Bacterial copper storage proteins

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Copper is essential for most organisms as a cofactor for key enzymes involved in fundamental processes such as respiration and photosynthesis. However, copper also has toxic effects in cells, which is why eukaryotes and prokaryotes have evolved mechanisms for safe copper handling. A new family of bacterial proteins uses a Cys-rich four-helix bundle to safely store large quantities of Cu(I). The work leading to the discovery of these proteins, their properties and physiological functions, and how their presence potentially impacts the current views of bacterial copper handling and use are discussed in this review.

Copper in biology

The utilization of metals by biological systems is highly paradoxical. On the one hand, metal ions provide proteins access to chemistry that would otherwise be impossible using the organic reactions that can be catalyzed by amino acid side chains. On the other hand, many of these metal ions can be toxic to cells. Copper is essential for most organisms as the cofactor for key enzymes involved in important processes such as respiration and photosynthesis (1–7). Ideas about the cellular toxicity of copper have developed in recent years, from solely being attributed to the generation of reactive oxygen species (ROS)2 (8–11). An emerging mechanism appears to be driven by the ability of copper to bind tightly at the active sites of metalloenzymes, particularly those containing iron-sulfur clusters. This not only destroys the reactivity of the mis-metallated protein but releases iron that can produce ROS (9). This toxicity is the reason why aquated (“free”) copper ions should not exist in cells and that copper is predicted to be highly restricted in eukaryotes (12) and prokaryotes (13). Copper availability appears to be largely constrained by the use of high-affinity sites in proteins (12–14), although “pools” of copper bound by other molecules are important (4, 5, 11, 15–18).

Approaches used by cells to enable safe copper handling, referred to as copper homeostasis, include sensors, transporters, chaperones, and insertion proteins with high affinity and specificity for copper (3–7, 12–14, 19–22). A well-characterized family of copper-homeostasis proteins are the copper-transferring P-type ATPases, which can remove this metal ion from the cytosol (4–7, 20–24). In eukaryotes, these copper-efflux pumps work with a cytosolic copper metallochaperone (ATOX1 in humans and Atx1 in yeast) to facilitate import into the trans-Golgi network for secreted copper enzymes (4, 5, 19, 24, 25). The two Cu-ATPases in humans (ATP7A and ATP7B) can relocate to the plasma membrane to remove excess intracellular copper when necessary (4, 24). In bacteria, the production of the copper-efflux pump CopA (23) is controlled by transcriptional regulators (sensors) such as CueR (13) and CsoR (26). CopA can work either alone or in concert with the ATOX1/Atx1 homologue CopZ to remove cytosolic copper (5–7, 20–23, 27, 28). It has recently been found that in bacteria not previously thought to possess this copper metallochaperone, such as Escherichia coli, CopZ can be made from the CopA gene by “programmed ribosomal frameshifting” (29).

It is emerging that the human immune system uses the toxicity of copper to attack invading pathogens. Previous minireviews in the “Thematic Series on Metals in Biology” have discussed copper biochemistry (3, 22), emphasizing its role in pathogenicity (30–34). We will therefore only touch on this issue briefly toward the end of our minireview. The main topic here is the recently discovered ability of bacteria to safely store copper using a highly novel approach (35). The more widespread and abundant class of the new family of bacterial proteins that can perform this function is cytosolic (36). This is somewhat controversial, as a widely accepted view is that bacteria have evolved not to use cytosolic copper enzymes as a way to help avoid the potential toxicity associated with their metabolism (6, 13, 37).

Discovery of a new bacterial copper storage protein and its characterization

Eukaryotes are able to store cytosolic copper using metallothioneins (MTs) (38–41). Related proteins have been characterized in pathogenic mycobacteria (42), but the idea that bacterial copper storage systems could be more common was unknown. This changed with the discovery of a new family of copper storage proteins, the Csp, in the methane-oxidizing bacterium (methanotroph) Methylosinus trichosporium OB3b (35). It is not surprising that such a finding about copper biochemistry was made in methanotrophs as these Gram-negative organisms are important (4, 5, 11, 15–18).
organisms use large amounts of copper to metabolize methane via the membrane-bound (particulate) methane monooxygenase (pMMO). This enzyme catalyzes the conversion of methane to methanol in almost all methanotrophs (17). pMMO, originally thought to have a dinuclear copper-active site, but which has very recently been suggested to be mononuclear (43), is housed on specialized intracytoplasmic membranes (17, 44) and can constitute a large proportion of total cellular protein. When copper levels are low, some methanotrophs (17, 45) have the ability to use the soluble MMO (sMMO), which has a dinuclear iron-active site (46). The switchover between these MMOs is copper-regulated, and more detail about this process and methanotroph classification and metabolism can be found in Ref. 17. Understanding how methanotrophs manage and use copper has immense environmental relevance due to methane being a highly potent greenhouse gas, and it is also essential for prospective biotechnological applications of these organisms and their MMOs (47–49).

