Acquisition of ionic copper by a bacterial outer membrane protein

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Abstract

Copper, while toxic in excess, is an essential micronutrient in all kingdoms of life due to its essential role in the structure and function of many proteins. Proteins mediating ionic copper import are known in eukaryotes, but have not yet been described in prokaryotes. Here we show that *Pseudomonas aeruginosa* OprC is a TonB-dependent transporter that mediates acquisition of ionic copper. Crystal structures of wild type and mutant OprC variants with silver and copper, as well as ICP-MS and electron paramagnetic resonance (EPR), suggest that binding of Cu(I) occurs via a surface-exposed methionine track leading towards an unprecedented CxxxM-HxM metal binding site that binds Cu(I) directly and can facilitate reduction of Cu(II) via the cysteine thiol. Together with quantitative proteomics and growth assays, our data identify OprC as an abundant component of bacterial copper biology that enables copper acquisition and potentially copper storage under a wide range of environmental conditions.

Introduction

Metals fulfil cellular functions that cannot be met by organic molecules and are indispensable for the biochemistry of life in all organisms. Copper is the third-most abundant transition metal in biological systems after iron and zinc. It has key roles as structural component of proteins or catalytic cofactor for enzymes¹, most notably associated with the biology of oxygen and in electron transfer. On the other hand, an excess of copper can be deleterious due to its ability to catalyse production of hydroxyl radicals^{2, 3}. Excessive copper may also disrupt protein structure by interaction with the polypeptide backbone, or via replacement of native metal cofactors from proteins, thus abolishing enzymatic activities via mismetallation^{4, 5}. Thus, cellular copper levels and availability must be tightly controlled. Bacterial copper homeostasis systems are well characterised⁶. Specific protein machineries are involved in fine-tuning the balance of intracellular copper trafficking, storage and efflux according to cellular requirement, in such a way that copper is always bound inside the cell. This control is executed by periplasmic and cytosolic metalloregulators, which activate transcription of periplasmic multi-copper oxidases, metallochaperones, copper-sequestering proteins^{7, 8} and transporters⁹⁻¹¹. To date, relatively few families of integral membrane proteins have been validated as copper transporters, and these have different structures and transport mechanisms¹². The P_{1B}-type ATPases such as CopA are responsible for Cu(I) efflux from the cytosol via several metal binding domains, using energy released from ATP hydrolysis¹³⁻¹⁵. A second class of copper export proteins are RND-type tripartite pumps such as CusABC, which efflux Cu(I) by utilising the proton-motive force¹⁶⁻¹⁸. Relatively few copper influx proteins have been identified. The inner membrane copper importer CcoA is a major facilitator superfamily (MFS)-type

transporter involved in fine-tuning the trafficking of copper into the cytosol and required for cytochrome c oxidase maturation^{19, 20}. The Ctr family of copper transporters is responsible for Cu(I) translocation into the cell without requiring external sources of energy²¹. However, Ctr homologs are found only in eukaryotes, and the molecular mechanisms by which copper ions enter bacteria is still a matter of debate. The exception is copper import via metallophores like methanobactin, a small Cu-chelating molecule that is secreted by methanotropic bacteria and most likely taken up via TonB-dependent transporters, analogous to iron-siderophores²².

Pseudomonas aeruginosa is a versatile and ubiquitous Gram-negative bacterium that promotes infections in several hosts and multiple sites. It is a notorious opportunistic pathogen in humans, and is mainly responsible for the development of chronic lung infection in cystic fibrosis patients^{23, 24}. *P. aeruginosa* has a number of TonB-dependent transporters (TBDTs) in the outer membrane (OM) dedicated to the acquisition of different iron-siderophore complexes such as pyochelin and pyoverdin²⁵. In addition, *P. aeruginosa* contains another TBDT, termed OprC (PA3790), whose function has remained enigmatic. Nakae *et al.* suggested that OprC binds Cu(II) with micromolar affinities²⁶. Transcription of OprC was recently found to be repressed in the presence of Cu(II) in the external medium under aerobic conditions²⁷, suggesting a role for OprC in copper acquisition. Very recently, the blue copper protein azurin was reported to be secreted by a *P. aeruginosa* Type VI secretion system and to interact with OprC, suggesting a role of the latter in Cu(II) uptake²⁷.

