Chaperone-mediated copper handling in the periplasm†

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Metal transport systems are broadly utilized to maintain low levels of metals to prevent cellular malfunction caused by an overabundance of metals. The CusCFBA Cu(I)/Ag(I) resistance system, commonly found in Gram-negative organisms, typically consists of a tripartite CBA transport complex that spans both the inner and outer membranes as well as a small periplasmic protein, CusF. In the CusCFBA system, CusF functions as a metallochaperone which transfers metal to the tripartite complex to aid in metal resistance. However, CusF-like proteins have also been observed in genomic contexts apart from the CBA-type transport systems, suggesting it could either play a role as a metallochaperone to other systems or have other roles than that of a metallochaperone. In this review, we focus on the molecular function of CusF in the CusCFBA transport system and discuss the metal transport pathway through this system. In addition we briefly discuss the potential functions of CusF-like proteins in other contexts.

1   Introduction

One can envision a timeline where life evolved under reducing, anaerobic conditions and later adapted to increasing concentrations of oxygen in the atmosphere. Under these early anaerobic conditions iron was readily available and served as a building block of iron–sulfur clusters in the active site of many enzymes. The soft Lewis acids, also termed soft metals, such as Cu(I), Ag(I), Cd(II) and Hg(II), which have a high affinity for soft Lewis bases such as thiolates,[1] were sequestered by dissolved sulfur compounds into mineral precipitates in the anaerobic, reducing environment of early Earth.

The redox chemistry of early life is preserved in the cytoplasm of all cells. However, the advent of rising oxygen concentrations challenged this arrangement not only by making iron less available but by solubilizing increasing amounts of soft metals.[2] Due to their affinity for reduced sulfur compounds the intracellular concentration of any free soft metal has to be held at extremely low levels.[3,4] At the same time, copper proved to be a very useful metal under aerobic conditions as organisms exploited the high polarizing power of copper in enzymes such as cytochrome oxidase, amine oxidase or multi-copper oxidases. This delicate balancing act for copper is achieved by compartmentalization, sequestration and, importantly, efflux.[5] Not only does copper efflux appear to be a requisite to live under aerobic conditions but most microorganisms do not possess copper-containing enzymes in their cytoplasm.[6] In most organisms, with the exception of photosynthetic bacteria, copper enzymes are usually membrane-bound or located in the periplasm.[6] Copper is often guided to various destinations via copper chaperones.[7,8]

In Escherichia coli copper toxicity appears to occur by largely the same mechanisms whether cells are grown under either aerobic or anaerobic conditions.[9] The strong soft metal Cu(I) displaces iron from iron–sulfur clusters of various enzymes. Cu(I) probably binds to coordinating sulfur atoms which then leads to a further degradation of the iron–sulfur cluster in enzymes such as isopropylmalate dehydratase, which is responsible for amino acid biosynthesis. Other iron–sulfur cluster-containing enzymes, such as fumarase A, are also readily inactivated by increasing cytoplasmic Cu(I) concentrations. Increased intracellular copper can be counteracted by CopA-mediated Cu(I) efflux from the cytoplasm to the periplasm[10] and through complexation by glutathione.[11]

In E. coli the periplasm can contain significantly higher copper concentrations than the cytoplasm.[12] Under aerobic conditions, periplasmic copper readily cycles between Cu(I) and Cu(II).

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Cupric ions can be reduced by periplasmic proteins, oxidation of cysteines (kept in a reduced state by DsbG[13]), and by quinones possibly linked to the cupric ion reductase Ndh-2.[14] Since Cu(I) is a prooxidant and can initiate generation of reactive oxygen species under aerobic conditions, periplasmic Cu(I) has to be kept at a low level. Maintaining low levels of periplasmic Cu(I) is also important as Cu(I) is the ion likely taken up by cells. One mechanism to achieve this is through the oxidation of Cu(I) to Cu(II) by the periplasmic multicopper oxidase CueO.[15] However, CueO is not active under anaerobic conditions and is vulnerable to inhibition by Ag(I), which has very similar properties to Cu(I) but cannot be oxidized by CueO (S. Singh and W. R. Montfort, in preparation). This makes additional mechanisms necessary to handle periplasmic Cu(I) (and Ag(I)).

