MINIREVIEW

Switch or Funnel: How RND-Type Transport Systems Control Periplasmic Metal Homeostasis[▽]

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Bacteria have evolved several transport mechanisms to maintain metal homeostasis and to detoxify the cell. One mechanism involves an RND (resistance-nodulation-cell division protein family)-driven tripartite protein complex to extrude a variety of toxic substrates to the extracellular milieu. These efflux systems are comprised of a central RND proton-substrate antiporter, a membrane fusion protein, and an outer membrane factor. The mechanism of substrate binding and subsequent efflux has yet to be elucidated. However, the resolution of recent protein crystal structures and genetic analyses of the components of the heavy-metal efflux family of RND proteins have allowed the developments of proposals for a substrate transport pathway. Here two models of substrate extrusion through RND protein complexes of the heavy-metal efflux protein family are described. The funnel model involves the shuttling of periplasmic substrate from the membrane fusion protein to the RND transporter and further on through the outer membrane factor to the extracellular space. Conversely, the switch model requires substrate binding to the membrane fusion protein, inducing a conformational change and creating an open-access state of the tripartite protein complex. The extrusion of periplasmic substrate bypasses the membrane fusion protein, enters the RND-transporter directly via its substrate-binding site, and is ultimately eliminated through the outer membrane channel. Evidence for and against the two models is described, and we propose that current data favor the switch model.

In Gram-negative bacteria, RND-driven tripartite protein complexes pump out a wide array of substrates. The RND (resistance-nodulation-cell division) protein superfamily can be divided into seven protein families with members being involved in transport of organic substances, transition metals, and polypeptides (55). Two RND families have been most extensively studied. The hydrophobe/amphiphile efflux family includes the RND proteins AcrB from *Escherichia coli* and MexB from *Pseudomonas aeruginosa*, both of which are involved in export of organic substances such as various antibiotics, bile salts, and other hydrophobic substances. The heavy metal efflux family contains metal transporters, such as CzcA from *Cupriavidus metallidurans* strain CH34 and CusA from *E. coli*. The nonspecificity of the RND proteins exporting organic substances has made elucidation of the actual transport pathway and pump regulation difficult. In contrast, metal-transporting RND proteins have a limited substrate spectrum, pumping out either the monovalent cations Cu(I) and Ag(I) or divalent cations of the transition metals Zn(II), Ni(II), Co(II), and sometimes the heavy metal cation Cd(II) (9, 25, 33). Since metals bind to very specific residues, the elucidation of the transport pathway should be easier.

RND proteins utilize the proton motive force to drive the efflux of the substrates (14, 32). They form homotrimers (29) or heterotrimers composed of two different RND polypeptides in a 2:1 ratio (18). Each RND monomer spans the inner membrane with 12 transmembrane alpha helices. The RND trimer contains a large hydrophilic portion that extends into the periplasmic space. Conformational changes for each monomer between three states (open access for substrate binding, substrate-bound, and extrusion to the outer membrane protein) drive proton-coupled export for these systems (28, 47).

The RND trimers are part of a larger tripartite transenvelope protein complex. The periplasmic portion of the RND trimer connects to the second component of the system, the trimeric outer membrane factor (19, 36). The trimer of the outer membrane factor forms a single tube that spans the outer membrane as a beta barrel. One end of this tube opens to the extracellular space and the other connects to an opening on top of the RND trimer.

The third component of the tripartite protein complex is the periplasmic membrane fusion protein (44), which forms a hexameric or trimeric ring around the RND trimer and outer membrane proteins, thereby stabilizing contact between these two trimers (2, 3, 29). In characterized examples of metal-transporting RND-driven protein complexes, CzcA and CusA are the RND proteins, CzcB and CusB the membrane fusion proteins, and CzcC and CusC the outer membrane factors (Fig. 1). Some systems additionally contain soluble periplasmic metal chaperone proteins, such as CusF for the CusCBA complex, which has been shown to deliver substrate to the membrane fusion protein (13, 23). Based on results of RND proteins belonging to the metal transporters and those for organic substances such as CzcCBA and TolC-AcrAB, a 3:6:3 polypeptide ratio of the CusCBA complex has been predicted (41, 50).

