Bacterial copper storage proteins

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ABSTRACT
Copper (Cu) is essential for most organisms as a cofactor for key enzymes involved in fundamental processes such as respiration and photosynthesis. However, Cu also has toxic effects in cells, which is why eukaryotes and prokaryotes have evolved mechanisms for safe Cu handling. A new family of bacterial proteins use a Cys-rich four-helix bundle to 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 on current views of bacterial Cu 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 catalysed by amino-acid sidechains. However, many of these metal ions can be toxic to cells. Copper (Cu) 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 Cu have developed in recent years, from solely being attributed to the generation of reactive oxygen species (ROS) (8-11). An emerging mechanism appears to be driven by the ability of Cu to bind tightly at the active sites of metalloenzymes, and particularly those containing iron-sulfur (Fe-S) clusters. This not only destroys the reactivity of the mis-metallated protein, but releases Fe that can produce ROS (9). This toxicity is the reason why aquated (‘free’) Cu ions should not exist in cells, and that Cu 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 Cu bound by other molecules are important (4, 5, 11, 15-18).

Approaches used by cells to enable safe Cu handling, referred to as Cu homeostasis, include sensors, transporters, chaperones and insertion proteins with high affinity and specificity for Cu (3-7, 12-14, 19-22). A well-characterized family of Cu-homeostasis proteins are the Cu-transporting P-type ATPases, which can remove this metal ion from the cytosol (4-7, 20-24). In eukaryotes, these Cu-efflux pumps work with a cytosolic Cu metallochaperone (the well-characterized ATOX1 in humans and Atx1 in yeast) to facilitate import into the trans-Golgi network for secreted Cu 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 Cu when necessary (4, 24). In bacteria, the production of the Cu-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 Cu (5-7, 20-23, 27, 28). It has recently been found that in bacteria not previously thought to possess this Cu metallochaperone such as E. 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 Cu to attack invading pathogens. Previous Minireviews in the Thematic...
Series on Metals in Biology in this journal have discussed Cu biochemistry (3, 22), emphasising its role in pathogenicity (30-34). We will therefore only touch on this issue briefly towards the end of our Minireview. The main topic here is the recently discovered ability of bacteria to safely store Cu using a highly novel approach (35). The more widespread and abundant class of the new family of bacterial proteins that can perform this function are cytosolic (36). This is somewhat controversial, as a widely accepted view is that bacteria have evolved not to use cytosolic Cu-enzymes as a way to help avoid the potential toxicity associated with their metalation (6, 13, 37).

The discovery of a new bacterial Cu storage protein and its characterization

Eukaryotes are able to store cytosolic Cu using metallothioneins (MTs) (38-41). Related proteins have been characterized in pathogenic mycobacteria (42), but the idea that bacterial Cu storage systems could be more common was unknown. This changed with the discovery of a new family of Cu storage proteins, the Csps, in the methanogenic bacterium (methanotroph) Methylosinus trichosporium OB3b (35). That such a finding about Cu biochemistry was made in methanotrophs is not surprising as these Gram negative organisms use large amounts of Cu to metabolise 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 Cu 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 Cu levels are low, some methanotrophs (17, 45) have the ability to use the soluble MMO (sMMO), which has a dinuclear Fe active site (46). Switchover between these MMsOs is Cu-regulated, and more detail about this process and methanotroph classification and metabolism can be found in ref. 17. Understanding how methanotrophs manage and use Cu has immense environmental relevance due to methane being a highly potent greenhouse gas, and is also essential for prospective biotechnological applications of these organisms and their MMsOs (47-49).

The ability to utilize large amounts of Cu results in methanotrophs having highly interesting Cu-handling systems. This includes methanobactin (Mbn) (17, 50-52), which has been considered comparable to certain Fe-binding siderophores (53), and thus termed a chalkophore (50). Mbn is a modified peptide (Fig. 1, A and B) that is part of a highly specialized Cu-uptake system, secreted to sequester Cu 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 lab has found that Mbn binds Cu(I) with affinities in the 1021 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 Mbns isolated from spent media in which methanotrophs were grown at low Cu 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 gel-filtration, chromatography, and fractions analysed for metals. A number of Cu-containing peaks were observed, but none contained Mbn.