Gram-negative bacteria such as *E. coli* utilize an arsenal of resistance systems in their struggle against excess metal concentrations, and the variety and general mechanisms of the transporters in prokaryotes have been reviewed elsewhere.[16] The CBA-type transporters are widely employed to handle detoxification of excess periplasmic metals. For Cu(I) and Ag(I)-transporting CBA-type systems, an additional periplasmic metal-binding component may provide additional protection to the periplasm, as described below.

2 CBA transporter overview

CBA transport complexes are commonly found in Gram-negative bacteria as mediators of efflux of a wide variety of substrates.[17] These efflux pumps are referred to as 'CBA' reflecting the three proteins usually involved in the complex. Note that these systems are distinct from the 'ABC' transporters which utilize ATP hydrolysis to drive transport.[18] In CBA-type transporters, the inner membrane component of the complex is an RND transporter, which is a member of a ubiquitous superfamily of permeases found in all kingdoms of life.[19] RND transporters drive the export of a variety of substrates using the proton gradient across the membrane as an energy source. RND transporters of the heavy metal efflux (HME) family are specifically involved in transport of metals across the cell membrane. In Gram-negative bacteria, the HME-RND transporter frequently forms a tripartite CBA system with an outer membrane factor and a periplasmic component of the membrane fusion protein (MFP) family. These three proteins form an extended complex than spans both the inner and outer membrane of Gram-negative bacteria (Fig. 1). The CBA components of the metal resistance systems show a good degree of similarity with the well-studied multidrug resistance systems, and likely are functionally similar in many respects. The mechanism of the multidrug resistance system, particularly with respect to the inner membrane protein, have been recently reviewed.[20–22] In the CBA-type metal resistance systems for which the substrates are expected to be monovalent species, a fourth periplasmic component is often involved.

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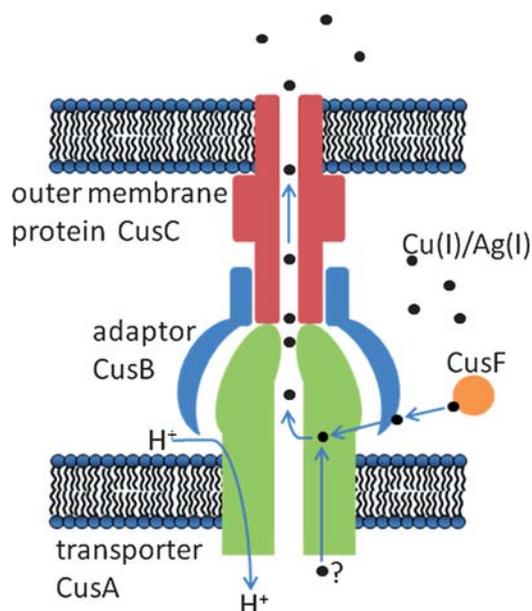
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Fig. 1 Model of the CusCFBA transport system.

3 Cus system identification and analysis

The CusCFBA system in *E. coli* is a CBA-type transporter with an additional fourth component. The Cus system consists of CusA, the inner membrane RND transporter, CusC, the outer membrane factor, CusB, the periplasmic adaptor protein, and CusF, the small periplasmic protein, all of which are encoded in a single operon. Early reports on the Cus system, which were published in rapid succession, established a role for the Cus system in *E. coli*'s metal-responsiveness. Munson *et al.* determined that the transcription of *cus* genes was controlled by a copper-responsive two-component system, CusRS.[23] Two-component systems, consisting of a sensory kinase and response regulator, are commonly found in bacterial systems and are utilized to regulate gene expression in response to environmental cues.[24] The *cus* locus, regulated by CusRS, was suggested to encode a copper efflux system.[23] Franke *et al.* demonstrated that the transcription of *cusC* increased in response to both silver and copper, but that under aerobic conditions, *E. coli* with a *cusA* deletion only showed a silver-sensitive phenotype but did not affect copper resistance.[25] Thus questions were raised as to which metals are substrates for this system. Further experiments clarified the metal-responsiveness issues through the determination that the genes encoding the Cus system were significantly upregulated under anaerobic conditions (where Cu(I) is the prevalent species) or under aerobic conditions when copper concentrations are extremely high.[26] Additionally, it was shown that the copper-resistance phenotype under aerobic conditions could be mimicked with strains containing a deletion of the gene encoding the multicopper oxidase, CueO, which converts Cu(I) to Cu(II).[27] In this strain, sensitivity is likely observed due to the presence of increased amounts of Cu(I), even under aerobic conditions. Under conditions of extreme copper stress, there is likely a significant proportion of Cu(I) even under aerobic conditions,[9] which may explain the induction of the Cus system under these conditions. Collectively, these findings suggest that Cu(I) is the likely copper species to which the Cus system confers resistance, in addition to Ag(I) with its similar chemical properties. No information is available as to whether CusF also binds the other group 11 element, gold.