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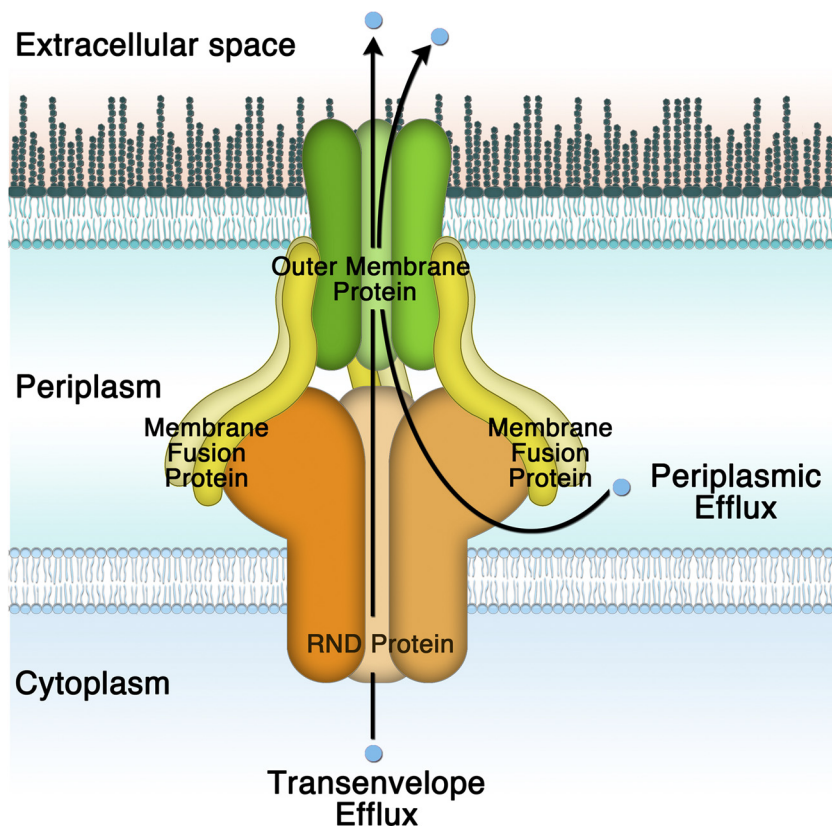
FIG. 1. Model of periplasmic and transenvelope efflux. Transenvelope efflux involves the detoxification of cytoplasmic substrates, which enter the complex from the cytoplasm and through the RND-driven complex protein to the extracellular milieu. Periplasmic efflux involves the extrusion of periplasmic substrates through the RND protein and out the outer membrane protein to the extracellular space.

This would be in contrast to previous modeling attempts where TolC-AcrAB was envisaged to display a 3:3:3 stoichiometry (53). The different models have recently been summarized (26).

The structures of components of the Cus system provide exciting perspectives for understanding the structure and function of RND-driven tripartite systems. The structure of the RND protein CusA (24) established that Cu(I) binds to three methionines in the periplasmic cleft of the inner membrane transport protein. Methionines essential for function of CusA were earlier identified at this position (13), making it probable that these residues form the periplasmic cation substrate-binding site of CusA. Moreover, this substrate-binding site is located in a similar position in the RND proteins AcrB and MexB, which transport organic substances (28, 38, 47). However, in contrast to the rather unspecific substrate binding sites in AcrB and MexB in CusA, a thioether-only binding site, formed by three methionines, confers specificity to Cu(I) and Ag(I) but not to other metals (43).

Two modes of action have been proposed for RND-driven tripartite efflux systems (33): periplasmic efflux and transenvelope efflux (Fig. 1). Periplasmic efflux involves the binding of periplasmic substrates by the RND inner membrane protein and subsequent export through the outer membrane factor to the extracellular space. In contrast, transenvelope efflux suggests transport of cytoplasmic molecules through the entire transmembrane region of the RND protein and the outer

membrane factor into the extracellular milieu, without releasing the substrate into the periplasm. It is very difficult to differentiate between both modes of action by biochemical experiments because any periplasmic efflux would decrease the periplasmic concentration of any substance, thereby decreasing uptake into the cytoplasm. To add more complexity, RND proteins may even mediate both processes, periplasmic efflux and transenvelope efflux. However, the question is, to what extent and under what conditions?

TRANSENVELOPE EFFLUX BY METAL-TRANSPORTING RND PROTEINS?

