporters, The first RND, AcrB from *E. coli*, was recently

Fig. 1 (Contd.)

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            501                                                       550
FieF(Eco)   I.DIVTSWPG VSGAHDLRTR QSGP.TRFIQ IHLEMEDS.L PLVQAHMVAD
     ZitB   KRRMCREIPE VRNVHHVHVW MVGE.KPVMT LHVQVIP... .PHDHDALLD
CzcD(Cme)   E.KQILATPG VKSFHDLHIW ALTSGKASLT VHVVND...T AVNPEMEVLP
    ZnT-1   V.KELRDVEG VEEVHELHVW QLAGSRIIAT AHIKCED... .PASYMQVAK
    CDF-1   K.KDLCSIVG VSKVEKLSVW TLCGQRIIAA AHVNICH... .PAVFPEAAY
     ZRC1   Q.REILAVPG VIAVHDFHVW NLTESIYIAS IHVQID...C APDKFMSSAK
    AtZAT   E.KGLLEMEE VVAVHELHIW AITVGKVLLA CHVNIR.... PEADADMVLN
     PAO1   R.EALLGIPG VTGLHDLHVW SITSGKISLT SHLVYD...P ALVDAEALLG
 CDF(Dra)   R.AELRALPG VQDVHDLHVW SVTGGVVNLT AHLVSD...R APAELLPAVH
 CDF(Sco)   R.AHILALDG VEDVHDLHAW TITSGMPVLS AHVVVDGEAL SAIGHEKMLH
CzcD(Sau)   I.TTIKKDSR IQSVHDCHVW TISNDMNALS CHVVVDHT.L TMKECELLLE
 CDF(Gst)   R.ADIRNIEG VEDVHDMHLW AISTDHYSLS AHVFVN.... EHIQPLCVIL

            551                                                       600
FieF(Eco)   QVEQAILRRF PGSDVIIHQD PCSVVPREGK RSMLS..... ..........
     ZitB   QIQHYLMDHY QIEHATIQME YQPCHGPDCH LNEGVSGHSH HHH.......
CzcD(Cme)   ELKQMLADKF DITHVTIQFE LAPCEQADAA QHFNASPALV GSKSLAAGGN
    ZnT-1   TIKDVFHNHG .IHATTIQPE FASVGSKSSV VPCELACRTQ CALKQCCGTR
    CDF-1   KIKNYFHDLG .VHSTTIEPT FEDTCMQSMR IMVKKVVDGK SIEEPVSVST
     ZRC1   LIRKIFHQHG .IHSATVQPE FVSGDVNEDI RRRFSIIAGG SPSSSQEAFD
    AtZAT   KVIDYIRREY NISHVTIQIE R......... .......... ..........
     PAO1   TVKALLHDRY EIEHSTLQLE TSACAQAEEP LAY....... ..........
 CDF(Dra)   EVAHGAG... .IEHVTVQVE PPGLHATDAA LHP....... ..........
 CDF(Sco)   ELQGCLGDHF DVEHCTFQLE PSGHAEHEAR LCR....... ..........
CzcD(Sau)   NIEHDLLHLN .IHHMTIQLE TPNHKHDESI ICSGTHSHSH NHHAHHHAHV
 CDF(Gst)   AVNEMLKEKY GIEHSTIQVE HAILHDHGSY GKAFLEKRKA SQP.......

            601                                                       650
FieF(Eco)   .......... .......... .......... .......... ..........

     ZitB   .......... .......... .......... .......... ..........
CzcD(Cme)   .......... .......... .......... .......... ..........
    ZnT-1   PQVHSGKEAE KAPTVSISCL ELSENLEKKP RRTKAEGSVP AVVIEIKNVP
    CDF-1   ENEITE.... .......... .......... .......... ..........
     ZRC1   SHGNTEHGRK KRSPTAYGAT TASSNCIVDD AVNCNTSNCL ..........
    AtZAT   .......... .......... .......... .......... ..........
     PAO1   .......... .......... .......... .......... ..........
 CDF(Dra)   .......... .......... .......... .......... ..........
 CDF(Sco)   .......... .......... .......... .......... ..........
CzcD(Sau)   H......... .......... .......... .......... ..........
 CDF(Gst)   .......... .......... .......... .......... ..........

