A four-helix bundle stores copper for methane oxidation

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Methane-oxidizing bacteria (methanotrophs) require large quantities of copper for the membrane-bound (particulate) methane monoxygenase1,2. Certain methanotrophs are also able to switch to using the iron-containing soluble methane monoxygenase to catalyse methane oxidation, with this switchover regulated by copper3,4. Methane monoxygenases are nature’s primary biological mechanism for suppressing atmospheric levels of methane, a potent greenhouse gas. Furthermore, methanotrophs and methane monoxygenases have enormous potential in bioremediation and for biotransformations producing bulk and fine chemicals, and in bioenergy, particularly considering increased methane availability from renewable sources and hydraulic fracturing of shale rock5,6. Here we discover and characterize a novel copper storage protein (Csp1) from the methanotroph Methylosinus trichosporium OB3b that is exported from the cytosol, and stores copper for particulate methane monoxygenase. Csp1 is a tetramer of four-helix bundles with each monomer binding up to 13 Cu(I) ions in a previously unseen manner via mainly Cys residues that point into the core of the bundle. Csp1 is the first example of a protein that stores a metal within an established protein-folding motif. This work provides a detailed insight into how methanotrophs accumulate copper for the oxidation of methane. Understanding this process is essential if the wide-ranging biotechnological applications of methanotrophs are to be realized. Cysotolic homologues of Csp1 are present in diverse bacteria, thus challenging the dogma that such organisms do not use copper in this location.

Biological exploits copper to catalyse a range of important reactions, but use of this metal has been influenced by its availability and potential toxicity7-9. In eukaryotes, excess copper is safely stored by cytosolic Cys-rich metallothioneins10-12. Prokaryotic cytosolic copper-requiring enzymes are not currently known, with most prokaryotes thought not to possess copper storage proteins. Copper-binding metallothioneins, such as those in eukaryotes, have been identified in pathogenic mycobacteria where they help detoxify Cu(I)13. Methanotrophs are Gram-negative bacteria that produce specialized membranes to harbour particulate methane monoxygenase (pMMO), which could either be discrete from or connected to the plasma membrane14-16. These organisms have the typical machinery for copper efflux from the cytosol17, but also possess the only currently characterized prokaryotic copper-uptake system; secreted modified peptides called methanobactins (mbtins)18-20, which bind Cu(I) with high affinity21,22, localize to the cytoplasm22, and are involved in soluble (s)MMO to pMMO switchover23.

While searching for internalized Cu(I)-mbtin in the switchover methanotroph M. trichosporium OB3b, large amounts of soluble, protein-associated copper were detected. This was unexpected as bioinformatics predicts the transcriptional activator CueR as the only soluble copper protein24. To identify the copper-binding proteins in the most abundant copper pool, soluble extracts from cells grown under elevated copper were separated by anion-exchange and gel-filtration chromatography. Copper peaks match the intensity profiles of a protein band just below the 14.4 kDa marker (Fig. 1a, b), which has been purified to near homogeneity (Extended Data Fig. 1a, b and Fig. 1c). This band was excised (Fig. 1b, c) and identified by peptide mass fingerprinting as an uncharacterized conserved hypothetical protein (herein Csp1, Extended Data Fig. 1c). The mature protein (122 amino acids) contains 13 Cys residues with a predicted molecular mass of 12,591.4 Da, consistent with its migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 1).

Recombinant apo-Csp1 (12,589.8 Da) elutes from a gel filtration column with an apparent molecular mass of ~50 kDa (Fig. 2a), indicating anion-exchange fraction (Extended Data Fig. 1a) was purified on a Superdex 75 column (Extended Data Fig. 1b), with the copper content and SDS–PAGE analyses of eluted fractions shown in c. The band of interest that migrates below the 14.4 kDa marker is indicated in each panel with an arrow, and protein identification was performed on the bands from the 7.0 ml (b) and 12.0 ml (c) fractions.

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Figure 2 | The structure of apo-Csp1. a, Analytical gel-filtration chromatograms of apo-Csp1 (red line) and protein to which 14.0 molar equivalents of Cu(I) were added (blue line) for samples (100 μM when injected) in 20 mM HEPES pH 7.5 containing 200 mM NaCl. The absorbance was monitored at 280 nm with the values for Cu(I)-Csp1 divided by 10 (see Extended Data Fig. 2a, b). Inset, SDS–PAGE analysis of the purified protein. b, Far-UV circular dichroism spectra of apo-Csp1 (red line) and Csp1 plus 14.0 equivalents of Cu(I) (blue line) at 39.6 and 35.7 μM respectively in 100 mM phosphate pH 8.0. c, The tetrameric arrangement in the asymmetric unit of the crystal structure of apo-Csp1, with the side-chains of the Cys residues that point into the core of the four-helix bundle shown as sticks for one monomer in d, and involves His36, Met40, Met43 (on the extended α1) and Met48.