The structure of the metal-transporting RND protein CusA suggests a possible methionine-lined pathway (M410-M501, M486-M403, M391-M1009) from the cytoplasm to the three conserved methionine residues (M573, M623, M672) that form the periplasmic substrate binding site (24). Such a putative copper transport pathway spanning the transmembrane alpha helices of CusA would argue for transenvelope efflux of copper ions. Indeed, transport of Ag(I) by membrane-reconstituted CusA has been demonstrated (24).

Other studies also have shown that RND proteins might transport substrates across the inner membrane. CzcA transports metals *in vitro* into proteoliposomes (14), and MexB of *Pseudomonas aeruginosa* similarly transports drugs (58). MexB even functions when expressed in Gram-positive bacteria that
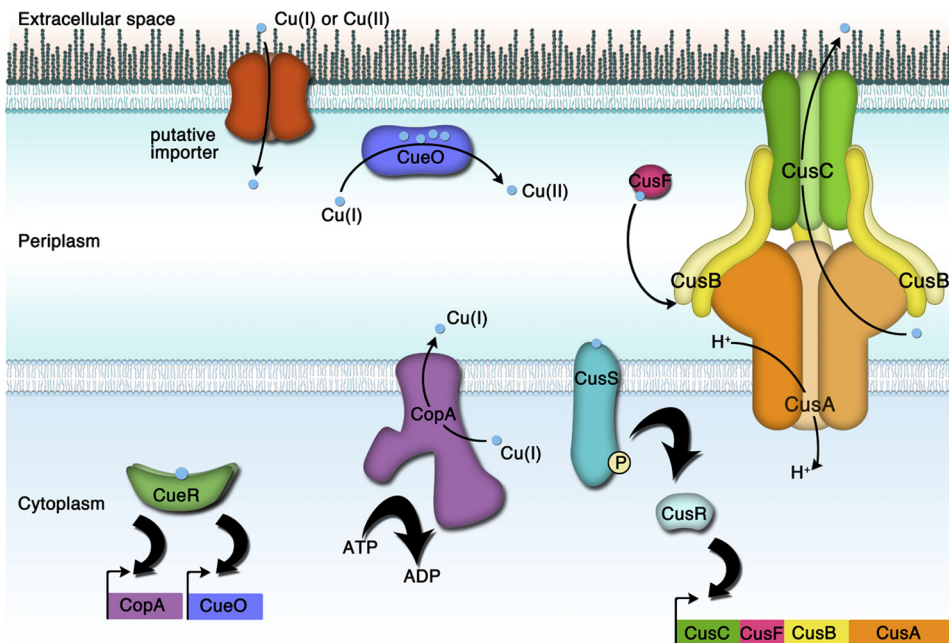
FIG. 2. Copper homeostasis mechanisms in *E. coli*. Several homeostatic mechanisms detoxify the cell of copper. Copper enters the bacterial cell through an unknown importer. CueO, multicopper oxidase; CusF, Cu(I)-binding metallochaperone; CusCBA, RND-driven tripartite complex; CopA, Cu(I)-translocating P-type ATPase; P, phosphate group. CusS, a histidine kinase, senses periplasmic Cu(I) and subsequently phosphorylates CusR, a response regulator, to activate transcription of *cusCFBA*. CueR senses copper and activates transcription of *copA* and *cueO*.

do not have a periplasm (58), since some of these Gram-positive bacteria contain genes for RND proteins in their genomes (33). Additionally, AcrB (10) and CzcA (14) may contain substrate binding sites located in the cytoplasm, and AcrD captures aminoglycosides from this cellular compartment (1). These data suggest transport across a single membrane, but are these results physiologically relevant?

Conversely, metal sensitivity and physiological evidence indicate that the metal-transporting RND proteins CusA and CzcA predominantly carry out efflux from the periplasm to the extracellular space. In *E. coli*, deletion of the gene encoding the Cu(I)-translocating P-type ATPase CopA, which exports cations from the cytoplasm to the periplasm, leads to a copper-sensitive phenotype (39) (Fig. 2). Copper sensitivity is not further increased by the additional deletion of *cusCFBA* (15) but is, however, by deletion of *cueO*, which encodes a periplasmic Cu(I) oxidase (15, 42, 49) (Fig. 2). Thus, CusCBA can be functionally substituted by an enzyme that detoxifies periplasmic Cu(I) by oxidation into the less toxic Cu(II) state, but CusCBA is not able to substitute CopA, the transporter that decreases the cytoplasmic Cu(I) concentration by export across the inner membrane.