            651     658
FieF(Eco)   ........
     ZitB   ........
CzcD(Cme)   ........
    ZnT-1   NKQPESSL
    CDF-1   ........
     ZRC1   ........
    AtZAT   ........
     PAO1   ........
 CDF(Dra)   ........
 CDF(Sco)   ........
CzcD(Sau)   ........
 CDF(Gst)   ........
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crystallized by two independent laboratories [37, 50]. As derived from experimental data and theoretical considerations [45], RND transporters accept their substrates from the periplasm (or from the cytoplasmic membrane in the case of hydrophobic substances such as dyes) rather than the cytoplasm [26, 30, 42, 43, 52] (Fig. 2).

In *C. metallidurans* CH34, the RND-type efflux complex CzcCBA is responsible for high-level resistance against cadmium, zinc and cobalt [40, 41, 54]. It has been shown that the Czc-system is an efflux pump energized by the proton-motive force [16, 38]. However, although expression of *czcCBA* results in extraordinarily high resistance against metals (in the high millimolar range) in wild type cells, the Czc-system by itself is probably not able to detoxify the cell from substrate metals. For a functional Czc-system, other transporters of the cytoplasmic membrane, such as CDF transporters or P-type ATPases, are needed.

Recently, the interplay of DmeF and FieF with CzcCBA in *C. metallidurans* was demonstrated [36]. The Co(II)-transporting CDF-protein DmeF was essential for CzcCBA-mediated Co(II) detoxification. Without DmeF, but in the presence of the two independent HME-RND systems Czc and Cnr (cobalt, nickel resistance), Co(II) resistance was reduced to less than 1% of that of wild-type. Resistance was further diminished when the gene for the CDF protein CzcD, which is part of the *czc*-determinant, was additionally deleted [36]. This effect was also demonstrated with the P-type ATPases ZntA and CadA from *C. metallidurans*. These proteins both mediate Zn(II) and Cd(II) tolerance in *E. coli*, and probably transport the same substrates in their indigenous host. When the genes of both P-type

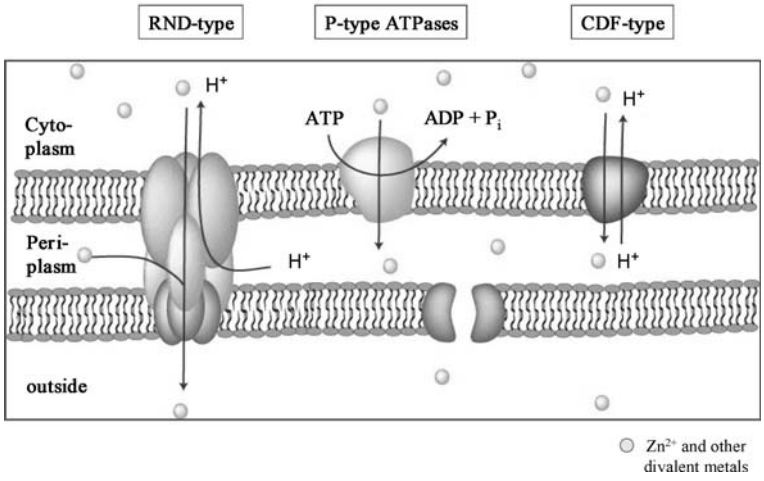

Fig. 2 Interplay of transporters of different metal efflux families in metal detoxification. High-level metal resistance is accomplished by the interplay of at least three families of metal efflux transporters in a two-step process. Cation diffusion facilitator (CDF) proteins translocate substrates across the cytoplasmic membrane utilizing the proton-motive force for energization. Likewise, CPx-type or P-type ATPases transport metal cations under ATP-hydrolysis from the cytoplasm to the periplasm. Tripartite HME-RND efflux complexes couple the antiport of protons with the efflux of periplasmic cations to the outside. Reentry of metal cations under high metal concentration might be prevented by the expression of selective porins in the outer membrane

ATPases were inactivated, cadmium resistance decreased even in the presence of CzcCBA [26]. This clearly indicates that, for metal-efflux, in addition to the HME-RND system, a second transporter is necessary that translocates metals across the cytoplasmic membrane because RNDs cannot acquire substrate directly from the cytoplasm.