Analysis of the components of the Cus system determined that all Cus components are required for full copper resistance.[28] The deletions of either *cusA* or *cusB* led to a complete loss of *cus*-mediated copper tolerance, though the effects were less dramatic for the *cusC* or *cusF* deletions. One explanation for the decrease in copper tolerance for the *cusC* deletion is that it is possible that another outer membrane factor in *E. coli* can partially substitute for CusC. The weakest effect on copper resistance is seen for the *cusF* deletion, demonstrating that CusF is not essential, though it contributes to full resistance.[28] Since homologs of CusF are not found in other well-characterized CBA-type transport systems, investigations were launched as to its role in the Cus system, as summarized below.

4 Possible roles for CusF

The small periplasmic protein CusF is found in operons encoding CBA transport systems predicted to handle monovalent metals. These findings provoke the question as to why a fourth component exists for this group of transporters, though not all CBA transporters with an RND transporter of the heavy metal efflux family have a CusF homolog. If the *cusF* gene is deleted, resistance to copper in *E. coli* is diminished, but not abolished, indicating that CusF is not an essential component of the Cus system.[28] What is the role of the CusF component in conferring metal resistance? CusF could be acting as a metallochaperone that binds metals in the periplasm and delivers them to the CusCBA components for export out of the cell. Another possibility is that CusF functions as a metal-dependent regulator of the CusCBA system, to enhance metal export when metals are present in the periplasm. In addition to those two potential functions, CusF may also provide protection to the periplasm as a Cu-buffer or by sequestering metals to prevent damage by free metals. Its potential function in these roles is discussed below.

5 Overall structure of CusF

In 2005 the crystal structure of apo-CusF was reported.[29] CusF is a small β-barrel protein with five β-strands forming a compact structure. The N-terminal 13 residues of CusF are disordered in solution, however in the apo-crystal structure, the N-terminal residues are stabilized by crystal packing such that they extend away from rest of the barrel structure.[29] The overall topology of CusF is related to proteins in the OB- (*o*ligonucleotide/*o*ligosaccharide *b*inding) fold family, which are characterized by a five-stranded β-barrel structure. Proteins in the OB-fold family have a diversity of functions, though many of them play a role in oligonucleotide or oligosaccharide binding. This fold is unrelated to other copper-binding proteins dominated by β-sheet structure, such as the cupredoxin fold. To date, no other proteins with OB-folds have characterized functions as copper binding proteins. The lack of a characterized structural and functional homolog has left many questions to be determined about the role of CusF-like proteins in metal resistance.

6 Does CusF bind Cu(II)?

Early reports of purified CusF suggested that it was a Cu(II) binding protein based on detecting Cu in ICP-mass spectra and observation of EPR spectra.[28,30] In NMR spectra, the addition of Cu(II), which is paramagnetic, resulted in line broadening for a few residues near the N-terminus of the protein and in residues in the C-terminal Strep affinity purification tag.[29,31] The residues at positions 3, 4, and 5 of the processed, mature CusF sequence from *E. coli* are all histidines, which in concert with the N-terminal amino group can form a divalent metal binding site termed an ATCUN motif.[32] A construct of CusF which lacks these N-terminal five residues is unable to bind Cu(II).[29] These residues are not conserved as histidines in CusF homologs, and the weak binding of divalent metals like Cu(II) at this site is not likely physiologically relevant.[31] Additionally, the earlier experiments were done with a construct of CusF which included a Strep affinity purification tag containing a histidine residue.[28–30] This tag was likely to play a role in increased Cu(II) binding since when CusF is purified in its naturally occurring form without the Strep affinity tag it does not show appreciable Cu(II) binding affinity by isothermal titration calorimetry or NMR experiments.[31]