Although Mbn was not found within cells in these metalloproteomic studies, the observation of soluble Cu pools in M. trichosporium OB3b extracts, whose abundance increased at higher Cu concentrations, is highly interesting. The complex mixture present in the major Cu-containing fraction was further purified to identify constituent Cu-binding proteins (35). Copper abundance in fractions matched the intensity of a band on an SDS-PAGE gel at ~12 kDa, which was purified to near homogeneity (Fig. 2A). The intensity profile of no other metal tested, including Mn, Fe and Zn, 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 Ala24 (Fig. 2B). Over-expressed Gly1 to Ala122 (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 sidechains. The protein can bind up to 12-14 Cu(I) ions per monomer in vitro, with an average Cu(I) affinity of ~1 × 10^{17} 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 Fe 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), whilst 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 sidechains), via which Cu(I) ions are presumed to enter and leave, have different coordination environments (Fig. 2E) (35). This includes the binding of Cu13 by His36, and Met48 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 Cu proteins (the Csps) was devised (35). The way Csps bind Cu(I) is very different to how a Cys-rich unstructured MT polypeptide folds around thiolate-coordinated clusters (Fig. 2F) (38, 39, 41). Ferritins, which store Fe, are also four-helix bundles, but use these to form a multimeric envelope that can be filled with thousands of Fe(III) ions (64). *M. trichosporium* OB3b possess three Csp homologues (Fig. 2G), and the first discovered as described above was called Csp1 (*MtCsp1* indicates it originates from *M. trichosporium* OB3b), and the others named *MtCsp2* and *MtCsp3*.

**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 section about Csp homologues in other methanotrophs), where they could perform different roles (see section about the 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* have been identified by metalloproteomics, although many of the other soluble Cu-containing fractions obtained from *M. trichosporium* OB3b are 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 to *MtCsp1*, are found in CXXXC motifs, and the protein also has no disulfide bonds present. Each monomer is therefore 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 His110 binds Cu18 along with Cys111, and His104 ligates Cu19 in addition to two thiolates (Fig. 3B) (36). The average Cu(I) affinity of *MtCsp3* (~2 × 10^{17} 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 McCsp3 and McCsp1 is the timescale of Cu(I) removal from their cores. Both have average Cu(I) affinities in the low $10^{17}$ M$^{-1}$ range, and assuming diffusion-controlled on-rates of $\sim 10^8$ M$^{-1}$s$^{-1}$, unassisted Cu(I) off-rates would be extremely slow ($\sim 10^{-9}$ s$^{-1}$). The physiological Cu(I) acceptor for any unassisted Cu(I) off-rates would be extremely slow in the Csps requires further investigation.

affinities in the low $10^{17}$ M$^{-1}$ range, and assuming removal from their cores. Both have average Cu(I) affinity molecule (54, and vide supra) has been investigated (see section about the functions of Csps). Stoichiometric concentrations of apo-Mbn removes all Cu(I) from McCsp1 in ~1h (35), whilst this process takes weeks to complete for McCsp3 (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 McCsp1 in ~1h (35), but only ~20% from McCsp3 in 85 h (36). There is a kinetic barrier to Cu(I) removal in McCsp3, not present in McCsp1, 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 if fast and slow Cu(I) removal is a distinguishing feature of Csp1s and Csp3s respectively (see section about the functions of Csps).