Against this background, the genetic organization of many plasmid-encoded HME-RND-determinants makes sense. In close vicinity to genes of HME-RNDs, genes encoding CDF-proteins, major facilitator superfamily (MFS) proteins, or P-type-ATPases that share the same, or very similar, substrate specificity as the neighboring HME-RND, can be found. Examples include *czcD* (CDF), *nreB* (MFS), *cnrT* (MFS) or *silP* (P-type), being part of the *czc*-Cd(II), Zn(II), Co(II)-, the *ncc*-Ni(II), Co(II), Cd(II)-, the *cnr*-Co(II), Ni(II)- or the *sil*-Ag(I)-determinant. Thus, such a determinant comprises a functional unit that can be horizontally transferred even into hosts lacking one component of the set of efflux systems, a crucial arrangement if the functions of these proteins are interdependent.

Conclusions

Despite their ubiquity, the structure and mechanism of action of CDF proteins remains largely unknown. Much of the most instructive data has emerged within the last few years. The available evidence supports the hypothesis that CDFs can couple antiported protons to the translocation of divalent metal cation substrates across a membrane, with a protein (dimer):metal:

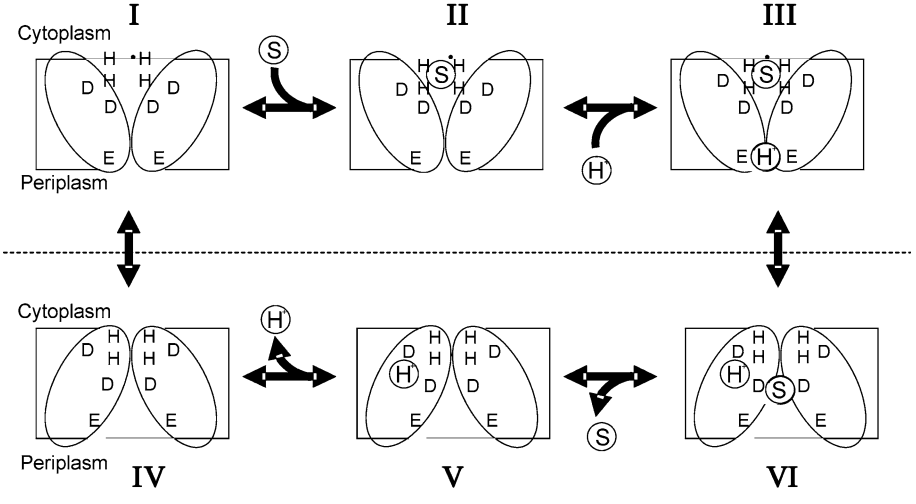

Fig. 3 Proposed CDF-transporter mechanism. Dimeric CDF-proteins located at the cytoplasmic membrane bind substrate metal cations (*S*) from the cytoplasm, probably via histidines in a "cytoplasm-open" confirmation. Protons (H^+) from the periplasm bind to negatively charged residues in trans to the substrate and a conformational change towards "periplasm-open" state is induced. Substrate and proton are subsequently released and the initial "cytoplasm-open" conformation is restored (adapted from Vazquez-Ibar et al. [58])

proton ratio of 1:1.23:1. This arrangement is electrogenic, creating a positive-outside electrochemical potential, suggesting that $\Delta\Psi$ also has a role in driving substrate transport, as demonstrated in the case of ZitB [9]. Additional experimental data using different CDF proteins, especially those derived from eukaryotes, are needed, both to solidify the universality of proton antiport and to determine if/how $\Delta\Psi$ contributes to CDF function in vivo. Important pieces of the available kinetic data are inconsistent, and the methods used to gather them still imperfect. Methods need to be devised that can account for such factors as protein orientation in the proteoliposomes and the metal-binding capacity of the systems themselves, if accurate K_m values are to be determined. Substrate binding, possibly the most important step of the process in terms of biotechnological applications, is thought to be a two-step process in ZitB, involving a rapid substrate-protein binding step followed by a slower, rate-limiting, conformational change, which releases the substrate trans to uptake [9]. This binding is likely facilitated by cysteine and histidine residues [9] (D. Fu, personal communication). The mechanism by which substrate reaches these residues is unclear. The charged C-terminus of most CDFs could act as a substrate accumulator or perhaps it serves as a chaperone-docking site, as studies on RAF1 have suggested [22].