7 Cu(I) and Ag(I) binding site of CusF

The molecular analysis of CusF identified two conserved methionines in CusF, residues 47 and 49 (using the numbering of the processed form of CusF) that were likely to be important in metal binding. If these residues were altered to isoleucines, the ability of cells to survive copper stress decreased.[28] Significant NMR chemical shift changes were observed in residues in the region of these methionines upon the addition of Cu(I) and Ag(I)[29,31] providing supporting evidence for their involvement in binding of the monovalent metals. Histidine 36, which shows complete conservation in CusF homologs, also shows significant NMR chemical shift effects and is located in close proximity to the methionine residues in the structure. Based on their proximity, conservation among CusF homologs, and NMR chemical shift changes upon the addition of Cu(I), His36, Met47 and Met49 were proposed to form the Cu(I) binding site of CusF.[29]

In the Cu(I) and Ag(I) bound crystal structures of CusF,[33,34] these three residues were shown to be coordinating the metals, and no significant overall structural changes were observed when the apo and metal bound structures were compared. However, surprisingly, a tryptophan sidechain (Trp44) was noted to be in close proximity of the bound metal (Fig. 2). The position of this tryptophan suggested the possibility of a novel Cu(I)-π (or Ag(I)-π) interaction, though the distance between the metals and CE3 and CZ3 carbons of the tryptophan ring were long compared to metal–arene complexes in the Cambridge Structural Database. However, there were other features that supported this interaction. These high resolution structures showed that the metal is displaced out of the plane formed by the two methionines and the histidine toward the indole ring of the tryptophan. Though X-ray absorption spectroscopy data were fit well by two sulfurs and a nitrogen residue, there was a slight improvement in the fit when a fourth scatterer was included.[33,34] More conclusively, spectral absorption changes between apo-CusF and CusF–Ag(I)[33,34] and
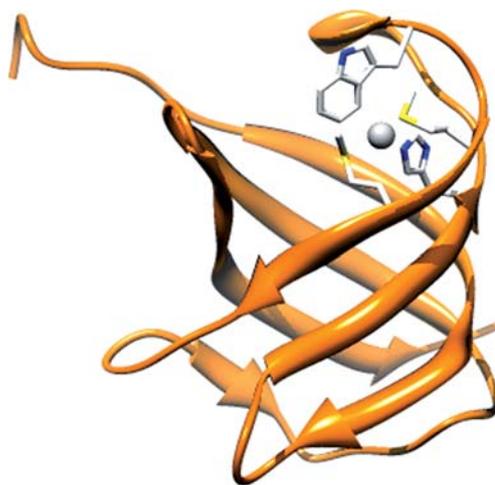

Fig. 2 Ribbon diagram of CusF. Residues at the metal binding site are shown in stick representation and Ag(I) is shown in silver (pdb code 2QCP[33]) The major conformations of the two methionine side chains are shown. Molecular graphics prepared with UCSF Chimera.[54]

UV resonance Raman spectroscopy[34] support the interaction of the metal with the tryptophan indole ring.

Methionine-rich motifs intermixed with nitrogen ligands are commonly found in proteins which bind Cu(I) in the oxidizing environment of the periplasm.[35–37] Methionine provides sulfur as a ligand, which is preferred by Cu(I) over oxygen or nitrogen, and is more stable to oxidation than cysteine. In the oxidizing environment of the periplasm, cysteine would form disulfides which would limit its ability to interact with metals. Additionally, while Cu(II) is generally tetrahedrally coordinated, Cu(I) generally favors lower coordination numbers. Thus, the metal-binding site of CusF clearly shows optimization for Cu(I) (or Ag(I) which has similar properties) *versus* Cu(II).