Csp homologues in other methanotrophs

Homologues of McCsp3 are present in 34 methanotrophs whose genomes have been sequenced, whilst McCsp1 homologues are found in 16 (Fig. 4A and Figs. S1 and S2). A single McCsp3 homologue is typically found in methanotrophs having this protein (two in Methylococcales bacterium NSP1-2 and Crenothrix polyspora), whilst two McCsp1 homologues are present in over half of the sequenced methanotrophs that have this protein (Methylocystis bryophila appears to have three exported Csps, 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 exported Csps it is not yet known whether these have distinct functions (vide infra). We have not established a way to differentiate between what we initially called McCsp1 and McCsp2 (35), and it may therefore be better to use Csp1a (McCsp1a) and Csp1b (McCsp1b) 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, whilst Csp3s are cytosolic. For the purpose of this review we will continue to use McCsp1 and McCsp2 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, and seven less, conserved in Csp3s (Fig. 4C). The sequence of the Tat signal peptide is highly similar in the Csp1s (Fig. 4B). Other conserved regions are found at the open end of both four-helix bundles, thought to be important for Cu(I) uptake and removal. The His36 and Met48 ligands in McCsp1 are present in many homologues, along with a number of the intervening residues in the $\alpha_1$(His36)-loop-$\alpha_2$(Met48) region. In addition, two residues (Phe97 and Pro98 in McCsp1) on the loop linking $\alpha_3$ and $\alpha_4$ are conserved in Csp1s, as is the Glu residue (Glu102 in McCsp1) at the start of $\alpha_4$ (Fig. 4B). Certain residues in the $\alpha_3$-loop-$\alpha_4$ region are also conserved in Csp3s, including the His110 ligand of McCsp3 (Fig. 3B). His104, which coordinates Cu18 in McCsp3, is less common, but the non-ligating His108 is more frequently present (Fig. 4C). Overall, the Cys ligands (Csp1s and Csp3s), the Tat signal peptide (Csp1s), the $\alpha_1$-loop-$\alpha_2$ (Csp1s) and $\alpha_3$-loop-$\alpha_4$ (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 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 also either a Csp1 or Csp3. Overlap mostly occurs in the closely related family of *Methylocysteae* (*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 the function of this protein is independent of Csp1 and Mbn (see section about the functions of Csps). Much attention has been paid to the role of Mbn in Cu acquisition and utilization by methanotrophs (17, 50-52, 54-60). However, the currently identified Mbn operon is only found in just over 10% of sequenced methanotroph genomes, suggesting other systems are important for these processes. Csps are present in over 40% of these methanotrophs.

**Csps homologues in non-methanotrophs**

Csps are found in many more bacteria than Csp1s (approximately 4000 versus 200 respectively are identified using the *M. trichosporium* OB3b proteins for searches), with Csp3s present in no less than 10 different bacterial phyla and Csp1s in at least five. Approximately 140 *Mt* Csp3 homologues are found in Archaea. It has recently been claimed (75) that Csps 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 section about the functions of Csps) and initial studies indicate its predicted mature Csp1 has similar Cu(I) binding and removal characteristics to *Mt*Csp1.

The more prevalent and widespread nature of Csp3s raises questions about Cu 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 *Mt*Csp3 (108 versus 133 amino acids, see Fig. S2), but has an additional Cys residue (giving 19). The structure of the apo-protein, including its tetrameric arrangement, is similar to *Mc*Csp3 (Fig. 3, A and C), again with little evidence of disulfide bond formation (36). The protein binds up to ~20 equivalents of Cu(I) in *vitro* with an average affinity of ~2 × 10^{17} M^{-1}, but removal is faster than for *Mc*Csp3 as ~85% of the Cu(I) core is acquired by BCS in 85 h (36). However, this is still very slow compared to *Mt*Csp1 (35), and the mouth of the four-helix bundle of Bscsp3 shows similarities to that of *Mc*Csp3 (Fig. 3, A and C) (36). The well-characterized cytosolic 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* Csps are similar to those of *Mt*Csp3 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.