Many fundamental structural questions remain unanswered, though not through lack of effort. Membrane proteins are notoriously difficult to prepare for X-ray crystallography, and CDF transporters are too large for most NMR applications, though individual regions such as the cytoplasmic C-termini of many CDF proteins, may lend themselves to the latter approach. Despite this, CDFs have been shown to exist as homodimers, the monomers of which generally consist of six transmembrane regions, with both termini cis to uptake. It remains to be seen whether exceptionally large CDFs, such as MSC2 and ZnT5, which contain 12 and 15 TMDs, respectively, also dimerize. Now that an accurate method has been devised for determining CDF mass, this question could soon be resolved [60].

The mechanism of substrate translocation remains an open question for all transport proteins, with the possible exception of bacteriorhodopsin and the lactose permease LacY from *E. coli*—currently the best characterized models [1, 24]. Since CDFs also have a proton translocating function, it is not unlikely they employ some of the same mechanisms as these two proteins. One possibility is that the conformational changes that allow the protons to proceed trans-cis (relative to metal-substrate uptake) simultaneously, create a similar cis-trans pathway for substrate translocation, or vice versa. This hypothesis requires either two channels or two paths within the same channel, as the substrate and protons would likely interfere with each other's interaction with pathway residues. Under the LacY paradigm, however, dual paths are not required because the substrates are translocated sequentially [1]. In ZitB, for instance, the protonated zinc-binding site would be open only to the cis face of the protein. After zinc binds, conformational changes "close" the cis face while opening the trans face for substrate release. The process is then reversed: the binding site, now on the trans face, is protonated, causing closure and re-opening at cis side of the membrane. Alternatively, cis substrate and trans proton binding could be simultaneously required to elicit the conformational changes required to open the trans face of the protein, with reversion to the prior state being dependent upon substrate release (Fig. 3). Data from FieF challenges these idealized models, as D157 is accessible to substrate from both sides of the membrane (D. Fu, personal communication). However, in the case of the sequential-binding model, this reside could be involved with both trans substrate release and subsequent proton binding, a situation that would allow binding from either side of the membrane. Determination of the spatial arrangement of intramembrane charged residues, particularly histidines, glutamates, and aspartates, is needed to begin to validate the possibility of either mechanism.

Most CDFs have, thus far, been studied out of their cellular context, with broad assumptions as to their utility made based on their cellular and/or organismal location. Recent data from DmeF and FieF (*C. metallidurans*) has put these proteins, and perhaps other bacterial CDFs, into a cellular context [36]. A likely scenario is that, in their perceived role as homeostatic transporters, CDFs effectively store small excesses of cations in the periplasm, where they can be readily reacquired as needed. Under conditions of higher metal stress, these periplasmic cations can be extracellularly removed from the periplasm by RND-type transporters. Combined with the potential Raf1/CDF1 interplay [22], these data suggest that CDFs may function as cogs in larger cellular processes, rather than as discreet systems unto themselves. However, as suggested above, this may be situation-dependent.

CDFs are only beginning to emerge as a thoroughly characterized protein family. While the focus for many researchers has been the eventual exploitation of these proteins in the bioremediation of metal-contaminated environments, it has become clear that the family is not limited to this end. From the potential drug-resistance implications of the CDF protein CepA from *K. pneumoniae* to their documented role in an oncogene pathway, CDFs appear to be involved in far more than just metal homeostasis [15, 22].