With a preference of Cu(I) for low coordination numbers and sulfur ligands, what role is the tryptophan playing in metal binding? The structures of CusF–Cu(I) and CusF–Ag(I) show that when bound to CusF, the metal is well shielded from solvent, which may play a role in protection from oxidation. XAS experiments support this hypothesis through demonstration that alteration of the tryptophan to an alanine allows some oxidation of the copper to occur and also permits access by a fourth ligand.[38] Substitution of tryptophan 44 by methionine, which naturally occurs in approximately ¼ of CusF homologs, increases the affinity of CusF for Cu(I) by approximately 1–3 orders of magnitude.[34,38] The substitution of Trp44 with alanine or methionine does not alter specificity of CusF. It is likely that the contribution of the Trp44 to metal binding is in maintaining a solvent shielded 3-coordinate environment of moderate binding affinity thus providing a protected environment for Cu(I) before it is removed from the cell by the CusCBA complex.

8 CusB

The periplasmic protein CusB is essential for metal resistance by the Cus system. CusB is a member of the membrane fusion protein (MFP) family, also called periplasmic adaptor proteins (reviewed in ref. 39). The proteins in this family stabilize the

association of the CBA efflux complexes through association with both the inner and outer membrane proteins as modeled in Fig. 1. These proteins are periplasmically localized and are commonly anchored to the inner membrane, either through a single membrane spanning helix or an acyl modification near the N-terminus of the protein.

CusB has some distinctive differences from periplasmic adaptor proteins that transport substrates that are not monovalent metals. First, CusB does not contain any motifs indicating association with the membrane and purifies as a soluble protein,[40] which suggests that it is not anchored to the inner membrane as are other proteins in this family. Importantly, though the inner membrane protein in drug resistance systems seems to serve as the major site for substrate recognition, the periplasmic adaptor protein CusB in the Cus system has a metal binding function.[40] The metal site in CusB was shown to consist of three methionines (21, 36, and 38, using the numbering from the first amino acid in the processed mature form of the protein) as determined from XAS, mutagenesis, and isothermal titration calorimetry. If any of the three metal binding methionines are altered to isoleucine, which significantly reduces *in vitro* metal binding, the ability of *E. coli* to survive copper stress is decreased. Thus, not only is CusB capable of binding Ag(I) with 1 : 1 stoichiometry, metal binding is functionally important for metal resistance.[40] The binding of silver by CusB causes a significant conformational change that is apparent by gel filtration chromatography.[40] These findings suggest that in the Cus system, CusB plays a more active functional role than that of simply an adaptor protein, and may be actively involved in the passage of metal through the Cus complex. This unique adaptation might be responsible for the astonishingly narrow substrate specificity of the Cus system.[41]

The partial crystal structure of CusB, consisting of 78% of the mature protein sequence, was recently determined to 3.4 Å resolution (Fig. 3A).[42] While the overall molecule is elongated as in the homologs from the multidrug resistance systems (Fig. 3B), the helical domain in CusB consists of a three helix bundle instead of a two-helix hairpin. The three β-domains individually display similarities between the homologs. The two CusB molecules in the asymmetric unit show domain movements with respect to each other, consistent with the domain flexibility that has been noted for the AcrA homolog[43] and the conformational changes seen in solution upon Ag(I) binding.[40] Thus flexibility is very likely a key feature of this periplasmic protein.