The ability to utilize large amounts of copper results in methanotrophs having highly interesting copper-handling systems. This includes methanobactin (Mbn) (17, 50–52), which has been considered comparable to certain iron-binding siderophores (53), and is thus termed a chalkophore (50). Mbn is a modified peptide (Fig. 1, A and B) that is part of a highly specialized copper-uptake system, secreted to sequester this metal ion under limiting conditions (17, 50). The mbnA gene, which codes for leader (cleaved) and core (modified) peptides, has been identified in an operon along with proteins either shown or suggested to be involved in modification reactions, apo-Mbn export, and re-incorporation of Cu(I)–Mbn (17, 51, 55–59). Related Mbn operons are present in some non-methanotrophic bacteria (17, 51, 59). Work in our laboratory has found that Mbn is bound Cu(I) with affinities in the 10^20 to 10^21 M^-1 range and have Cu(II) affinities that are ~6–10 orders of magnitude weaker (52, 54, 60). We suggested oxidation could assist removal of the metal ion in cells (52), although a conformational change at the N terminus of the peptide now appears to be the most likely mechanism to promote release (60). Having characterized a range of Mbn isolated from spent media in which methanotrophs were grown at low copper concentrations (52, 54, 60), including determination of their high-resolution crystal structures (Fig. 1, A and B) and analysis of the Cu(I)–Mbn uptake process (Fig. 1, C and D) (52), understanding the fate of internalized Cu(I)–Mbn became our next aim. To try to isolate intracellular Cu(I)–Mbn, soluble extracts from the model switchover methanotroph M. trichosporium OB3b were separated using anion-exchange, followed by size-exclusion chromatography, and fractions were analyzed for metals. A number of copper-containing peaks were observed, but none contained Mbn.

Although Mbn was not found within cells in these metalloproteomic studies, the observation of soluble copper pools in M. trichosporium OB3b extracts, whose abundance increased at higher copper concentrations, is extremely interesting. The

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**Figure 1. Structures of Cu(I)–Mbns and Mbn-mediated copper uptake.** A and B, the crystal structures of the Cu(I)–Mbns from M. trichosporium OB3b (A, PDB file 2XHJ) (54) and Methylocystis hirsuta CSC1 (B, PDB file 2YGI) (52). Below the structures are the sequences of the leader (black and underlined) and core peptides that make up MbnA. Core peptides are modified to give the Mbn, and the M. hirsuta CSC1 Cu(I)–Mbn structure is of a form with the three C-terminal residues cleaved (amino acids are numbered according to the sequence of the core peptides). The Cu(I) ions are shown as orange spheres ligated by the sulfur atoms (S1 and S2) from thioamide/enethiol groups, and two oxazolone (oxa) ring nitrogens in M. trichosporium OB3b Cu(I)–Mbn, with the N-terminal coordinating heterocycle being a pyrazinediol (pyra^a) in M. hirsuta CSC1 Cu(I)–Mbn. Other differences include a sulfate-modified Thr side chain in M. hirsuta CSC1 Cu(I)–Mbn and the overall hairpin-like structure of this Cu(I)–Mbn compared with the more compact M. trichosporium OB3b Cu(I)–Mbn. Also shown are copper uptake by (lines) and relative sMMO activity of (bars) M. trichosporium OB3b (C) and M. hirsuta CSC1 (D) cells after the addition of M. trichosporium OB3b Cu(I)–Mbn (open gray triangles and gray bars) and M. hirsuta CSC1 Cu(I)–Mbn (open cyan circles and cyan bars) to sMMO-active cells. In both cases, copper uptake and switchover from sMMO to pMMO is faster with the native Cu(I)–Mbn (52).
complex mixture present in the major copper-containing fraction was further purified to identify constituent copper-binding proteins (35). Copper abundance in fractions matched the intensity of a band on an SDS polyacrylamide gel at ~12 kDa, which was purified to near homogeneity (Fig. 2A). The intensity profile of no other metal tested, including manganese, iron, and...
zinc, corresponded with this band, identified by peptide mass fingerprinting as an uncharacterized conserved hypothetical protein possessing 13 Cys residues (Fig. 2B). The protein has a predicted twin-arginine translocase (Tat) signal peptide, suggesting it is folded prior to export from the cytosol (61), and cleavage is likely (62) after Ala-24 (Fig. 2B). Overexpressed Gly-1 to Ala-122 (no signal peptide) forms a tetramer of four-helix bundles (Fig. 2C), with all Cys residues pointing into the cores of the monomers (Fig. 2D) (35). The protein has no disulfide bonds due to the Cys residues all being found on α-helices in a fold that constrains the side chains. The protein can bind up to 12–14 Cu(I) ions per monomer in vitro, with an average Cu(I) affinity of \( \sim 1 \times 10^{17} \text{M}^{-1} \) (35).