Figure 3 | [Cu(I)]-binding by Csp1. a, Plot of [Cu(BCA)3]+ concentration against the [Cu(I)]/[Csp1] ratio upon titrating Cu(I) into apo-Csp1 (2.43 μM) in the presence of 103 μM BCA (same buffer as for Fig. 2a). [Cu(BCA)3]+ starts forming after ~12 equivalents of Cu(I) are added. b, Analytical gel-filtration chromatogram of Csp1 (116 μM) mixed with ~25 equivalents of Cu(I) in the same buffer. Csp1 (Bradford, red squares), copper (atomic absorption spectroscopy, blue triangles) and Cu(I) (BCS in the presence of 7.6 M urea, open cyan circles) concentrations are shown. The main Csp1-containing fractions bind 11.8–12.9 equivalents of Cu(I). c, The structure of Cu(I)-Csp1 (chain A) including the anomalous difference density for copper contoured at 3.5σ (orange mesh). The copper ions (Cu1–Cu13 correspond to A1123–A1135 in the PDB file 5AJF) are represented as dark grey spheres, and with bond distances (in Å) to the Cu(I) ion. At other Cu(I) sites the Cys ligands originate from adjacent α-helices (for example, Cu2, Cu3 and Cu4 in Fig. 3d and Cu10, Cu12, Cu13, Cu11, Cu12, Cu13 and Cu14 in Fig. 3e). d, The opening into the core of the four-helix bundle is facing out in d, and e with bond distances (in Å) in red.
with bond angles ranging from 102° to 142° (Fig. 3e). Cu13 is also coordinated (Fig. 3e) by Met48 (2.6 Å), as well as by His36 (N8, 2.0 Å) and Cys37 (2.2 Å). These two atypical Cu(I) sites (Cu11 and Cu13) are found at the open end of the bundle, and with the nearby Met40 and Met43 (Fig. 3c) potentially help to recruit the metal.

Metal storage within an established protein-folding motif has not previously been observed. Iron is stored by ferritins using polymeric four-helix bundles, but with monomers forming a protein envelope that surrounds a ferric-oxide mineral core. Storing multiple Cu(I) ions within a four-helix bundle in Csp1 provides a stark contrast to unstructured apo-metallothioneins that fold around metal clusters. For example, a truncated form of yeast metallothionein binds a Cu(I)-thiolate cluster using ten Cys residues with three-coordinate and two two-coordinate sites. A four-helix bundle is formed upon Cu(I) addition to a synthetic peptide possessing a CXXC motif, and binds a Cu5S4 cluster. The arrangement of the Cu(I) ions within Csp1 is unprecedented in biology and inorganic chemistry.

Tetrameric Csp1 is capable of binding up to 52 Cu(I) ions, consistent with a role in copper storage. The major copper-requiring protein in M. trichosporium OB3b is pMMO. Regardless of the uncertainty about the structure of the specialized metalloenzymes that house pMMO, cytosolic copper must cross a membrane before incorporation into this enzyme. Csp1 and its closely related homologue Csp2 possess signal peptides (Extended Data Figs 1c and 1d), predicted as targeting the twin arginine translocation (Tat) machinery, and therefore locate outside the cytosol. To test whether Csp1 and Csp2 store copper for pMMO, the Csp1/csp2 double mutant strain of M. trichosporium OB3b was constructed. Switchover to sMMO for cells transferred from high to low copper is significantly faster in Csp1/csp2 than in the wild-type strain, and sMMO activity is 1.8 times greater in the former after almost 28 h (Extended Data Fig. 4). These data are not inconsistent with Csp1 and Csp2 storing, and potentially also chaperoning, copper for pMMO, thus allowing M. trichosporium OB3b to use this enzyme longer for growth on methane when copper becomes limiting, although this hypothesis has not been explicitly tested.

An important attribute of a metal storage protein is its metal affinity. Upon increasing the concentration of BCA, Cu(I) starts to be withheld from Csp1 (Fig. 4a and Extended Data Fig. 5a–c). The buffering of free Cu(I) by ligands such as BCA and the tighter chromophoric Cu(I) ligand bathocuproine disulfonate (BCS; log KBCS = 20.8 (ref. 25), see Extended Data Fig. 6a, b) has been used to obtain an average Cu(I) affinity for Csp1 of ~1 × 1017 M⁻¹ (Fig. 4b, c and Extended Data Fig. 6c, d). Mbttin, the copper-chelating ligand produced by M. trichosporium OB3b, has a much tighter Cu(I) affinity, and stochiometrically removes Cu(I) from Csp1 in ~1 h (Fig. 4d and Extended Data Fig. 7a–d). This high affinity makes copper removal from imported mbttin potentially problematical (for example apo-Csp1 cannot directly acquire Cu(I) from mbttin; Extended Data Fig. 7e), and Cu(I)-mbttin may need to be degraded within a cell to deliver the metal to, pMMO, an enzyme of great environmental importance that has tremendous biotechnological potential for the utilization and mitigation of methane. The prediction would be that Csp3 can store copper in the cytosol, not only in M. trichosporium OB3b but in many other bacteria. This raises the possibility that there are cytosolic copper-requiring enzymes in bacteria still to be discovered.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions C.D. and K.J.W. designed the research, N.V. and S.J.A. performed in vitro characterization of Csp1 and Cu(I) binding. S.P. isolated Csp1 from M. trichosporium OB3b and crystalized the protein, A.B. and N.G.P. solved the crystal structure, and A.T.C. and J.C.M. constructed and characterized the Acsp1::csp2 M. trichosporium OB3b strain. C.D. wrote the paper with help from all authors.