The three methionine residues, Met21, Met36, and Met38, which make up the metal binding site[40] were outside the region for which the CusB crystal structure was determined,[42] so a complete structural picture of metal-bound CusB is still lacking. The region missing from the CusB crystal structure, the N-terminal 60 residues and C-terminal 22 residues, may form an additional domain that is not present in the homologs from the multidrug resistance systems which lack an equivalent region at the N-terminus. While the crystal structure report also described Cu(I)- and Ag(I)-bound CusB structures,[42] it is not clear that these sites are physiologically relevant. The metal sites in these structures are not formed from conserved residues and are not consistent with the XAS data showing metal coordination by 3 sulfurs or the mutagenesis and functional data that support a methionine metal site.[40] The metal-binding sites in the crystals
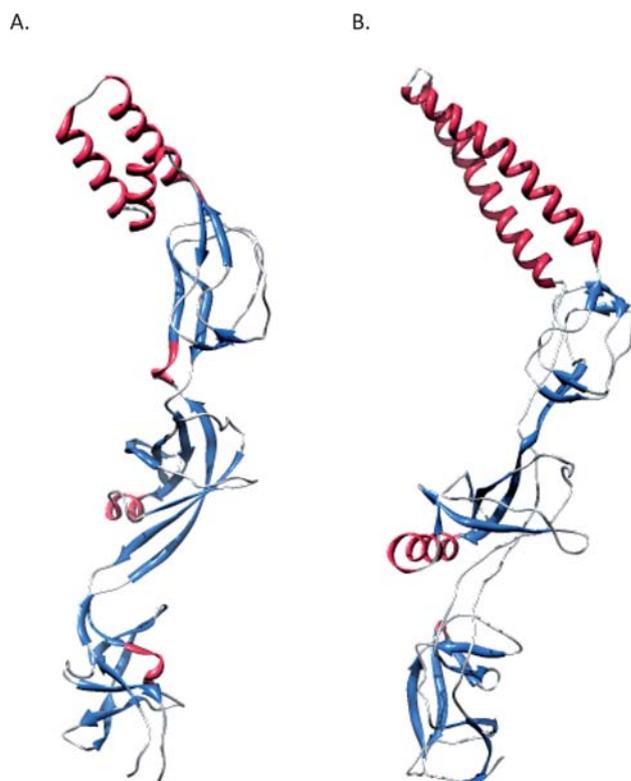

Fig. 3 Ribbon diagram colored according to secondary structure of (A) CusB (pdb code 3H9I)[42] and (B) the periplasmic adaptor protein, MexA, of a multidrug resistance system (pdb code 2V4D).[55] The models are oriented similarly to the view shown in the cartoon in Fig. 1, such that the top of the molecule is closer to the outer membrane, while the bottom is closer to inner membrane. The metal binding site consisting of Met21, Met36, and Met38[40] is outside the region for which the CusB crystal structure has been determined.[42] Based on the location of the termini, the missing regions consisting of residues 1–60 and 358–379 are expected to be at the bottom of the figure as shown. Molecular graphics prepared with UCSF Chimera.[54]

may represent adventitious binding sites resulting from the high metal concentrations in which the crystals were soaked. A crystal structure of the entire protein in its metal-bound state is still needed to structurally characterize the three-methionine metal site and overall conformational changes on metal binding.

9 Evidence for CusF as a metallochaperone

If CusF serves as a metallochaperone, it is expected to transfer metal ions to an accepting protein, of which the likely candidates are proteins in the CusCBA complex. Yeast two-hybrid experiments demonstrated potential interactions between CusF and CusB.[28] Due to the identification of CusB as a metal-binding protein,[40] it makes a likely target to be the protein to which CusF transfer its metal. Experimental evidence for metal transfer between CusF and CusB came from XAS experiments in which the metal binding sites of CusF and CusB were distinguished by incorporation of selenomethionine into one of the two proteins.[44] Thus in a mixture of the two proteins the location of the copper can be identified. These experiments demonstrated Cu(I) transfer

from CusF to CusB, as well as transfer in the reverse direction from CusB to CusF. In both cases, the copper is distributed to the same extent between the two proteins, which is likely a reflection of their similar binding affinities for metal. In the cell, where the other components of the Cus system are also present, the model is that copper is expected to be removed from CusB for export by the Cus system, thus driving the transfer of copper in the direction of CusF to CusB with little transfer in the reverse direction.