In the crystal structure (35) each monomer binds 13 Cu(I) ions (Fig. 2E) in an unprecedented arrangement along the core of the four-helix bundle, a motif commonly found in metalloproteins (35, 63), including that binding the iron site of sMMO (46). Four of the Cu(I) ions are coordinated by two thiolates on the same α-helix in CXXXC motifs (Fig. 2B), whereas the majority of the other sites are ligated by two Cys residues on different helices. The solvent-accessible sites at the mouth of the bundle (the opposite end contains a number of hydrophobic side chains), by which Cu(I) ions are presumed to enter and leave, have different coordination environments (Fig. 2E) (35). This includes the binding of Cu13 by His-36 and Met-48 acting as a bridging ligand between Cu11 and Cu13. A tetramer capable of accommodating up to 52 largely solvent-protected Cu(I) ions is consistent with a role in storage, and hence the name of this novel family of copper proteins (the Csps) was devised (35).

Comparison of Csp homologues in M. trichosporium OB3b

MtCsp1 and MtCsp2 have high identity (∼60% sequence conservation), and both possess predicted (61) Tat signal peptides. The presence of two exported Csps within the same methanotroph is not uncommon (see under “Csp homologues in other methanotrophs”), where they could perform different roles (see under “Functions of Csps”). MtCsp3 has no signal peptide (Fig. 2G), is therefore cytosolic, and has lower sequence identity to MtCsp1 and MtCsp2 (∼20% conserved residues). Neither MtCsp2 nor MtCsp3 has been identified by metalloproteomics, although many of the other soluble copper-containing fractions obtained from M. trichosporium OB3b have yet to be thoroughly investigated. MtCsp2 has not been studied due to its high sequence similarity to MtCsp1 (Fig. 2G). In vitro studies of MtCsp3 show it is also a tetramer of four-helix bundles having 18 Cys residues pointing into the core of each monomer (Fig. 3A) (36). The additional Cys residues, compared with MtCsp1, are found in CXXXC motifs, and the protein also has no disulfide bonds. Each monomer is able to bind more metal ions within its core and has 19 Cu(I) sites in the crystal structure (Fig. 3B). Most of these are coordinated by two thiolates, largely alternating between Cu(I) ions bound by Cys residues from the same α-helix (in CXXXC motifs) and inter-helical sites. Atypical coordination is again found at the mouth of the bundle where His-110 binds Cu18 along with Cys-111, and His-104 ligates Cu19 in addition to two thiolates (Fig. 3B) (36). The average Cu(I) affinity of MtCsp3 (∼2 × 10^{17} \text{M}^{-1}) is similar to that of MtCsp1 (35, 36).

Important differences are found in how MtCsp3 and MtCsp1 bind Cu(I). In the case of MtCsp3, Cu(I) binding gives rise to relatively intense fluorescence at ∼600 nm upon excitation within the S(Cys) → Cu(I) ligand-to-metal charge transfer bands below 400 nm (35, 36). Such emission has been associated with Cu(I)–Cu(I) interactions in proteins binding solvent-protected Cys-coordinated Cu(I) clusters, such as the MTs (38, 42, 66). The fluorescence from MtCsp3, which reaches a maximum value when it is approximately half-loaded, may be related to the formation of solvent-protected tetranuclear Cu(I) clusters within its central core (67). Similar structures do not occur in MtCsp1 due to it having fewer Cys residues. Furthermore, and functionally more important, Cu(I) binding is cooperative in MtCsp1 (35), but not in MtCsp3 (36). This could also be related to discrete cluster formation in MtCsp3 (67), but the exact cause of both of these aspects of Cu(I) binding in the Csps requires further investigation.