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References

1. Abramson J, Iwata S, Kaback HR (2004) Lactose permease as a paradigm for membrane transport proteins (Review). Mol Membr Biol 21:227–236

2. Andrews SC, Robinson AK, Rodriguez-Quinones F (2003) Bacterial iron homeostasis. FEMS Microbiol Rev 27:215–237
3. Anton A, Grosse C, Reissmann J, Pribyl T, Nies DH (1999) CzcD is a heavy metal ion transporter involved in regulation of heavy metal resistance in *Ralstonia* sp. strain CH34. J Bacteriol 181:6876–6881
4. Anton A, Weltrowski A, Haney CJ, Franke S, Grass G, Rensing C, Nies DH (2004) Characteristics of zinc transport by two bacterial cation diffusion facilitators from *Ralstonia metallidurans* CH34 and *Escherichia coli*. J Bacteriol 186:7499–7507
5. Banerjee S, Wei B, Bhattacharyya-Pakrasi M, Pakrasi HB, Smith TJ (2003) Structural determinants of metal specificity in the zinc transport protein ZnuA from *Synechocystis* 6803. J Mol Biol 333:1061–1069
6. Blaudez D, Kohler A, Martin F, Sanders D, Chalot M (2003) Poplar metal tolerance protein 1 confers zinc tolerance and is an oligomeric vacuolar zinc transporter with an essential leucine zipper motif. Plant Cell 15:2911–2928
7. Bloß T, Clemens S, Nies DH (2002) Characterization of the ZAT1p zinc transporter from *Arabidopsis thaliana* in microbial model organisms and reconstituted proteoliposomes. Planta 214:783–791
8. Bruinsma JJ, Jirakulaporn T, Muslin AJ, Kornfeld K (2002) Zinc ions and cation diffusion facilitator proteins regulate Ras-mediated signaling. Dev Cell 2:567–578
9. Chao Y, Fu D (2004a) Kinetic study of the antiport mechanism of an *Escherichia coli* zinc transporter, ZitB. J Biol Chem 279:12043–12050
10. Chao Y, Fu D (2004b) Thermodynamic studies of the mechanism of metal binding to the *Escherichia coli* zinc transporter YiiP. J Biol Chem 279:17173–17180
11. Conklin DS, McMaster JA, Culbertson MR, Kung C (1992) COT1, a gene involved in cobalt accumulation in *Saccharomyces cerevisiae*. Mol Cell Biol 12:3678–3688
12. Delhaize E, Kataoka T, Hebb DM, White RG, Ryan PR (2003) Genes encoding proteins of the cation diffusion facilitator family that confer manganese tolerance. Plant Cell 15:1131–1142
13. Eide DJ, Bridgham JT, Zhao Z, Mattoon JR (1993). The vacuolar H^+-ATPase of *Saccharomyces cerevisiae* is required for efficient copper detoxification, mitochondrial function, and iron metabolism. Mol Gen Genet 241:447–456
14. Eng BH, Guerinot ML, Eide D, Saier MH Jr (1998) Sequence analyses and phylogenetic characterization of the ZIP family of metal ion transport proteins. J Membr Biol 166:1–7
15. Fang CT, Chen HC, Chuang YP, Chang SC, Wang JT (2002) Cloning of a cation efflux pump gene associated with chlorhexidine resistance in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 46:2024–2028
16. Goldberg M, Pribyl T, Juhnke S, Nies DH (1999) Energetics and topology of CzcA, a cation/proton antiporter of the resistance-nodulation-cell division protein family. J Biol Chem 274:26065–26070
17. Grass G, Otto M, Fricke B, Haney CJ, Rensing C, Nies DH, Munkelt D (2005) FieF (YiiP) from *Escherichia coli* mediates decreased cellular accumulation of iron and relieves iron stress. Arch Microbiol 183:9–18
18. Grunberg K, Wawer C, Tebo BM, Schuler D (2001) A large gene cluster encoding several magnetosome proteins is conserved in different species of magnetotactic bacteria. Appl Environ Microbiol 67:4573–4582
19. Guffanti AA, Wei Y, Rood SV, Krulwich TA (2002) An antiport mechanism for a member of the cation diffusion facilitator family: divalent cations efflux in exchange for K^+ and H^+. Mol Microbiol 45:145–153
20. Hoyos B, Imam A, Korichneva I, Levi E, Chua R, Hammerling U (2002) Activation of c-Raf kinase by ultraviolet light. Regulation by retinoids. J Biol Chem 277:23949–23957