10 Transport of metal through the Cus system

Transport of metal from CusF to CusB is likely to be followed by removal of metal through the CBA transport complex. However, there are likely to be significant differences between the monovalent-metal transporting systems and the multidrug-resistance systems in the path the metal takes through the complex because of the differing degrees of specificity and different chemical natures of the substrates that are transported by these systems. In the CBA-type multidrug-resistance systems, which have very broad substrate specificity, significant evidence suggests that substrates are bound by the inner membrane RND transporters before subsequent efflux (reviewed in ref. 22 and 45), and the periplasmic adaptor protein is unlikely to play a substrate binding function. The multidrug resistance systems often transport hydrophobic compounds which must likely be captured by the RND component from their location in the inner membrane. Thus the substrate specificity and chemical properties of the substrates are quite different for these classes of transporters. In the CusCFBA system, as well as in homologous monovalent metal resistance systems, the binding of metal substrates by CusF and CusB may be a significant factor in the high degree of substrate specificity shown by the Cus system,[41] as well as serving as a mechanism for capturing substrates from the aqueous environment of the periplasm.

Once metal is bound to CusB, it is likely transferred to the inner membrane RND protein CusA. Several conserved methionine residues are apparent in the CusA sequence, and the mutation of some of these residues to isoleucine affects the ability of *E. coli* to survive metal stress.[28] This work is suggestive that the methionines in CusA could form a Cu(I) or Ag(I) binding site, though direct metal binding has not yet been demonstrated for CusA. The homology model of CusA based on the structures of AcrB[46,47] positions these methionines in a cleft of CusA that could potentially be accessible by CusB for metal transfer, but structural data that definitively place the methionines in proximity has not yet been obtained. Analysis of the sequence of the outer membrane protein CusC and its homologs does not identify any potential metal binding residues. This portion of the complex may serve simply as a passageway through the outer membrane without a specific site for metal interaction. This hypothesis is consistent with the proposal that other *E. coli* outer membrane proteins might partially substitute for CusC, as some copper resistance is still seen in *cusC* deletions.[28] Thus in the CusCFBA metal-resistance system, metal discrimination probably takes place in the metal binding sites of the CusF, CusB and CusA proteins as metal is transported by the Cus system, as shown by the arrows in Fig. 1.

11 Prevalence and genomic context of CusB and CusF

Combining the genomic composition and function of CusF and CusB-like proteins further elucidates potential roles of these proteins and ultimately improves our understanding of how metal ions play a role in the biology of microorganisms. To determine the prevalence and potential roles of CusB and CusF-like proteins, BLAST analysis was performed of predicted amino acids sequences from 1081 sequenced bacterial genomes. CusB (gi: 89107439) and CusF (gi: 89107438) amino acid sequences were queried using blastp with default parameters. Sequence alignment hits with *E*-values less than 0.001 and sequence percent identity higher than 25% were further analyzed. Subsequently, these sequences were scanned for metal-binding motifs, $M_{21}M_{36}M_{38}$ for CusB and $H_{36}W_{44}M_{47}M_{49}$ or $H_{36}M_{44}M_{47}M_{49}$ for CusF. Of the 1081 sequences searched, approximately 27% contained CusB-like proteins and 16% contained CusF-like proteins. Organisms that lack CusCFBA homologs may have functional substitutes, such as the CueP system in *Salmonella*.[48] Interestingly, CusB-like and CusF-like proteins containing metal binding motifs were only found in the Proteobacteria phylum, which is comprised of Gram-negative bacteria with extreme metabolic diversity (Table 1). The majority represent known Gram-negative bacteria of medical, industrial, and agricultural significance.

Surprisingly, the arrangement of *cusF* within its genomic context is rather varied. In many instances, *cusF* is flanked by genes encoding CusCBA-like components, which occurred in 47% (80) of the 171 sequence hits. This cluster of *cusCFBA*-like genes is present in the α, β, δ, and γ proteobacterial classes. Specifically, the Enterobacteriales order, composed of microbes such as *Escherichia*, *Shigella*, and *Enterobacter* spp., is completely dominated by *cusF*-like genes flanked only by *cusCBA*-like genes. In some cases, genes encoding CusF-like proteins appear to be alone. Approximately 3% of genes encoding CusF-like proteins are not flanked by any genes encoding putative copper resistance systems, and these are usually flanked by transposases. Their role is not known. However, since *cusF* of *E. coli* has been shown to have the strongest response *via* transcript levels upon copper shock of all known copper determinants,[49,50] and constitutive expression of CusF increases silver resistance in an *E. coli* strain,[51,52] it is possible that CusF alone could exert a protective effect through metal sequestration.