Author Information Atomic coordinates have been deposited in the Protein Data Bank under accession numbers 5AJE and 5AJF for apo- and Cu(I)-Csp1 respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to C.D. (christopher.dennison@ncl.ac.uk).
Identification and purification of copper proteins from *M. trichosporium* OB3b. *M. trichosporium* OB3b cultures were grown as described\(^\text{22}\) at 27 °C in a 5 l fermentor agitated at 250 r.p.m. in nitrate minimal salts (NMS) medium supplemented with 10 μM iron and typically 5 μM copper. Cultures were analysed for sMMO activity as described\(^\text{33}\). Cells harvested at an absorbance, A\(_{600\text{nm}}\), typically between 1.1 and 2.2 were collected by centrifugation (4 °C) at 9,000 g and pellets washed with 20 mM HEPES pH 8.8 followed by the same buffer containing 10 mM EDTA. The cell pellet was resuspended in 20 mM HEPES pH 8.8 and lysed by freeze grinding in liquid nitrogen\(^\text{34}\). The lysate was allowed to thaw in an anaerobic chamber (BLB technology, \(\mathcal{O}_2 \leq 2 \text{ ppm}\), oxygen scavenging into biotinylating tubes sealed in the anaerobic chamber and centrifuged at 160,000 g for 1 h at 10 °C. The supernatant was recovered inside the anaerobic chamber, diluted fivefold and loaded (either 59 or 90 mg protein from 10 and 16 l of cells, respectively) onto a 5 ml Hitrap Q HP anion-exchange column (GE Healthcare). For the purification of extracts from 10 l of cells, the Hitrap column was eluted with a linear NaCl gradient (0–200 mM) using either a stirred cell or a centrifugal filter device (Millipore) with a linear NaCl gradient (0–200 mM, total volume 100 ml). Csp1-containing fractions, identified by SDS–PAGE using Oriole fluorescent gel stain (Bio-Rad). All images of fluorescently labelled gels were inverted to make bands clearer in print. Gel-filtration chromatography of copper-containing fractions was performed on a Sephadex G100 (Sigma) packed column (1 cm × 20 cm) inside the anaerobic chamber, or on a Superdex S75 10/300 GL (GE Healthcare) column in 20 mM HEPES pH 7.5 plus 200 mM NaCl (thoroughly degassed and nitrogen-purged for the Superdex 75 column that was attached to an Akta Purifier) and at flow rates of 0.35 and 0.8 ml min\(^{-1}\) respectively (fraction size 1 ml). Proteins whose intensity on SDS–PAGE gels correlated with copper concentration profiles were excised from gels and underwent peptide mass fingerprinting\(^\text{36}\). Digestion with trypsin was performed at an E/S ratio of 1:100 overnight in 50 mM NH\(_4\)HCO\(_3\) pH 8. The resultant peptides were resuspended in 0.1% aqueous trifluoroacetic acid and desalted using C18 ZipTips (Millipore). Peptides were then separated on a NanoAcquity liquid chromatography system (Waters) using a 75 μm × 100 mm C18 capillary column (Waters). A linear gradient from 1 to 50% acetonitrile in 0.1% aqueous formic acid was applied over 30 min at a flow rate of 0.3 ml min\(^{-1}\). Eluted peptides were identified using a linear trap quadrupole Fourier transform mass spectrometer (Thermo Electron) in positive ionization mode with scans over 700–1,000 m/z in data-dependent mode and a Fourier transform mass spectrometry (MS) resolution setting of 50,000. The top five ions in the parent scan were subjected to MS/MS analysis in the linear ion trap region. The proteins from which detected peptides originated were identified using the Mascot MS/MS ion search tool (Matrix Science) by comparison against the entire database of proteobacteria in NCBI. SignalP and TatP were used to identify putative signal sequences\(^\text{32}\). Therefore, for most Cu(I)-Csp1 samples the number of Cu(I) equivalents quoted are based on the apo-protein concentration determined by DTNB (the Cu(I)-Csp1 concentrations were determined by Bradford assay (Coomassie Plus protein assay kit, Thermo Scientific) with BSA standards (0–1,000 μg ml\(^{-1}\)). The ratio of apo-Csp1 concentration obtained using the Bradford and DTNB assays (Bradford:DTNB ratio) was 1.11 ± 0.12 (\(n = 27\) for samples not treated with DTNB and 1.03 ± 0.04 (\(n = 9\) for samples that were reduced before these assays. Incubation with DTNB does not have a significant effect on the thiol content and this step was excluded from all subsequent experiments as contamination of apo-Csp1 with even trace amounts of Cu(II) would influence quantification using DTNB. Investigating Cu(I)-binding to Csp1. Cu(I) stock solutions (50–100 mM [Cu(II)Cl\(_2\)F\(_2\)] in 100% acetonitrile) were diluted to ~1–12 mM in 20 mM HEPES pH 7.5 plus 200 mM NaCl in the anaerobic chamber\(^\text{15}\). Copper concentrations were determined by AAS, and Cu(I) was quantified anerobically by UV–vis with the chromophoric high-affinity Cu(I) ligands BCS and BCA by monitoring formation of [Cu(BCS)]\(^{+}\), and [Cu(BCA)]\(^{3+}\) at 483 nm (\(\epsilon = 12,500 \text{ M}^{-1} \text{ cm}^{-1}\)) and 562 nm (\(\epsilon = 7,700 \text{ M}^{-1} \text{ cm}^{-1}\)) respectively\(^\text{38, 39}\). Cu(I) was added to apo-Csp1 by mixing the appropriate amount of the buffered Cu(I) solution with apo-Csp1 (–2–20 μM) that had been quantified by DTNB, in 20 mM HEPES pH 7.5 plus 200 mM NaCl in the anaerobic chamber. Using DTNB in urine is a more precise method for quantifying apo-Csp1 than the Bradford assay, particularly at low protein concentrations, and was therefore used routinely. However, the DTNB assay in urine is very slow and is only suitable for use in the Extended Data Fig. 5i. Therefore, for most Cu(I)-Csp1 samples the number of Cu(I) equivalents quoted are based on the apo-protein concentration determined by DTNB (the Cu(I)-Csp1 concentrations take into account dilution of the sample due to the addition of Cu(I), and the number of Cu(I) equivalents quoted are those in the final sample). The [Cu(I)]/Csp1 ratio was routinely checked using protein (Bradford assay) and copper (AAS and with 2.5 mM BCS both in the absence and presence of ~7 mM urea, compared in Extended Data Fig. 2d) quantifications, with good agreement. For titrations (performed more than 10 times), Cu(I) from the buffered solution was added to apo-Csp1 (–2–20 μM) in 20 mM HEPES pH 7.5 plus 200 mM NaCl in a final concentration of Cu(I) ranging from 0.1 to 5 μM. The immediate appearance of ligand-to-metal charge transfer (LMCT) bands, characteristic of Cu(I) coordination by thiolate\(^\text{40, 32}\) was monitored in the UV region. Emission spectra were acquired on a Cary Eclipse fluorescence spectrophotometer (Varian) by exciting at 280 nm and following the emission in the 400–700 nm range using emission and excitation monochromators.
excitation slits of 20 and 10 nm respectively. The concentration of the Cu(I) solution was regularly checked during titrations, usually with BCA, and replaced as required.