The most striking difference between MtCsp3 and MtCsp1 is the time scale of Cu(I) removal from their cores. Both have average Cu(I) affinities in the low 10^{17} \text{M}^{-1} range, and assuming diffusion-controlled off-rates of ∼10^6 \text{M}^{-1} \text{s}^{-1}, unassisted Cu(I) off-rates would be extremely slow (∼10^{-9} \text{s}^{-1}). The physiological Cu(I) acceptor for any Csp is currently unknown. As M. trichosporium OB3b produces an Mbn, removal by this high Cu(I)-affinity molecule (54, and vide supra) has been investigated (see under “Functions of Csps”). Stoichiometric concentrations of apo-Mbn removes all Cu(I) from MtCsp1 in ∼1 h (35), while this process takes weeks to complete for MtCsp3 (36). Comparative Cu(I) removal studies have also been carried out with well-characterized chromophoric Cu(I)-chelating molecules such as bicinechonic acid and particularly bathocuproine disulfonate (BCS). These ligands have routinely been used to measure how tightly Cu(I) binds to a range of proteins (26, 68–71), including determination of the Cu(I) affinities of Mbns (52, 54) and the average values for the Csps (35, 36). They have also been implemented as model acceptors for investigating Cu(I) removal from homeostasis proteins (68, 72). Using a large excess of the higher-affinity ligand BCS results in complete removal of Cu(I) from MtCsp1 in ∼1 h (35), but only ∼20% from MtCsp3 in 85 h (36). There is a kinetic barrier to Cu(I) removal in MtCsp3, not present in MtCsp1, most likely related to structural alterations at, and particularly the amino acid residues around, the mouths of their four-helix bundles, a number of which coordinate Cu(I) (Figs. 2E and 3B). Work is underway to determine the cause of this difference and to understand whether fast and slow Cu(I) removal is a distinguishing feature of Csp1s and Csp3s, respectively (see under “Functions of Csps”).
Csp homologues in other methanotrophs

Homologues of MtCsp3 are present in 34 methanotrophs whose genomes have been sequenced, and MtCsp1 homologues are found in 16 (Fig. 4A and Figs. S1 and S2). A single MtCsp3 homologue is typically found in methanotrophs having this protein (two in Methylococcaceae bacterium NSP1–2 and Crenothrix polyspora), and two MtCsp1 homologues are present in over half of the sequenced methanotrophs that have this protein (Methylocystis bryophila appears to have three, although one of these has only seven Cys residues). A different name for a protein implies an alternative function. However, for organisms with two or more MtCsp1 homologues it is not yet known whether these have distinct functions (see below). We have not established a way to differentiate between what we initially called MtCsp1 and MtCsp2 (35), and it may therefore be better to use Csp1a (MtCsp1a) and Csp1b (MtCsp1b) to signify exported Csp homologues when found within the same organism. This approach provides the clear definition that Csp1s are the exported members of this family of proteins, whereas Csp3s are cytosolic. For the purpose of this review, we will continue to use MtCsp1 and MtCsp2 for the exported proteins in M. trichosporium OB3b.

Bioinformatics also highlight residues and regions of Csp1s and Csp3s that are conserved in methanotrophs (Fig. 4, B and C, and Figs. S1 and S2). This includes the Cu(I)-coordinating Cys residues; all 13 are highly conserved in the Csp1s (Fig. 4B), with 15 highly conserved in Csp3s (Fig. 4C). The sequence of the Tat signal peptide is highly similar in the Csp1s (Fig. 4B). Conserved regions are found at the open end of both four-helix bundles, thought to be important for Cu(I) uptake and removal. The
His-36 and Met-48 ligands in MtCsp1 are present in almost all homologues, along with a number of the intervening residues in the α1(His-36)–loop–α2(Met-48) region. In addition, two residues (Phe-97 and Pro-98 in MtCsp1) on the loop linking α3 and α4 are highly conserved in Csp1s, as is the Glu residue (Glu-102 in MtCsp1) at the start of α4 (Fig. 4B). Certain residues in the α3–loop–α4 region are conserved in Csp3s. This includes the His-110 ligand of MtCsp3 (Fig. 3B) and the non-ligating His-108, but these are predominantly present only in Methylocystaceae family (Methylosinus, Methylocystis, and Pleonormphononas genera) strains. His-104, which coordinates Cu18 in MtCsp3, is also conserved in these strains, but is replaced by an Ile residue in most other methanotrophs that have a MtCsp3 homologue (Fig. 4C and Fig. S2). Overall, the Cys ligands (Csp1s and Csp3s), the Tat signal peptide (Csp1s), the α1–loop–α2 (Csp1s) and α3–loop–α4 (Csp1s and certain Csp3s) regions are conserved features of these proteins in methanotrophs.