In contrast to the *cusF*-like genes flanked by *cusCBA*-like genes mentioned above, 28% (48) of the 171 proteins contain genes encoding CusF-like domains that are fused to CusB-like proteins at the C-terminus, and many of these have an additional gene encoding a CusF-like protein along with CusCBA-like components. The function of the CusF–CusB fusion protein is not well understood. The structure of CusB[42] suggests the N- and C-termini of CusB are likely in proximity, thus one can hypothesize that the metal binding site of the fused CusF-like domain may interact and possibly deliver metal ions to the three conserved methionines at the N-terminus of CusB. Additionally, a gene was found encoding a CusF-like protein fused to a CusA-like protein in *Oligotropha carboxidovorans*. It may be possible that this CusF-like domain could transfer metal directly to CusA, however, delivery of the metal ion to CusA *via* this route is not known.

Table 1 Prevalence of CusB-like and CusF-like proteins

	Genomes	CusB-like Proteins MMM[a]	CusF-like Proteins HWMM[b]	HMMM[c]
Actinobacteria	128	0	0	0
Bacteroidetes	89	0	0	0
Chlamydiae	7	0	0	0
Cyanobacteria	43	0	0	0
Firmicutes				
Bacillales	52	0	0	0
Clostridia	109	0	0	0
Lactobacillales	50	0	0	0
Mollicutes	23	0	0	0
Others	89	0	0	0
Proteobacteria				
Alpha				
Rhizobiaceae	8	5	4	3
Rickettsiales	31	0	0	0
Others	112	58	30	9
Beta				
Bordetella	5	2	6	0
Burkholderiaceae	25	41	0	3
Neisseriaceae	12	0	0	0
Others	33	32	30	22
Delta	37	3	2	0
Epsilon	28	0	0	0
Gamma				
Enterobacteriales	55	53	35	0
Pasteurellaceae	12	0	0	0
Pseudomonadaceae	14	11	5	0
Vibrionaceae	16	27	1	0
Xanthomonadaceae	8	3	1	0
Others	93	54	18	1
Others	2	2	1	0
Spirochaetales	19	0	0	0

[a] Sequence hits contained the motif MMM, which is the CusB metal binding motif. [b] Sequence hits contained the motif HWMM, which is the *E. coli* CusF metal-binding motif. [c] Sequence hits contained the motif HMMM, which is an alternative CusF metal-binding motif.

The relative abundance of sequences encoding CusB-like proteins over CusF-like proteins support the observation that CusF is not essential for the CusCBA complex and may be involved in alternative copper homeostatic mechanisms. By examining the genomic context of genes encoding CusF-like proteins, we uncovered its presence in other putative copper resistance systems, ultimately revealing potential roles within these systems. Of the 171 proteins, 19% (33) are flanked by genes encoding copper resistance systems different than the CusCBA complex within the α, β, and δ-proteobacterial classes and the Pseudomonadales order. Within the vicinity of genes encoding CusF-like proteins lie genes for putative "blue" (type 1) copper oxidases, multicopper (type 1, 2 & 3) oxidases and outer membrane proteins. Similar systems containing an oxidoreductase, a "blue" copper protein, and an outer membrane protein have been shown to promote copper resistance in *Xanthomonas campestris*. Mutations in any of these genes rendered *X. campestris* copper sensitive.[53] It appears that CusF-like proteins may be involved in the delivery of metal ions to these detoxifying systems and is likely contributing to copper homeostasis. However, the role of CusF-like proteins in these copper resistance systems has yet to be fully investigated.

12 Conclusions

CusF is a small protein with a big role in metal resistance. CusF functions as a metallochaperone for the CusCBA transport system which aids in detoxification of metal through efflux from the periplasm. It is possible that CusF may also exert a protective effect against high concentrations of metal simply through metal sequestration. Because the CusF signature sequence is found in a diverse set of genomic surroundings, this suggest that its role is not limited to that of a metallochaperone for the CusCBA-like systems, and there is yet more to be discovered about its function in enabling bacteria to survive environments with high copper and silver concentrations.

13 Acknowledgements

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