A similar scenario emerged when studying the function of the metal-transporting RND-driven tripartite protein complex CzcCBA from *C. metallidurans* CH34 (conferring cobalt, zinc, and cadmium resistance). Deletion of two Zn(II)/Cd(II)-translocating P-type ATPases led to loss of the ability of CzcCBA to confer Cd(II) resistance (22). A deletion of two Co(II)/Zn(II) transporters of the cation diffusion facilitator (CDF) protein family (36) resulted in a failure of CzcCBA to mediate cobalt resistance (27). A quadruple mutant strain devoid of the known P-type ATPases and CDF proteins able to export

Zn(II) across the inner membrane into the periplasm is highly zinc sensitive but can be complemented to wild-type zinc resistance levels by overexpression of *czcCBA* (45). Although this result may be an argument in favor of transenvelope efflux, it is not: overexpression of *czcCBA* in a *C. metallidurans* strain carrying a Δ*zupT* deletion led to growth impairment because the cells are unable to acquire zinc (M. Herzberg, A. Kirsten, and D. Nies, unpublished data). ZupT is the main zinc uptake system for *C. metallidurans* under conditions of low zinc availability but not the only zinc uptake system. Nevertheless, overexpression of *czcCBA* seems to decrease the periplasmic zinc concentration to a level that is not sufficient for further zinc uptake by these other systems, which also protects the cell when environmental zinc concentrations are very high, even in the absence of all known inner membrane efflux systems.

In *C. metallidurans* CH34 a deletion of *czcA* led to a strong expression of a *czc-lacZ* reporter fusion, which is under the control of a two-component regulatory system CzcRS (composed of a membrane-bound histidine kinase sensor and cytoplasmic response regulator) (45). In contrast, deletion of the gene encoding the main Co(II)-exporting CDF protein DmeF did not yield upregulation of *czcCBA*. Thus, CzcS is sensing the metal concentration in the same cellular compartment that is detoxified by CzcCBA, which is the periplasm. Moreover, although CzcA is able to transport Zn(II) into proteoliposomes, the K_m value of the transport is about 6.6 mM (14), 33-fold higher than the total cellular zinc content of 0.2 mM (34). Finally, metal transport by CzcA into proteoliposomes was abolished in the presence of glutathione (D. Nies, unpublished data) whereas glutathione would be present in physiologically relevant amounts in the cytoplasm.

Thus, transport of metal cations by CusA and CzcA from the

cytoplasm to the periplasmic substrate-binding sites can be demonstrated but probably does not contribute to metal resistance, which is predominantly based on periplasmic efflux by the respective RND-driven tripartite transenvelope complexes. What could be the physiological relevance of an RND-mediated transport across the inner membrane?

One possible answer is that rather than a primary function in transport across the inner membrane, the transmembrane methionine pairs (M410-M501, M486-M403, and M391-M1009) of CusA regulate the extrusion of substrate under various metal concentrations. CusCBA and CzcCBA export surplus cations (copper and zinc, respectively) that are essential at lower levels. Moreover, copper and zinc are necessary in both the periplasmic and the cytoplasmic compartments. While most zinc-containing proteins reside in the cytoplasm (30), the periplasmic alkaline phosphatase, for instance, is transported into the periplasm as an unfolded polypeptide via the Sec general excretion pathway (37). Consequently, zinc insertion must take place in the periplasm. Similarly, although most copper-containing enzymes of *E. coli* are periplasmic or have their copper sites exposed to the periplasm, the presence of cytoplasmic copper proteins cannot be excluded. Examples of these are YhcH (54) and factors involved in molybdenum cofactor biosynthesis (46). Therefore, flux control is needed for these two RND-driven efflux pumps to prevent situations of metal starvation, similar to the flux control that fine-tunes the activity of other metal transporters (31). Flux control of RND-driven systems would need two checkpoints, one in the periplasm and one in the cytoplasm, because metal homeostasis in both compartments has to be protected.