The Mbn operon is present in 12 methanotroph genomes, and due to overlap, less than half of the sequenced methanotrophs (38 of 89) possess either the Mbn operon, Csp1, or Csp3 (Fig. 4A). Nine methanotrophs possess all three, with none having the Mbn operon without either a Csp1 or Csp3. Overlap mostly occurs in the Methylosinus and Methylocystis genera, but there is not sufficient evidence to suggest the functions of Csp1, Csp3, and Mbn are directly related. However, the observation that Csp3 is present alone in 22 methanotrophs (Csp1 is rarely present on its own) could indicate that the func-
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It has recently been claimed (75) that Csp3s are present in shows Cu(I)-binding properties and structure of has been studied. Therefore, (108 versus 108) in plants and another in a soil-dwelling fungus according to Fig. Csp1 BCs in 85 h (36). However, this is still very slow compared with 4622 J. Biol. Chem. OB3b proteins for searches), with Csp3s present in no less than 10 different bacterial phyla and Csp1s in at least five. Approximately 140 MtCsp3 homologues are found in Archaea. It has recently been claimed (75) that Csp3s are present in eukaryotes. However, the three proposed eukaryotic Csps (two in plants and another in a soil-dwelling fungus according to Fig. S1 in Ref. 75) are identical to bacterial Csp sequences and are therefore most likely not from the organism indicated but are due to contamination with bacterial DNA. This is not surprising given that the bacteria in question are either soil dwelling or widely distributed in the environment. The Csp1s identified in non-methanotrophs are found in Gram-negative bacteria. A notable example is found in Neisseria gonorrhoeae (see under “Functions of Csps”), and initial studies indicate its predicted mature Csp1 has similar Cu(I) binding and removal characteristics to MtCsp1.

The more prevalent and widespread nature of Csp3s raises questions about copper handling in bacteria that have this protein. Copper homeostasis has been extensively investigated in certain Csp3-possessing non-methanotrophs, with probably the best example being Bacillus subtilis (21, 28, 68, 76, 77). Therefore, the Csp3 from this model Gram-positive bacterium has been studied in vitro (36). BsCsp3 is smaller than MtCsp3 (108 versus 133 amino acids, see Fig. S2), but it has an additional Cys residue (giving a total of 19). The structure of the apo-protein, including its tetrameric arrangement, is similar to MtCsp3 (Fig. 3, A and C), again with little evidence of disulfide bond formation (36). The protein binds up to ~20 eq of Cu(I) in vitro with an average affinity of ~2 × 10^{-17} M^{-1}, but removal is faster than for MtCsp3 as ~85% of the Cu(I) core is acquired by BCS in 85 h (36). However, this is still very slow compared with MtCsp1 (35), and the mouth of the four-helix bundle of BsCsp3 shows similarities to that of MtCsp3 (Fig. 3, A and C) (36). In particular, three His residues corresponding to His-104, His-108 and His-110 are present, and these are more conserved in non-methanotrophs (36). The cytotoxic Cu(I) metallochaperone CopZ is present in B. subtilis. Removal of Cu(I) by this potential physiological partner is also slow with BsCopZ acquiring ~40% of Cu(I) from BsCsp3 in 64 h (36). The in vitro Cu(I)-binding properties and structure of Streptomyces lividans Csp3 are similar to those of MtCsp3 and BsCsp3 (36), although the average Cu(I) affinity appears to be an order of magnitude weaker (75). Crystal structures of the apo-Csp3s from Pseudomonas aeruginosa (3KAW) and Nitrosospira multiformis (3LMF) have been deposited by a structural genomics consortium. These are similar to those of other Csp3s (Fig. 3, A and C), with all Cys residues pointing into the cores of their four-helix bundle folds, and no disulfide bonds present.