21. Huang L, Kirschke CP, Gitschier J (2002) Functional characterization of a novel mammalian zinc transporter, ZnT6. J Biol Chem 277:26389–26395
22. Jirakulaporn T, Muslin AJ (2004) Cation diffusion facilitator proteins modulate Raf-1 activity. J Biol Chem 279:27807–27815
23. Kambe T, Narita H, Yamaguchi-Iwai Y, Hirose J, Amano T, Sugiura N, Sasaki R, Mori K, Iwanaga T, Nagao M (2002) Cloning and characterization of a novel mammalian zinc transporter, zinc transporter 5, abundantly expressed in pancreatic beta cells. J Biol Chem 277:19049–19055
24. Lanyi JK (1997) Mechanism of ion transport across membranes. Bacteriorhodopsin as a prototype for proton pumps. J Biol Chem 272:31209–31212
25. Lee SM, Grass G, Haney CJ, Fan B, Rosen BP, Anton A, Nies DH, Rensing C (2002) Functional analysis of the *Escherichia coli* zinc transporter ZitB. FEMS Microbiol Lett 215:273–278
26. Legatzki A, Grass G, Anton A, Rensing C, Nies DH (2003) Interplay of the Czc system and two P-type ATPases in conferring metal resistance to *Ralstonia metallidurans*. J Bacteriol 185:4354–4361
27. Li L, Kaplan J (2001) The yeast gene MSC2, a member of the cation diffusion facilitator family, affects the cellular distribution of zinc. J Biol Chem 276:5036–5043
28. Liesegang H, Lemke K, Siddiqui RA, Schlegel HG (1993) Characterization of the inducible nickel and cobalt resistance determinant *cnr* from pMOL28 of *Alcaligenes eutrophus* CH34. J Bacteriol 175:767–778
29. Liuzzi JP, Blanchard RK, Cousins RJ (2001) Differential regulation of zinc transporter 1, 2, and 4 mRNA expression by dietary zinc in rats. J Nutr 131:46–52
30. Lomovskaya O, Zgurskaya HI, Nikaido H (2002) It takes three to tango. Nat Biotechnol 20:1210–1212
31. MacDiarmid CW, Milanick MA, Eide DJ (2002) Biochemical properties of vacuolar zinc transport systems of *Saccharomyces cerevisiae*. J Biol Chem 277:39187–39194
32. Mergeay M, Nies D, Schlegel HG, Gerits J, Charles P, Van Gijsegem F (1985) *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J Bacteriol 162:328–334
33. Michalczyk A, Varigos G, Catto-Smith A, Blomeley RC, Ackland ML (2003) Analysis of zinc transporter, hZnT4 (Slc30A4), gene expression in a mammary gland disorder leading to reduced zinc secretion into milk. Hum Genet 113:202–210
34. Michaud NR, Fabian JR, Mathes KD, Morrison DK (1995) 14-3-3 is not essential for Raf-1 function: identification of Raf-1 proteins that are biologically activated in a 14-3-3-independent and Ras-independent manner. Mol Cell Biol 15:3390–3397
35. Morrison DK, Cutler RE (1997) The complexity of Raf-1 regulation. Curr Opin Cell Biol 9:174–179
36. Munkelt D, Grass G, Nies DH (2004) The chromosomally encoded cation diffusion facilitator proteins DmeF and FieF from *Wautersia metallidurans* CH34 are transporters of broad metal specificity. J Bacteriol 186:8036–8043
37. Murakami S, Nakashima R, Yamashita E, Yamaguchi A (2002) Crystal structure of bacterial multidrug efflux transporter AcrB. Nature 419:587–593
38. Nies DH (1995) The cobalt, zinc, and cadmium efflux system CzcABC from *Alcaligenes eutrophus* functions as a cation-proton antiporter in *Escherichia coli*. J Bacteriol 177:2707–2712
39. Nies DH (2003) Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev 27:313–339
40. Nies D, Mergeay M, Friedrich B, Schlegel HG (1987) Cloning of plasmid genes encoding resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus* CH34. J Bacteriol 169:4865–4868
41. Nies DH, Nies A, Chu L, Silver S (1989) Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*. Proc Natl Acad Sci USA 86:7351–7355
42. Nikaido H, Basina M, Nguyen V, Rosenberg EY (1998) Multidrug efflux pump AcrAB of *Salmonella typhimurium* excretes only those *beta*-lactam antibiotics containing lipophilic side chains. J Bacteriol 180:4686–4692