**Competition between Csp1 and chromophoric ligands.** The binding of Cu(I) by Csp1 in the presence of either BCA or BCS was investigated in a variety of ways. Additions of Cu(I) to Csp1 (1.2–3.0 μM) were performed in the presence of ~90–110 μM BCA in 20 mM MES at pH 5.3 (the pKa of BCA is 3.8 (ref. 25) and its \( β_1 \) value is therefore hardly affected at this pH value) and 6.5, HEPES at pH 7.5, TAPS at pH 8.5 and CHES at pH 9.5, all plus 200 μM NaCl. [Cu(BCA)]1− concentrations were determined under anaerobic conditions as described above. This experiment was repeated twice at pH 6.5 and at least three and up to six times at other pH values, except at pH 7.5, when it was performed more than ten times. Apart from at pH 5.5, equilibration typically took less than 10 min (~20 min at ~11.5 Cu(I) equivalents). During these titrations the concentration of the Cu(I) solution was regularly checked and replaced as required. For experiments at pH 5.5 (MES), and at higher concentrations of BCA (up to ~12.2 μM and using ~2.0–3.7 μM apo-Csp1), UV–vis spectra were acquired between 4 and 48 h after mixing, to ensure equilibration had occurred (experiments at pH 6.5 and 9.5 were repeated two and four times respectively, while the experiment at pH 7.5 was repeated six times). The final pH values of samples were checked at the end of incubation for up to 67 h. A comparison of the ability of Csp1 to compete with BCA and BCS was performed at least three times by incubating Cu(I)-Csp1 (~2.4–2.7 μM) loaded with ~10–13 equivalents of Cu(I) with various concentrations of either ligand in 20 mM HEPES pH 7.5 plus 200 mM NaCl in the anaerobic chamber. [Cu(BCS)]2− and [Cu(BCA)]2− concentrations were determined by UV–vis under anaerobic conditions of mixtures incubated for various times (4–48 h) with very little change. Furthermore, apo-Csp1 (~2.5–2.8 μM) plus BCS (~100 or 250 μM) was incubated anaerobically with 0 to ~22 Cu(I) equivalents in 20 mM HEPES pH 7.5 plus 200 mM NaCl (repeated four times). The absorbance at 483 nm was monitored anaerobically after 4 h and up to 43 h after mixing (no variation observed). The kinetics of Cu(I) removal from Cu(I)-Csp1 (~0.3–1.6 μM) loaded with ~11–14 equivalents of Cu(I) by ~2.5–5.0 μM BCS was compared (five times) in the absence and presence of ~7 M urea monitored anaerobically at 483 nm in 20 mM HEPES pH 7.5 plus 200 mM NaCl.