Functions of Csps

The predicted Tat-exported MtCsp1 acts as a copper store for methane oxidation for the following reasons. 1) copper-bound MtCsp1 is isolated (35) from M. trichosporium OB3b grown in 5 μM copper (Fig. 2A) i.e. using pMMO to oxidize methane. None of the other metals analyzed in the metalloproteomic studies (including manganese, iron, and zinc) co-eluted with MtCsp1. Furthermore, of the metal ions tested in our laboratory, only Cu(I) binds tightly in vitro. 2) The deletion of both genes for the exported Csps (MtCsp1 and MtCsp2) results in significantly faster switchover from pMMO to sMMO in M. trichosporium OB3b cells transferred from high to low copper (35). 3) In gene expression studies (see Fig. S5 in Ref. 58 and see also Ref. 78) MtCsp1 is up-regulated in a similar manner to pMMO at a copper concentration resulting in switchover (10 – 12.5 μM). 4) The copper peak and the MtCsp1 SDS-PAGE band (Fig. 2A) are absent in sMMO-active M. trichosporium OB3b cells. 5) The structure of MtCsp1 as a tetramer of Cys-lined four-helix bundles allows the binding of 52 Cu(I) ions. Collectively, these data provide extensive evidence that MtCsp1 stores Cu(I) for pMMO allowing continued growth on methane using this enzyme when copper becomes limiting. Given the similarities of the structures and Cu(I)-binding properties of MtCsp1 homologues, many having an even greater capacity for metal ions, it is almost implicit that other members of this new family of proteins are able to bind and store Cu(I).

The cellular destination of exported Csp1s depends on the cellular structure of methanotrophs (see below). The periplasmic multi-copper oxidase CueO, which is involved in copper homeostasis in E. coli, is also predicted to be Tat-exported (79). As this is the pathway for folded protein secretion, it had been assumed that CueO acquired the four copper ions it needs for activity in the cytosol. However, it is now thought this protein is exported in a copper-free “incomplete folding” state and requires copper in the periplasm (79). A number of other bacterial copper proteins are predicted to be Tat-exported (7), and further work is needed to determine whether these acquire copper in the cytosol. However, it seems highly unlikely that Csp1s are exported in a partially folded state as this would potentially promote disulfide bond formation in such Cys-rich proteins, and Tat export may be required to prevent this from occurring. Csp1s therefore most likely fold completely and acquire Cu(I) in the cytosol prior to export.

Although the prevailing view is that the intracytoplasmic membranes housing pMMO are invaginations of the plasma membrane (Fig. 4D), most evidence is either out-dated or indirect (80 – 82). If these membranes are discrete from the plasma membrane (Fig. 4D), pMMO would be only the second example, after plastocyanin in the thylakoid compartments of cyanobacteria (83), of a bacterial cytoplasmic copper-requiring protein. If this is the case, in a methanotroph such as M. trichosporium OB3b having two exported Csps, MtCsp1 could deliver Cu(I) to the intracytoplasmic membranes for pMMO while MtCsp2 transfers Cu(I) to the periplasm for other copper-requiring enzymes (Fig. 4D). MtCsp2 is not up-regulated by
10 μM copper (78), and this suggested function may not require copper-regulated expression (see below), or it may occur at higher copper concentrations than those causing switchover (it is also possible that MtCsp2 could act as a Cu(I) store for pMMO at higher copper concentrations). In methanotrophs that have a single Csp1, we assume this will only store Cu(I) for pMMO if the enzyme is housed in cytoplasmic compartments. However, if the intracytoplasmic and plasma membranes are contiguous, a single Csp1 could store Cu(I) for pMMO and other destinations in the periplasm. As already stated, most Csp1 possessing methanotrophs also have Mbn (Fig. 4A), which they produce under copper-limiting conditions (17, 50), when the Cu(I) from a Csp would be required. Therefore, during switchover from pMMO to sMMO it is possible that apo-Mbn may play a role in removing Cu(I) from MtCsp1, a process that readily occurs in vitro (35), to aid delivery to pMMO. In non-methanotrophs, Csp1 will deliver Cu(I) to the periplasm. Export of a protein that can store large amounts of Cu(I) (there is little sign of oxidation upon prolonged exposure of Cu(I)-MtCsp1 in air) will provide and stabilize a source of cuprous ions outside the cytosol, which may otherwise be difficult in the more oxidizing periplasm. This could be the oxidation state of copper required for insertion into certain enzymes, as appears to be the case for pMMO.