**The functions of Csps**

**The predicted Tat-exported MtCsp1 stores Cu for pMMO,** for the following reasons. (1) Cu bound MtCsp1 is isolated (35) from *M. trichosporium* OB3b grown in 5 μM Cu (Fig. 2A) i.e. using pMMO to oxidize methane. None of the other metals analyzed in metalloproteomic studies (including Mn, Fe, and Zn) co-eluted with MtCsp1. Furthermore, of the metal ions tested in our lab only Cu(I) binds tightly *in vitro*. (2) The deletion of both genes for the exported Csps (*Mt*Csp1 and *Mt*Csp2) results in significantly faster switchover from pMMO to sMMO in *M. trichosporium* OB3b cells transferred from high to low Cu (35). (3) In transcriptomic studies (Fig. S5 in ref. 58 and 78) MtCsp1 is up-regulated in a similar manner to pMMO at a Cu concentration resulting in
switchover (10-12.5 µM). (4) The Cu 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 Cu 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 (vide infra). The periplasmic multi-Cu oxidase CueO, which is involved in Cu 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 Cu ions it needs for activity in the cytosol. However, it is now thought this protein is exported in a Cu-free ‘incomplete folding’ state and acquires Cu in the periplasm (79). A number of other bacterial Cu proteins are predicted to be Tat exported (7), and further work is needed to determine if these acquire Cu 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 (Tat-export may be required to prevent this 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 Cu-requiring protein. If this is the case, in a methanotroph such as M. trichosporium OB3b having two exported Csp1s, MtCsp1 could deliver Cu(I) to the intracytoplasmic membranes for pMMO, whilst MtCsp2 transfers Cu(I) to the periplasm for other Cu-requiring enzymes (Fig. 4D). MtCsp2 is not up-regulated by 10 µM Cu (78) and this suggested function may not require Cu-regulated expression (vide infra), or may occur at higher Cu concentrations than those causing switchover (it is also possible that MtCsp2 could act as a Cu(I) store for pMMO at higher Cu 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 Cu-limiting conditions (17, 50), when the Cu(I) from a Csp would be required. Therefore, during switchover from pMMO to sMMO (due to decreased Cu availability) 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 Cu 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 whilst preventing toxicity is presumed. Bioinformatics (Fig. 4A), and the more widespread nature of Csp3s both in methanotrophs and other bacteria, indicate that the function of Csp3 is not directly linked to Csp1. Preliminary in vivo studies on the csp3-delete strain of B. subtilis show a weak, and unusual, Cu-dependent phenotype (36). Growth in LB media is inhibited relative to wild type (WT) B. subtilis in the range of ~1.5 to 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 Cu concentration. Transcriptional studies have shown that BsCsp3 is up-regulated under spor-forming and stress-inducing conditions, including elevated NaCl concentrations (84), but the response to Cu was not tested. Interestingly, both Csp1 and Csp3 are up-regulated
when the methanotroph Methylocystis sp. SC2 is grown in 0.75% NaCl (85), although the relevance of salt stress on Csp expression remains unclear. The multi-Cu oxidase CotA is one of very few predicted Cu 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 Cu efflux pump that is not required for Cu tolerance in P. aeruginosa, but is suggested to be involved in export coupled to Cu 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 known Cu efflux systems, including CopA, are also up-regulated (90).

Gene expression studies show that McCsp3 is not up-regulated at the relatively low Cu 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 Cu toxicity when over-expressed in both the copA delete strain (ΔcopA) (Fig. 3D) (36) and also WT Escherichia coli (Fig. 3E). In both cases, the cells over-expressing BsCsp3 accumulate more Cu than control cells, and Cu(I)-BsCsp3 is observed. As well as being able to complement the phenotype caused by deletion of the Cu-efflux pump (23), over-expressed BsCsp3 provides an additional growth advantage at elevated Cu 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 Cu levels (75).

When considering the functional properties of Csp3 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 (Fig. 2, E and G, Fig. 3B, Fig. 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 Csp3s in cells is unknown. Many Cu exporters, chaperones, sensors (homeostasis proteins) and Cu target enzymes/proteins have higher Cu(I) affinities, typically in the 10^{17} to 10^{21} M^{-1} range (13, 26, 40, 68-71). 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 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 relevant (as may differences in Cu(I)-binding cooperativity), and suggests that the exported Csp3s act as a more temporary store of Cu(I) for pMMO, whilst Csp3 plays a role in longer-term storage (consistent with the suggestion that the functions of these proteins are not directly related). 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.