Indeed, an important result (24) supports this proposal: when the three methionine pairs (M410-M501, M486-M403, and M391-M1009) in CusA that are suggested to be part of the methionine-lined pathway in the transmembrane helices were changed into isoleucine residues, the mutant strains displayed decreased copper resistance. However, this result is not necessarily due to the ineffective transport of substrate from the cytoplasm to the periplasm.

An alternative explanation is that CusA contains a cytoplasmic exposed methionine pair (M410 and M501) that serves as a flux control switch. In this case, binding and transport of cytoplasmic copper may be a sensing mechanism. The importance of cytoplasmic sensing may be to signal extreme copper stress that could be alleviated in part by decreasing periplasmic copper through export by the Cus system. Full CusCBA activity would be attained only if sufficient copper is present in the cytoplasm. The switch could be reset by residual transport of copper from one methionine pair to the next, eventually to the three methionine-coordinating binding sites (M573, M623, and M672) and subsequent export. Based on the available evidence, a cytoplasmic flux control should be carefully considered.

CusB MAY ACT AS A SWITCH OR A FUNNEL

The membrane fusion protein CusB of *E. coli* contains three methionines, M21, M36, and M38, at positions similar to those that are conserved in all known Cu(I)- and Ag(I)-transporting MFP proteins (16) (Fig. 3). The three methionines have been shown to specifically bind Cu(I) and Ag(I) and are also able to

accept these cations when delivered by the metallochaperone CusF (6). Upon metal binding, CusB undergoes a conformational change (5). Replacing any of the three conserved methionines with isoleucine in both the substrate binding sites in the periplasmic cleft of CusA (M573I, M623I, M672I) and in the N terminus of CusB (M21I, M36I, M38I) led to a loss of CusCBA/CusF-mediated copper resistance (5, 13, 24). Other putative copper binding residues, M190 and M324 in CusB, identified by metal binding of the crystals (52), were shown not to be physiologically relevant (E.-H. Kim, C. Rensing, and M. M. McEvoy, unpublished data). What is the role of metal binding by the membrane fusion proteins? Here, the significance of the metal binding site of membrane fusion proteins in two proposed models is discussed (Fig. 3).

In the funnel model, metal is transferred from one protein metal binding site to the next utilizing the metal binding site in CusB as one step in the transfer pathway. In this case, CusF binds Cu(I) in the periplasm and transfers Cu(I) to the three methionines of the CusB proteins of the complex. Cu(I) would then be transferred to the three methionines of a CusA molecule presently in the open access state to make it a ligand-binding conformation (Fig. 3). An advantage of this model would be that it could enhance substrate specificity through three successive checkpoints via the metal binding sites in CusF, CusB, and CusA. Other interfering metals would not be bound by CusF and CusB and thus would not interfere with CusA-mediated extrusion of Cu(I) and Ag(I).

The switch model predicts that the role of the membrane fusion protein is to bind metal, resulting in an "on" conformational state. Here, the substrate is not transferred from the membrane fusion protein to the RND inner membrane protein, but substrate enters the pump directly from the periplasm to the RND protein (Fig. 3). In this case, the metal binding site of the membrane fusion protein could be a periplasmic flux control switch. This could be achieved by the CusB subunits undergoing a conformational change triggered by binding of Cu(I) cations, which in turn leads to the activation of CusA or the opening of the outer membrane factor CusC. As described for AcrB (28, 47), the substrate-binding site of the trimeric CusA would alter between three states: open access, ligand binding, and ligand extrusion into the CusC outer membrane factor tube. Open access would only be allowed if CusB is in the correct "on" conformation. Alternatively, a Cu(I)-linked conformational change in CusB might lead to an opening up of CusC, which may be closed at the periplasmic end when not transporting substrates (4, 20). Since membrane fusion proteins contact the C-terminal domain of the outer membrane factors with an alpha-helical hairpin domain (7, 8, 17, 57), such a pairwise conformational change is possible.