The precise function of a cytosolic Csp3 is currently unknown, although a general role in Cu(I) storage while preventing toxicity is presumed. Bioinformatics (Fig. 4A), and the more widespread nature of Csp3s both in methanotrophs and other bacteria, suggest that the function of Csp3 is not directly linked to Csp1. Preliminary in vivo studies on the csp3-deletion strain of B. subtilis show a weak, and unusual, copper-dependent phenotype (36). Growth in LB media is inhibited relative to WT B. subtilis in the range of ~1.5–2 mM added Cu(II), but only after cells have been grown for more than 12 h in the presence of the metal. Obtaining this phenotype reproducibly is difficult, being sensitive to growth conditions and particularly copper concentration. Transcriptional studies have shown that BsCsp3 is up-regulated under spore-forming and stress-inducing conditions, including elevated NaCl concentrations (84), but the response to copper was not tested. Interestingly, both Csp1 and Csp3 are up-regulated when the methanotroph Methylocystis sp. SC2 is grown in 0.75% NaCl (85). The relevance of salt stress on Csp expression remains unclear. The multi-copper oxidase CotA is one of very few predicted copper enzymes in B. subtilis (2) and is a component of the spore coat where it is thought to be involved in pigment production (86), and BsCsp3 could store Cu(I) for this enzyme. The P. aeruginosa Csp3 (the protein was incorrectly called a Csp1, but it does not possess a signal peptide and is cytosolic) is not induced (87) by the addition of 0.5 mM Cu(II). However, neither is CopA2, a second copper efflux pump that is not required for copper tolerance in P. aeruginosa, but is suggested to be involved in export coupled to copper acquisition by cytochrome c oxidase (88). The S. lividans Csp3 is up-regulated by 0.4 mM Cu(II) and in a csoR (copper-sensitive operon repressor) deletion mutant (89). A transcriptomic study of the Gram-negative bacterium Sphingobium sp. ba1 has shown up-regulation of Csp1 and Csp3 in response to 10 mM Ni(II), but under these conditions copper resistance systems, including CopA, are also up-regulated (90).

Gene expression studies show that MtCsp3 is not up-regulated at the relatively low copper concentrations (10 μM) required for switchover (78). However, putative CopAs in M. trichosporium OB3b are also not up-regulated under these conditions. Copper detoxification is not the proposed primary function of Csp3s, but BsCsp3 can provide protection against copper toxicity when overexpressed in both the copA delete strain (ΔcopA) (Fig. 3D) (36) and also WT E. coli (Fig. 3E). In both cases, the cells overexpressing BsCsp3 accumulate more copper than control cells, and Cu(I)-BsCsp3 is observed. As well as being able to complement the phenotype by deletion of the copper-efflux pump (23), overexpressed BsCsp3 provides an additional growth advantage at elevated copper to having CopA alone. Furthermore, BsCsp3-bound Cu(I) can be withheld from the efflux pump. It has also been found that in S. lividans Csp3 enables growth at higher copper levels (75).

When considering the functional properties of Csps, it is important to keep in mind key in vitro results (35, 36). Csp3s can generally bind a greater number of Cu(I) ions than Csp1s, due to usually having more Cys residues (Figs. 2, E and G, 3B, and 4, B and C, and Figs. S1 and S2 for methanotrophs). Csp1s and Csp3s have similar average Cu(I) affinities (~10^{17} M^{-1}) yet exhibit dramatic differences in terms of Cu(I) removal rates. How Cu(I) is extracted from Csps in cells is unknown. Many copper homeostasis proteins and copper target enzymes/proteins have higher Cu(I) affinities, typically in the 10^{12} to 10^{15} M^{-1} range (13, 26, 40, 68–71), and their ability to acquire Cu(I) from Csps is thermodynamically favored. Faster Cu(I) unloading by small molecule Cu(I) ligands occurs for Csp1s (35), but a kinetic barrier to removal is present in Csp3s (36). The interplay between thermodynamics and kinetics in copper homeostasis is currently not well understood. Furthermore, how many of the proteins involved in this process acquire copper is unknown (apart from the CopZ–CopA interaction). Csp1s are expected to be exported after acquiring Cu(I), and Csp3s kinetically trap Cu(I) in the cytosol. These proteins may therefore have evolved different approaches to enable them to bind and maintain a store of Cu(I) even in the presence of proteins with higher affinities (CopZ acquires Cu(I) very slowly from BsCsp3). In methanotrophs, which can have both a Csp1 and Csp3, such as M. trichosporium OB3b, the dramatic variation in removal rates could be more important (as may differences in Cu(I)-binding cooperativity) and suggests that the exported Csps act as a more temporary store of Cu(I) for pMMO, whereas Csp3 plays a role in longer-term storage. Whether this distinction between Cu(I) removal rates exists for all Csp1s and Csp3s has to be established. If slow Cu(I) removal is a conserved feature of Csp3s, then the requirement for a longer-term store needs to be understood, as well as how the kinetic barrier to removal is overcome when Csp3-bound Cu(I) is required.