**Estimation of the average Cu(I) affinity of Csp1.** The average Cu(I) affinity of Csp1 was estimated by determining the Cu(I) occupancy of Csp1 as a function of the concentration of free Cu(I) ([Cu(I)]free) buffered using either BCA or BCS24−26. Apo-Csp1 (~2.7 and ~3.6 μM respectively) in 20 mM HEPEs pH 7.5 plus 200 mM NaCl was mixed anaerobically with increasing Cu(I) concentrations in the presence of BCS (101 μM) or BCA (1,210 and 2,000 μM). Mixtures were incubated anaerobically with 0 to ~22 Cu(I) equivalents in 20 mM HEPES pH 7.5 plus 200 mM NaCl (repeated four times). The absorbance at 483 nm was monitored anaerobically after 4 h and up to 43 h after mixing (no variation observed). The kinetics of Cu(I) removal from Cu(I)-Csp1 (~0.3–1.6 μM) loaded with ~11–14 equivalents of Cu(I) by ~2.5–5.0 μM BCS was compared (five times) in the absence and presence of ~7 M urea monitored anaerobically at 483 nm in 20 mM HEPES pH 7.5 plus 200 mM NaCl.

**Fractional Cu(I) occupancy** was calculated using the maximum value observed in a particular experiment (see below) and plots against [Cu(I)]free were fitted to the nonlinear form of the Hill equation (3) to obtain the average dissociation constant of Csp1 for Cu(I) (\( K_{Cu} \)) and Hill coefficient (n value):
The Cu(I)-Csp1 crystal for the X-ray absorption near-edge spectrum shown in Extended Data Fig. 2c was obtained as above but using 600 nl of protein plus 300 nl of 0.025 M MgCl$_2$, 0.025 M McA$_7$, 0.1 M Tris-Bicine pH 8.5. 13.5% v/v 2-methyl-2,4-pentanediol (racing) plus 13.5% PEG 1000 and 13.5% PEG 3350 (80 μl well solution). Crystals were frozen directly in the reservoir solution.

Diffraction data were collected at the Diamond Light Source, UK, on beamlines I02 (apo-Csp1, λ = 0.9795 Å) and I24 (Cu(I)-Csp1, λ = 1.377 Å) at 100 K, processed and integrated with DIALS and scaled using Aimless$^{44}$. For both data sets, space groups were determined using Pointless and later confirmed during refinement$^{45}$. The phase was solved by single-wavelength anomalous dispersion using copper, but was complicated by poorly resolved low-resolution reflections. The omission of data from 44.42 to 10.00 Å was required for successful heavy-atom location and the calculation of initial phases. Phasing, density modification and initial model building were performed using PHASER$^{46}$ through the CCP4 interface$^{47}$, using SHELDX$^{48}$, PARRTOT$^{49}$ and Buccaneer$^{50}$. The model of Cu(I)-Csp1 was used as the search model for molecular replacement in Molrep$^{51}$ to solve the apo-protein data set. The first 11 residues could not be modelled in both structures (His12 is close to the open end of an adjacent monomer in the Csp1 tetramer). Solvent molecules were added using COOT and checked manually. Simple solvent scaling was used for the apo-Csp1 model and Babinet solvent scaling was used for the Cu(I)-Csp1 model. All other computing used the CCP4 suite of programs$^{46}$. Five per cent of observations were randomly selected for the $R_{max}$ set. The models were validated using MolProbity$^{52}$ and data statistics and refinement details are reported in Extended Data Table 1. In a Ramachandran plot, 100% of residues are in most favoured regions for both models, and chain A of apo- and Cu(I)-Csp1 overlay with a root mean squared deviation of 0.2 Å. In the structure of Cu(I)-Csp1 (5AF), the Csp1 ions referred to herein as Cu–Cu13 are numbered A1123–A1135 in chain A and the corresponding sites in chain B are numbered B1123–B1135.

**X-ray absorption near-edge spectroscopy.** X-ray absorption near-edge spectroscopy was conducted on beamline I24 at the Diamond Light Source, UK, using a Vertex-EX detector (Hitachi). X-ray fluorescence was measured on a fresh Cu(I)-Csp1 crystal between 8,948 and 9,030 eV with an acquisition time of 3 s per data point and a constant step of 0.5 eV for the spectrum shown in Extended Data Fig. 2c (measurements were made on at least two other crystals giving very similar spectra).