A periplasmic flux control switch seems to be required in *C. metallidurans*, as evidenced by the growth impairment of a ΔzupT mutant strain that overexpresses *czcCBA*. In this case, however, efficient flux control is hampered by the absence of the three periplasmic metal chaperones CzcI, CzcJ, and CzcE of the Czc system and the artificially high expression level of *czcCBA* (21). The membrane fusion protein CzcB possesses two putative metal-binding sites that are required for full CzcCBA-mediated metal resistance in *C. metallidurans* (41) and could be involved in this regulatory process.
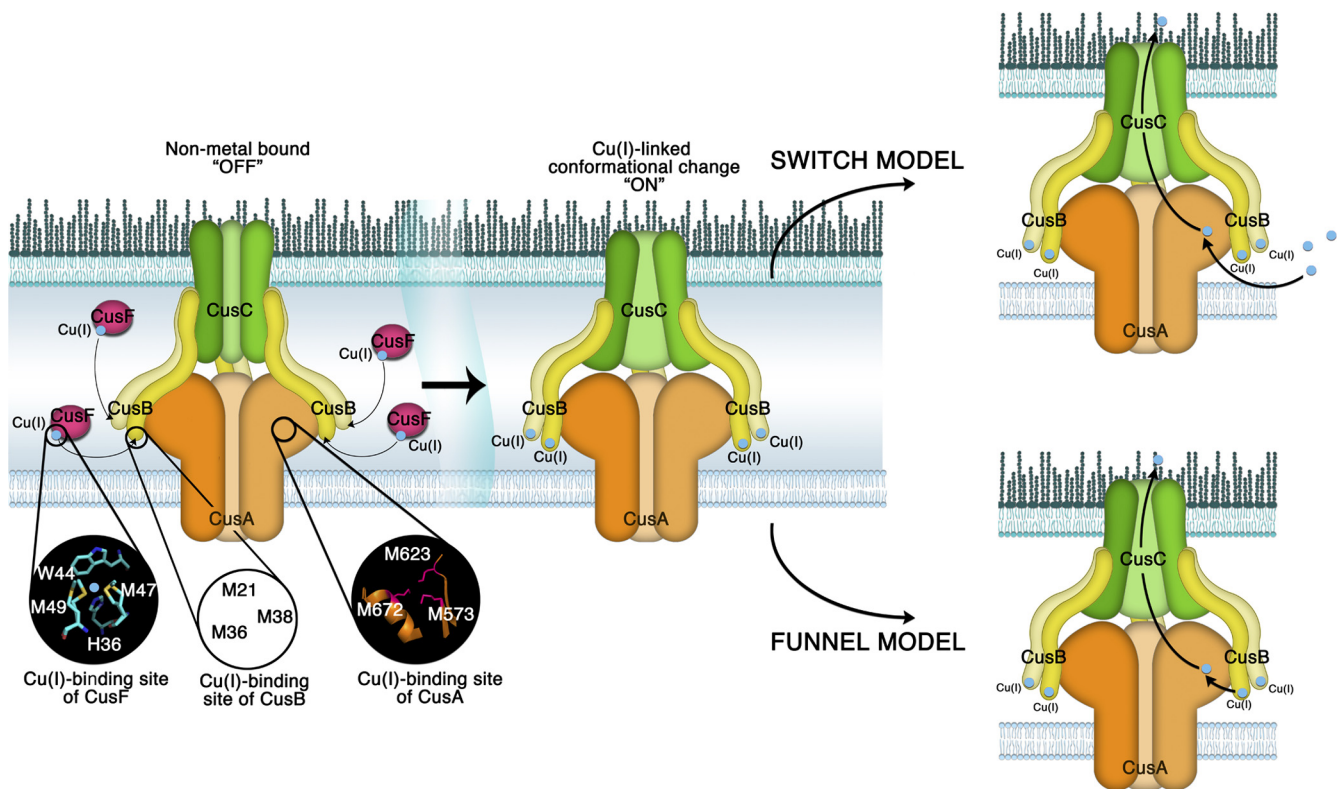
FIG. 3. Funnel and switch model of CusCFBA-mediated Cu(I)/Ag(I) transport. CusF coordinates Cu(I)/Ag(I) via $H_{36}W_{44}M_{47}M_{49}$ (23) and delivers metal to the metal-binding site of the membrane fusion protein CusB ($M_{21}M_{36}M_{38}$) (5), thereby inducing a conformational change and creating an open-access state of the CusCBA complex. In the switch model, periplasmic Cu(I)/Ag(I) binds to the metal-binding site of CusA ($M_{573}M_{623}M_{672}$) (13, 24) and then exits through CusC to the extracellular space. However, in the funnel model, Cu(I)/Ag(I) is transferred from CusB directly to the metal-binding site of CusA and then exits through CusC to the extracellular space.