**Possible link between Csps and pathogenicity?**

As mentioned in the Introduction, and covered in previous minireviews in this series, the interplay between copper homeostasis systems in a pathogen and host is beginning to be rec-
Bacterial copper storage proteins

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References

6. Fung, D. K., Lau, W. Y., Chan, W. T., and Yan, A. (2013) Copper efflux is essential for the survival of Salmonella enterica /H9004 and Salmonella enterica bilizing iron-sulfur cluster formation in M. trichosporium OB3b possesses three homologues: two closely related proteins having predicted Tat signal peptides and a cytosolic version. Exported MtCsp1 stores Cu(I) for pMMO. The more widespread occurrence of cytosolic Csps complicates the current conceptually simplistic idea that these proteins, when produced at relatively high levels, can take the place of copper-efflux pumps, known virulence factors (30, 88, 92, 93), in providing protection against elevated copper levels. The only other characterized bacterial copper-storing protein is the MT-like MymT found in pathogenic mycobacteria (42), but this does not appear to be required for infection. Whether a Csp would help a pathogen fight against host-based copper attack remains to be established.

Concluding remarks

The Csps were identified in methanotrophs, bacteria with atypically high copper demands, which they use for methane oxidation, and M. trichosporium OB3b possesses three homologues: two closely related proteins having predicted Tat signal peptides and a cytosolic version. Exported MtCsp1 stores Cu(I) for pMMO. The more widespread occurrence of cytosolic Csps complicates the current conceptually simplistic idea that these organisms have evolved not to use copper in this compartment (20). A lot more work is needed to understand copper storage and removal for the exported Csps and the cytosolic Csp3s. Csps are only found in ~40% of methanotrophs, whereas pMMO is nearly always present, and although Csp3s are widespread in bacteria, they are far from ubiquitous. The discovery of the Csps leads to the intriguing question of whether there are other bacterial copper-storage systems yet to be found. Even if this is not the case, the presence of Csp3s indicates that as predicted for other metalloproteomes (100), the possibility exists that there are cytoplasmic copper-requiring enzymes yet to be discovered.

The presence of bacterial copper storage proteins seems consistent with a number of other observations as follows: 1) that copper efflux and sequestration, including a Cu(II)-binding siderophore (34, 94). Csps are present in pathogenic bacteria, such as N. gonorrhoeae (Csp1), Streptococcus pneumoniae (Csp3), Salmonella enterica sv. Typhimurium (Csp3), and the opportunistic pathogen P. aeruginosa (Csp3). The ability of Csps to bind large quantities of Cu(I) would make them ideal to defend pathogens against copper attack by a host. The Cu(I)-buffering ability of Csp3 that prevents toxicity in the ΔcopA strain of E. coli (Fig. 3, D and E) demonstrates that these proteins, when produced at relatively high levels, can take the place of copper-efflux pumps, known virulence factors (30, 88, 92, 93), in providing protection against elevated copper levels. The only other characterized bacterial copper-storing protein is the MT-like MymT found in pathogenic mycobacteria (42), but this does not appear to be required for infection. Whether a Csp would help a pathogen fight against host-based copper attack remains to be established.

The Csps were identified in methanotrophs, bacteria with atypically high copper demands, which they use for methane oxidation, and M. trichosporium OB3b possesses three homologues: two closely related proteins having predicted Tat signal peptides and a cytosolic version. Exported MtCsp1 stores Cu(I) for pMMO. The more widespread occurrence of cytosolic Csps complicates the current conceptually simplistic idea that these organisms have evolved not to use copper in this compartment to help avoid toxicity. A role for these proteins in Cu(I) storage is currently the most logical suggestion for their function, but in many cases what they are storing copper for remains unknown. The presence of bacterial copper storage proteins seems consistent with a number of other observations as follows: 1) that bacterial copper-import systems exist (6, 7, 17, 21, 52, 56, 77, 95), including into the cytosol; 2) that endogenous pools of the metal are available in bacteria (11, 15, 16, 18, 96); and 3) that E. coli grown in both LB and minimal medium accumulates copper (97). It also suggests that there are alternative mechanisms to using different cellular compartments to prevent mismetallation of proteins by copper (37). Furthermore, the ability of bacteria to store copper in the cytosol could provide further insight into the observation that certain periplasmic proteins are loaded with copper that has passed through the cytosol (88, 98, 99). Most of the organisms in which this has been reported possess a Csp3.

A lot more work is needed to understand copper storage and removal for the exported Csps and the cytosolic Csp3s. Csps are only found in ~40% of methanotrophs, whereas pMMO is nearly always present, and although Csp3s are widespread in bacteria, they are far from ubiquitous. The discovery of the Csps leads to the intriguing question of whether there are other bacterial copper-storage systems yet to be found. Even
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