43. Nikaido H, Zgurskaya HI (2001) AcrAB and related multidrug efflux pumps of *Escherichia coli*. J Mol Microbiol Biotechnol 3:215–218
44. Outten CE, O'Halloran TV (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292:2488–2492
45. Palmer M (2003) Efflux of cytoplasmically acting antibiotics from gram-negative bacteria: periplasmic substrate capture by multicomponent efflux pumps inferred from their cooperative action with single-component transporters. J Bacteriol 185:5287–5289
46. Palmiter RD, Findley SD (1995) Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. EMBO J 14:639–649
47. Paulsen IT, Saier MH Jr (1997) A novel family of ubiquitous heavy metal ion transport proteins. J Membr Biol 156:99–103
48. Persans MW, Nieman K, Salt DE (2001) Functional activity and role of cation-efflux family members in Ni hyperaccumulation in *Thlaspi goesingense*. Proc Natl Acad Sci USA 98:9995–10000
49. Pilon-Smits E, Pilon M (2002) Phytoremediation of metals using transgenic plants. Crit Rev Plant Sci 21:439–456
50. Pos KM, Diederichs K (2002) Purification, crystallization and preliminary diffraction studies of AcrB, an inner-membrane multi-drug efflux protein. Acta Crystallogr D Biol Crystallogr 58):1865–1867
51. Ramsay LM, Gadd GM (1997) Mutants of *Saccharomyces cerevisiae* defective in vacuolar function confirm a role for the vacuole in toxic metal ion detoxification. FEMS Microbiol Lett 152:293–298
52. Rensing C, Grass G (2003) *Escherichia coli* mechanisms of copper homeostasis in a changing environment. FEMS Microbiol Rev 27:197–213
53. Rensing C, Mitra B, Rosen BP (1997a) The *zntA* gene of *Escherichia coli* encodes a Zn(II)-translocating P-type ATPase. Proc Natl Acad Sci USA 94:14326–14331
54. Rensing C, Pribyl T, Nies DH (1997b) New functions for the three subunits of the CzcCBA cation-proton antiporter. J Bacteriol 179:6871–6879
55. Saier MH Jr, Tam R, Reizer A, Reizer J (1994) Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. Mol Microbiol 11:841–847
56. Stein WD (1990) Channels, carriers and pumps: an introduction to membrane transport. Academic Press, San Diego, Calif.
57. Vaneechoutte M, Kampfer P, De Baere T, Falsen E, Verschraegen G (2004) *Wautersia* gen. nov., a novel genus accommodating the phylogenetic lineage including *Ralstonia eutropha* and related species, and proposal of *Ralstonia* [*Pseudomonas*] *syzygii* (Roberts et al. 1990) comb. nov. Int J Syst Evol Microbiol 54:317–327
58. Vazquez-Ibar JL, Guan L, Weinglass AB, Verner G, Gordillo R, Kaback HR (2004) Sugar recognition by the lactose permease of *Escherichia coli*. J Biol Chem 279:49214–49221
59. Vik SB, Antonio BJ (1994) A mechanism of proton translocation by F1F0 ATP synthases suggested by double mutants of the a subunit. J Biol Chem 269:30364–30369
60. Wei Y, Li H, Fu D (2004) Oligomeric state of the *Escherichia coli* metal transporter YiiP. J Biol Chem 279:39251–39259
61. Wiebe CA, Rieder C, Young PG, Dibrov P, Fliegel L (2003) Functional analysis of amino acids of the Na^+/H^+ exchanger that are important for proton translocation. Mol Cell Biochem 254:117–124