A possible link between Csp3s and pathogenicity?

As mentioned in the introduction, and covered in previous Minireviews in this Series, the interplay between Cu homeostasis systems in a pathogen and host is emerging as important for virulence (30-34, 91). Compared to nutritional immunity used to withhold other essential metal ions, hosts are thought to expose invading pathogens to Cu (32, 33, 91). In mammalian hosts ATP7A pumps Cu into the phagolysosomal compartment and Cu homeostasis systems can protect the pathogen against this attack (30-33, 91-
A number of possible defence approaches have been identified, such as Cu efflux and sequestration, including by a Cu(II)-binding siderophore (34, 94). Csps are present in pathogenic bacteria including *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) could make them ideal to defend pathogens against Cu attack by a host. The Cu(I)-buffering ability of Csp3 that prevents toxicity in the Δ*copA* strain of *E. coli* (Fig. 3, D and E) demonstrates that these proteins, when produced at relatively high levels, can take the place of Cu-efflux pumps, known virulence factors (30, 88, 92, 93), in providing protection against elevated Cu levels. The only other characterized bacterial Cu 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 Cu-attack remains to be established.

**Concluding remarks**

The Csps were identified in methanotrophs, bacteria with atypically high Cu 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 Cu 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 Cu for remains unknown. The presence of bacterial Cu storage proteins seems consistent with a number of other observations: (1) that bacterial Cu-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 Cu (97). It also highlights that there are alternative mechanisms to using different cellular compartments to prevent mis-metallation of proteins by Cu (37). Furthermore, the ability of bacteria to store Cu in the cytosol could provide further insight into the observation that certain periplasmic proteins are loaded with Cu that has passed through the cytosol (88, 98, 99). Most of the organisms in which this has been observed possess a Csp3.

A lot more work is needed to understand Cu storage and removal for the exported Csp1s and the cytosolic Csp3s. Csps are only found in ~40% of methanotrophs, whilst 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 Cu-storage systems yet to be found. Even if this is not the case, the presence of Csp3s indicates that as predicted for other metallproteomes (100), there are likely to be cytoplasmic Cu-requiring enzymes yet to be discovered.