**Construction of strain Δcsp1Δcsp2 of M. trichosporium OB3b.** A double mutant, strain Δcsp1Δcsp2, was constructed by sequential deletion of csp1 followed by csp2, from M. trichosporium OB3b using a previously described method$^{53}$ with minor modifications. In each case, using genomic DNA from M. trichosporium OB3b by con-
Extended Data Figure 1 | Purification of proteins from *M. trichosporium* OB3b and the amino-acid sequence of Csp1. a, The copper content of anion-exchange fractions (NaCl gradient shown as a dashed line) and the SDS–PAGE analysis of selected fractions (1 ml) from the purification of soluble extract from *M. trichosporium* OB3b cells. The band just below the 14.4 kDa marker, indicated with an arrow, is present. Fraction 32 was judged to have the lowest level of contaminating proteins and was further purified by gel-filtration chromatography on a Superdex 75 column (b). Csp1 is present in the peak that elutes at ~11 ml and contains considerable copper (see Fig. 1c). b, The amino-acid sequence of Csp1 showing the predicted Tat leader peptide (the first 24 residues of the pre-protein) in italics. The 13 Cys residues are highlighted in yellow, and His36 (cyan), Met40, Met43 and Met48 (magenta) are also indicated (the numbering of these residues refers to the mature protein). The CXXC and CXXXC motifs are underlined. The region in bold corresponds to the single tryptic fragment identified on two separate occasions in MS analysis, representing 11% sequence coverage of the mature protein (Mascot search of peptide mass fingerprint, expectation value = 1.9 × 10^{-2}). The sequence of this fragment was confirmed by liquid chromatography/MS/MS (data not shown). This is the only tryptic peptide from the mature protein that would be anticipated to be readily detected by MS (owing to either small mass or presence of Cys residues in all other theoretical tryptic fragments) and is unique to this protein among all proteobacterial protein sequences in the NCBInr database.
Extended Data Figure 2 | Cu(I) binding to Csp1. **a**, UV–vis difference spectra upon the addition of Cu(I) to apo-Csp1 (5.32 μM) showing the appearance of S(Cys)→Cu(I) LMCT bands.**b**, Plots of absorbance at 250 nm (filled squares), 275 nm (filled circles) and 310 nm (open circles) against [Cu(I)]/[Csp1] ratio taken from the spectra in **a**. The absorbance rises steeply until ~11–15 Cu(I) equivalents but continues to rise, particularly at lower wavelengths, making binding stoichiometry difficult to determine precisely with this approach. Systems that bind multiple Cu(I) ions in clusters such as those found in metallothioneins, typically give rise to luminescence at around 600 nm (refs 10, 13). However, limited luminescence is observed at 600 nm during the titration of Cu(I) into Csp1 (data not shown). **c**, X-ray absorption near-edge spectrum of a fresh crystal of Cu(I)-Csp1 at 100 K. **d**, Plots of [Cu(BCS)₂]⁺ formation against time after the addition of Cu(I)-Csp1 (0.93 μM) loaded with 11.8 equivalents of Cu(I) to 2.510 μM BCS either in the absence (dashed line) or presence (solid line) of 7.9 M urea. Cu(I) is removed faster in urea and is limited by the rate of Cu(I)-Csp1 unfolding (Extended Data Fig. 5i). The presence of urea has little effect on the end point for this reaction. Experiments in **a**, **b** and **d** were all performed in 20 mM HEPES pH 7.5 containing 200 mM NaCl.
Extended Data Figure 3 | Sequence comparison of Csp1 homologues in M. trichosporium OB3b.

The M. trichosporium OB3b genome possesses two genes that code for Csp1 homologues, Csp2 and Csp3, having 58 and 19% sequence identity to Csp1, respectively. The predicted Tat leader peptides of Csp1 (MERRDFVTAFGALAAAAAASSAFA) and Csp2 (MERRQFVAAIGAAAAAASASRAFA) are omitted. The Cys residues (13 in Csp1 and Csp2 and 18 in Csp3) are highlighted in yellow with CXXXC and CXXC motifs underlined. A CXXXC motif in an α-helix allows both of the Cys residues to coordinate the same Cu(I) ion (Fig. 3d, e), which is not the case for a CXXC motif. This is consistent with the observation that a synthetic peptide containing a CXXC motif binds a Cu₄S₄ cluster via a four-helix bundle made from four peptides, with coordination involving only one Cys per peptide²⁸,⁵⁶.