WHAT IS THE EVIDENCE FOR THE SWITCH MODEL VERSUS THE FUNNEL MODEL?

Based on the structures for AcrB, MexB and CusA, the central substrate binding domain of RND proteins is in the periplasmic cleft (24, 48, 59). If the membrane fusion protein of the tripartite protein complex delivers metal to the substrate binding region in the periplasmic cleft of the RND protein, as postulated in the funnel model, metal binding to the membrane fusion protein must take place in close proximity to the cleft of the RND protein. However, based on available knowledge of metal binding sites in membrane fusion proteins and periplasmic clefts of RND proteins, this is not the case. In ZneB, a membrane fusion protein from an RND-driven tripartite system from *C. metallidurans* CH34, the zinc binding site is located between the beta barrel and membrane proximal domain, which is predicted to be distant from the periplasmic cleft; therefore direct substrate transfer is unlikely (11). In CzcB, two putative metal-binding sites immediately follow the hydrophobic membrane anchor of the membrane fusion protein (41) so that these sites are distant from the periplasmic cleft of the RND protein CzcA. In CusB, the three methionines that bind Cu(I) are also near the N terminus, proximal to the outer face of the inner membrane. Some CusB-like proteins have two repeats of the conserved $MX_{13}MXM$ motif, such as in the obligate methanotroph *Methylosinus trichosporium* strain OB3b. Thus, the location and number of metal

binding sites in the membrane fusion proteins lend support to the switch model. Analogous to the situation in metal regulatory proteins (25), binding of a metal cation with a subsequent conformational change in the membrane fusion protein may serve to fine-tune the transport activity of the RND-driven tripartite protein complex, thereby ensuring proper metal homeostasis in the periplasm. This is especially important in light of the need to have metal concentrations within acceptable concentrations in the various compartments of the cell to ensure that the correct metal is incorporated into the respective enzyme (56).

Further support of the switch model for tripartite RND-efflux systems comes from the analysis of mutations in the metal binding residues of the CusCBA system. When M21, M36, and M38 in CusB were replaced with cysteines rather than isoleucines, two mutant proteins (M36C and M38C) mediated some residual copper resistance. However, the M21C mutant protein was unable to contribute to copper resistance. In CusA, replacement of M573, M623, or M672 with cysteine resulted in copper sensitivity (Kim et al., unpublished). This would indicate that there is little flexibility in the substrate binding and transport region of CusA. In contrast, CusB may have more flexibility because Cu(I) only needs to be bound to induce the conformational change that leads to activation of the transport activity of the CusCBA efflux complex.

WORKING MODEL OF CusCFBA

Together, the structural models and functional data suggest the following for bacterial metal homeostasis as described below for the *E. coli* systems. Copper is sensed by CueR and CusRS (Fig. 2). CueR regulates expression of the Cu(I)-translocating P-type ATPase CopA and the multicopper oxidase CueO (40). While CopA pumps out excess copper from the cytoplasm, CueO oxidizes Cu(I) to Cu(II) within the periplasm, thereby preventing Cu(I)-mediated toxicity (Fig. 2). However, under anaerobic conditions CueO is not active and is also readily poisoned by minute amounts of Ag(I) making expression of *cusCFBA* necessary (35; S. K. Singh et al., unpublished data). The periplasmic metallochaperone CusF is strongly upregulated by excess copper as indicated by microarray data (12) and would initially function to sequester excess Cu(I) rapidly in the periplasm. This would then allow for the assembly of the CusCBA complex. Metal binding by CusB could trigger activation of the CusCBA complex, which pumps Cu(I) from the periplasm to the extracellular milieu. Once the Cu(I) concentration dips below a level needed to fill the CusB binding sites, the pump is turned off. Additional protection may arise from transport of cytoplasmic copper through the Met pairs of CusA as proposed by Su et al. (51), or if these methionine pairs act as a flux control site, absence of Cu(I) from these sites could also downregulate activity. However, these residues are not conserved in all putative CusA-like RND transporters, making an essential role unlikely. Following CusA, Cu(I) could be transferred through the CusC channel to the outside of the cell.

In conclusion, CusCFBA appears to have at least two sites of allosteric control regulating transport of periplasmic copper to the extracellular milieu. This may be a general feature of all metal-transporting RND proteins, which may thus be controlled at the transcriptional level and by flux control.

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