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FOOTNOTES
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The abbreviations used are: Cu, copper; ROS, reactive oxygen species; Fe-S, iron-sulfur; MTs, metallothioneins; CspS, Cu storage proteins, Csp1, exported Csp; Csp3, cytosolic Csp; pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase; Mbn, methanobactin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tat, twin-arginine translocase; BCS, bathocuproine disulfonate; WT, wild type; ΔcopA, copA delete strain.
Figure 1. Structures of Cu(I)-Mbns and Mbn-mediated Cu uptake. The crystal structures of the Cu(I)-Mbns from *M. trichosporium* OB3b (A, PDB file 2XJH) (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) 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 to the more compact *M. trichosporium* OB3b Cu(I)-Mbn. Also shown are Cu-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 grey triangles and grey bars) and *M. hirsuta* CSC1 Cu(I)-Mbn (open cyan circles and cyan bars) to sMMO-active cells. In both cases, Cu uptake and switchover from sMMO to pMMO is faster with the native Cu(I)-Mbn (52).
Figure 2. Discovery and characterization of Csp1 in *M. trichosporium* OB3b. A, Copper content of anion-exchange soluble extracts of *M. trichosporium* OB3b purified on a gel-filtration column, and SDS-
PAGE analysis of the fractions eluted between 8 and 15 ml (35). The intensity profile of the band indicated with an arrow matches that of the main Cu peak. B, The sequence of this 146 amino-acid residue protein (MtCsp1) has a predicted Tat signal peptide (bold) and 13 Cys residues (highlighted yellow) largely present in CXXXC and CXXC motifs (underlined). C, The tetrameric arrangement in the crystal structure (PDB file 5FJD) (35) of the over-expressed predicted mature form of MtCsp1 (Gly1 to Ala122) with the pink (top left) four-helix bundle monomer (helices numbered) shown in detail (D), highlighting the Cys residues that all point into the core, and residues around the mouth of the bundle, in stick representation. E, The structure (PDB file 5FJE) of Cu(I)-MtCsp1 (35) with the metal ions represented as grey spheres and numbered. F, the crystal structure (PDB file 1RJU) of a truncated form of Saccharomyces cerevisiae MT binding eight Cu(I) ions via 10 Cys residues (39) is shown for comparison. G, A sequence alignment of the three Csp homologues present in M. trichosporium OB3b created in T-coffee (65) using the predicted mature forms of MtCsp1 and MtCsp2.
Figure 3. Structural and functional studies of Csp3s. A, The crystal structure of the apo-MtCsp3 (PDB file 5ARM) tetramer (36). The side chains of the 18 Cys residues pointing into the core, and three His residues at the mouth, are shown as sticks in the pink (top left) four-helix bundle monomer. B, The crystal structure (PDB file 5ARN, 36) of Cu(I)-MtCsp3 (αN omitted) with the metal ions as grey spheres and numbered. C, The crystal structure of the apo-BsCsp3 (PDB file 5FIG) tetramer of four-helix bundles (36) using the same representation as in (A), with 19 Cys residues pointing into the core of the pink (top left) four-helix bundle monomer. D, the growth (37 °C) of ΔcopA E. coli in the absence of (black circles) and plus 1.0 mM (blue circles) added Cu(NO₃)₂ are compared with ΔcopA cells over-expressing BsCsp3 in the absence of (red triangles) and plus 1.0 mM (cyan triangles) added Cu(NO₃)₂ (36). E, the growth (37 °C) of WT E. coli in the absence of (black circles) and plus 3.4 mM (blue circles) added Cu(NO₃)₂ are compared.
with WT cells over-expressing BsCsp3 in the absence of (red triangles) and plus 3.4 mM (cyan triangles) added Cu(NO₃)₂. Over-expressed BsCsp3 can protect ΔcopA (D) and WT (E) E. coli from Cu toxicity.
Figure 4. Bioinformatics of Csp1s, Csp3s and the Mbn operon in methanotrophs. The 89 methanotroph genomes currently available in the NCBI database were interrogated with pBLASTp using MtCsp1 and MtCsp3 as search queries. A. A Venn diagram of the distribution of Csp1, Csp3 and Mbn operons (identified by the presence of homologues of M. trichosporium OB3b MbnA, MbnB and MbnC) in methanotroph genomes. Alignments of 26 and 36 sequences (see Figs. S1 and S2) were used to produce WebLogos (73) for Csp1s (B) and Csp3s (C) respectively. In these the overall height of the stack at a particular position represents the degree of conservation, whilst the height of the symbol for an amino-acid residue (green for polar, purple for neutral, blue for basic, red for acidic and black for hydrophobic) within the stack signifies relative frequency. Widths are unscaled so less frequently occupied positions in the alignment (see Figs. S1 and S2) are not represented by narrower stacks (composition adjustment was left to the default value for typical amino acid usage). Signal peptides were identified using SignalP (74) and TatP (62), and this region is labelled in (B). D. A schematic of the model methanotroph M. trichosporium OB3b showing two possible arrangements of the intracytoplasmic membranes that house pMMO, and the location and potential roles of MtCsp1, MtCsp2 and MtCsp3. The established cytosolic Cu sensing (CueR) and efflux (CopA) system, and the known locations and interactions of Mbn (MbnT is a Ton-B-dependent transporter (56) and MbnE is suggested to bind Mbn in the periplasm (58)) are also included. A much more detailed model of Cu homeostasis and Cu-regulated switchover in a methanotroph can be found in ref. 59. This includes the Csps, but does not discuss their importance.
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