The alignment was produced using the T-coffee alignment tool⁵⁷. Asterisks indicate fully conserved sequence positions; the ‘*’ and ‘.’ symbols indicate strongly and weakly similar sequence positions respectively.
Extended Data Figure 4 | sMMO activity of wild-type *M. trichosporium* OB3b and the Δcsp1/csp2 strain. Purple colour, indicating sMMO activity, is evident at 19.25 h in the Δcsp1/csp2 strain (tubes 4–6), but not until 24.5 h in the wild type (WT, tubes 1–3) when using a qualitative assay. When quantified spectrophotometrically at 27.75 h, the average sMMO activity in the Δcsp1/csp2 strain (grey) is 1.8-fold greater ($P = 0.04$, one-tailed t-test) than that of the wild type (WT, white), as shown in the bar chart (mean ± s.d. of three replicates).
Extended Data Figure 5 | The dependence on pH of competition between Csp1 and BCA for Cu(I), and far-UV circular dichroism spectra showing pH stability and unfolding of Csp1 in urea. a, Plots of $\left[\text{Cu(BCA)}_2\right]^{2-}$ concentration against $[\text{Cu(I)}/[\text{Csp1}]]$ ratio for the addition of Cu(I) to apo-Csp1 $(2.38–2.56 \, \text{mM})$ in the presence of $103 \, \text{mM}$ BCA in $20 \, \text{mM}$ buffer (see Methods) at pH 5.5 (filled squares), 6.5 (filled circles), 7.5 (filled triangles), 8.5 (open circles) and 9.5 (open squares) plus $200 \, \text{mM NaCl}$. Equilibration is fast (<20 min) at pH 6.5 and higher and the data shown are from titrations of Cu(I) into apo-Csp1. At pH 5.5 equilibration is slower and the data are for mixtures incubated for 21 h. Also shown are results for mixtures of Cu(I) with apo-Csp1 $(3.31–3.67 \, \text{mM})$ at pH 6.5 (b) and 9.5 (c) in the presence of $120 \, \text{mM}$ (filled squares), $300 \, \text{mM}$ (open circles), $450 \, \text{mM}$ (stars), $600 \, \text{mM}$ (filled triangles) and $900 \, \text{mM}$ (open squares) BCA, all after incubation for 15 h. At lower BCA concentrations, Csp1 is able to compete effectively for Cu(I) in the pH range 6.5–9.5, giving Cu(I) binding stoichiometries of 12–14 (see also Figs 3a and 4a). At pH 5.5, Csp1 competes less effectively with BCA for Cu(I), most probably because of the protonation of Cys ligands 37. This is consistent with greater competition by $600 \, \text{mM}$ BCA at pH 6.5 (b) compared with pH 7.5 (Fig. 4a) because of deprotonation of the Cys ligands 37. g–i, Far-UV circular dichroism spectra of apo-Csp1 $(7.94 \, \text{mM}, 0.10 \, \text{mg ml}^{-1})$ except that spectra were acquired at 0, 15, 30, 45 and 60 min (solid lines) after addition of urea (7 M); unfolding is significantly faster at lower protein concentrations and is consistent with the reaction with DTNB in urea being complete in 20 min at Csp1 concentrations <4 \, \text{mM}. h, Far-UV circular dichroism spectra of Csp1 incubated with 14.0 equivalents of Cu(I) $(19.9 \, \text{mM}, 0.25 \, \text{mg ml}^{-1})$ as in g but at 0, 60, 240 and 480 min and 24 h (solid lines) after addition of urea (7 M) compared with the spectrum for Cu(I)-Csp1 in buffer with no urea (dashed line). The arrow in g to i indicates how the spectrum changes with time.
Extended Data Figure 6 | Competition for Cu(I) between Csp1 and chromophoric ligands and the determination of the apparent average Cu(I) dissociation constant for Csp1 using BCS. a, Plots of [Cu(L)₂]^{2+} concentration against [L] (BCA or BCS) after the incubation of Cu(I)-Csp1 (2.59 μM) loaded with 10.4 equivalents of Cu(I) with different concentrations of BCA (filled circles) and BCS (filled squares) for 17 h. b, Plots of [Cu(BCS)₂]^{3−} concentration against [Cu(I)] for apo-Csp1 (2.71 μM) in the presence of 99.2 μM (open squares) and 248 μM (filled squares) BCS incubated with increasing concentrations of Cu(I) (0, 4.38, 11.0, 15.3 and 21.9 equivalents; data shown after 17 h incubation). BCS competes much more effectively with Csp1 for Cu(I) than BCA, and [Cu(BCS)₂]^{3−} is stoichiometrically formed at 248 μM BCS. c, Plot of [Cu(BCS)₂]^{3−} concentration against the [Cu(I)]/[Csp1] ratio for mixtures of Cu(I) plus apo-Csp1 (2.70 μM) in the presence of 101 μM BCS (open squares) for 19 h. For comparison, the data from b (2.71 μM Csp1 in the presence of 99.2 μM BCS for 17 h) are also shown (filled squares). The data in a–c were all acquired in 20 mM HEPES plus 200 mM NaCl at pH 7.5. d, Fractional occupancy of Cu(I)-binding sites in Csp1 (maximum value is 11.3 equivalents in this experiment) at different concentrations of free Cu(I) for the experiment shown in c. The solid line shows the fit of the data to the nonlinear Hill equation giving $K_{Cu} = (1.3 \pm 0.1) \times 10^{-17}$ M ($n = 2.7 \pm 0.2$). Hill coefficients larger than 1 indicate positive cooperativity for Cu(I) binding by Csp1. Confirmation, and the cause, of this effect will be the subject of further studies.
Extended Data Figure 7 | Cu(I) exchange between Csp1 and mbtin. UV–vis spectra of apo-mbtin (dashed lines) and at various times up to 360 min (thick lines) after the addition of either Cu(I)-Csp1 or Cu(I). Cu(I)-Csp1 (1.02 μM) loaded with 13.0 equivalents of Cu(I) was added to either 13.4 μM (a) or 27.4 μM (c) apo-mbtin. Cu(I) alone (13.3 μM) was added to 13.4 μM (b) or 27.1 μM (d) apo-mbtin. Plots of absorbance at 394 nm against time for a–d are shown in Fig. 4d. Mbtin from *M. trichosporium* OB3b has a Cu(I) affinity of (6–7) × 10^20 M^-1 at pH 7.5 (determined using a logβ₂ value of 19.8 for [Cu(BCS)]^2+, but is an order of magnitude tighter if the more recent logβ₂ value of 20.8 (ref. 25) is used) and stoichiometrically removes Cu(I) from Csp1 within 1 h. e, UV–vis spectra of Cu(I)-mbtin (2.71 μM, black line) immediately after mixing with apo-Csp1 (234 μM, green line) and after incubation under anaerobic conditions for 1 h (blue line) and 20 h (red line). Small increases in absorbance are observed because of the absorbance of apo-Csp1 at these wavelengths and precipitation. The latter was more of a problem at longer incubation times and the sample at 20 h required filtering before running the spectrum shown. The small changes observed are not consistent with the formation of apo-mbtin. All experiments were performed in 20 mM HEPES pH 7.5 plus 200 mM NaCl.
Extended Data Figure 8 | Sequence comparison of Csp homologues from diverse bacteria. Homology searches show that Csp homologues are encoded in the genomes of diverse bacteria. Multiple sequence alignment of the three *M. trichosporium* OB3b proteins (OB3b Csp1, OB3b Csp2 and OB3b Csp3) with a selection of these proteins, including one member (from *Neisseria gonorrhoeae*) that also possesses a putative Tat signal sequence (underlined), shows that the Cys residues (highlighted in yellow) are highly conserved. The alignment was produced using the T-coffee alignment tool 57. Asterisks indicate fully conserved sequence positions; the ‘:’ and ‘.’ symbols indicate strongly and weakly similar sequence positions respectively. *N. gonorrhoeae* sequence: open reading frame (ORF) NGAG_01502, UniProt accession C1I025; *P. aeruginosa* sequence: ORF PA96_2930, UniProt accession X5E748 (PDB accession number 3KAW); *Streptomyces coelicolor* sequence: ORF SCO3281, UniProt accession Q9X8F4; *N. multiformis* sequence: ORF NmuI_A1745, UniProt accession Q2Y879 (PDB accession number 3LMF); *Rhizobium leguminosarum* sequence: ORF RLEG_20420, UniProt accession W0IHZ3; *Ralstonia metallidurans* sequence: ORF Rmet_5753, UniProt accession Q1LB64; *Salmonella enterica* sv. Typhimurium sequence: ORF STM14_1521, UniProt accession D0ZVJ6; *Bacillus subtilis* sequence: ORF BSU10600, UniProt accession O07571; *Legionella pneumophila* sequence: ORF LPE509_p00081, UniProt accession M4SK87.
## Extended Data Table 1 | Data collection and refinement statistics

|                          | Apo-Csp1       | Cu(I)-Csp1     |
|--------------------------|----------------|----------------|
| **Data collection**      |                |                |
| Space group              | P2₁            | P2             |
| Cell dimensions          |                |                |
|   $a$, $b$, $c$ (Å)      | 40.9, 105.9, 48.7 | 44.4, 41.4, 53.1 |
|   $\alpha$, $\beta$, $\gamma$ (°) | 90.0, 112.5, 90.0 | 90.0, 92.6, 90.0 |
| Resolution (Å)           | 44.95-1.50     | 53.06-1.90     |
|   (1.53-1.50)*           | 7.0 (50.5)     | 8.7 (43.3)     |
| $R_{merge}$ (%)          | 10.9 (2.6)     | 5.6 (2.1)      |
| Completeness (%)         | 99.7 (99.8)    | 99.1 (97.1)    |
| Redundancy               | 3.7 (3.7)      | 2.8 (2.4)      |
| **Refinement**           |                |                |
| Resolution (Å)           | 1.50           | 1.90           |
| No. reflections          | 60896 (3056)   | 15212 (990)    |
| $R_{work}$/$R_{free}$    | 12.2/17.9      | 19.8/23.2      |
| No. atoms                |                |                |
|   Protein                | 3209           | 1575           |
|   Ligand/ion             | 0              | 28             |
|   Water                  | 406            | 116            |
| B-factors                |                |                |
|   Protein                | 16.2           | 40.2           |
|   Ligand/ion             |               | 41.4           |
|   Water                  | 27.0           | 47.7           |
| R.m.s deviations         |                |                |
|   Bond lengths (Å)       | 0.020          | 0.016          |
|   Bond angles (°)        | 1.8            | 1.6            |

*Highest resolution shell is shown in parenthesis.
Extended Data Table 2 | Primers used for cloning Csp1 and making the Δcsp1/csp2 M. trichosporium OB3b strain

| Primer   | Sequence (5’ to 3’)* |
|----------|----------------------|
| Csp1_F   | GCCCATATGGGAGAGGATCCTCATTGC |
| Csp1_R   | GCCGCAATACTGGCAGGGACACTCTATGGC |
| 684AF    | ATATCCCGGGTAAAGGGTGAG ACCGCCCATCAG |
| 684AR    | GATCGTCGACACGACCGAGCAACCTAAAC |
| 684BF    | GATCGTCGACCTAAAGGGTGCCGCTCCTAGTTTC |
| 684BR    | GATCAAGCTTCGCCTCGCGTCCGTATTTC |
| 1592AF   | CATCAAGCTTCGCTGCGCAGCATCCTCCTC |
| 1592AR   | CATCCTGCAAGGCTGTCGCTCCGTCGTTC |
| 1592BF   | TAATGGGATCCGACGCTGTCGAGCTGAAC |
| 1592BR   | ATTAGAATTCGCGGAGCCCGCTGGAAG |
| 684TF    | CACATCGCCGCTGATCAG |
| 684TR2   | CGACCACGATCCTACAG |
| 1592TF   | ACCCTTCTACGCAATCCC |
| 1592TR   | ACGTGGATCGGCTCCTCCTC |

* Introduced restriction sites are underlined when relevant.