To clarify the role of OprC in copper biology, we have determined X-ray crystal structures of wild type and mutant OprC proteins in the absence and presence of copper and silver, and characterised metal binding via ICP-MS, EPR and quantum chemistry calculations. OprC indeed has the typical structure of a TBDT, and differences between the Cu-loaded and Cu-free protein demonstrate changes in tertiary structure that likely lead to TonB interaction. Strikingly, metal binding experiments show that while OprC can bind to both Cu (I) and Cu (II) *in vitro*, only Cu (I) is present at the principal CxxxM-HxM metal binding site, suggesting that Cu(I) is the imported redox state and that reduction of Cu(II) may be mediated by OprC.

Figure 1. OprC is a TonB-dependent transporter. Cartoon representation of (a,c) Cu-loaded OprC and (b,d) Cu-free OprC (OprC_{AA}). The N-terminal plug domain is shown separately for both forms (e,f). Structures are shown in rainbow from N-terminus (blue) to C-terminus (red); copper is represented as a magenta sphere. The arrow in (f) highlights the visibility of the Ton box in apo-OprC.

Results

OprC is a TonB-dependent transporter that binds ionic copper

The structure of OprC crystallised under aerobic conditions in the presence of 2 mM CuCl₂ was solved using single wavelength anomalous dispersion (SAD), using data to 2.0 Å resolution (Methods; Supplementary Table 1; Supplementary Fig. 1). As indicated by the successful structure solution, OprC contains bound copper and shows the typical fold of a TBDT, with a large 22-stranded β-barrel occluded by an N-terminal ~15 kDa plug domain (Fig. 1). The copper binding site comprises residues Cys143 and Met147 in the plug domain and His323 and Met325 in the barrel wall. The CxxxM-HxM configuration, which coordinates the copper in a tetrahedral manner (Fig. 2a, b), is highly unusual and has, to our knowledge, not been observed before in copper homeostasis proteins. The most similar site is present for one of the copper ions of the valence-delocalised Cu_A dimer in cytochrome c oxidase, where the

copper ion is coordinated by 2Cys+1Met+1His^{28, 29}. The other relevant sites are class I Type I copper proteins like pseudoazurin and plastocyanin, where copper is coordinated by 2His+1Cys+1Met³⁰. Interestingly, and unlike class I Type I copper proteins, concentrated solutions and OprC crystals are colourless in the presence of Cu(II). Another notable feature of the OprC structure becomes apparent when analysing the positions of the methionine residues. As shown in Fig. 2d, out of the 15 visible methionines in OprC, 10 are organised in such a way that they form a distinct "track" leading from the extracellular surface towards the copper binding site. An additional two methionines are not visible due to loop disorder, but given their positions (Met448, Met558) they will be a part of the methionine track. We propose that the methionine track serves to bind copper with low affinity and may guide the metal towards the principal binding site. It should be noted that the anomalous difference maps of OprC crystallised in the presence of Cu(II) do not show any evidence for weaker, secondary copper sites (Supplementary Fig. 1).

Figure 2. OprC has an unusual CxxxM-HxM binding site and a methionine binding track.
a, Stick models of copper-coordinating residues Cys143, Met147, Met325 and His323. Electron density in gray mesh (2Fo-Fc map contoured at 2.0 σ , carve = 2.0) is shown for the binding site residues C/M-H/M and the copper atom (anomalous difference map shown in

magenta, contoured at 3.0σ , carve = 2.25). **b**, Distances between coordinating residues and metal show that copper is coordinated via 1 thiolate (from Cys), two thioethers (from Met), and one imidazole nitrogen from His. **c**, Mutation of binding site residues Cys143 and Met147 to alanines abolishes copper binding (2Fo-Fc map contoured at 2.0σ , carve = 2.0). **d,e**, OM plane (**d**) and extracellular views (**e**) showing the thioether atoms of all methionine residues present in OprC as yellow spheres.

Copper binding by OprC is highly specific and near-irreversible.

Following structure determination of copper-bound OprC, several attempts were made to produce a structure of copper-free OprC. First, the protein was purified and crystallised without added copper; this gave a structure that was identical to the one already obtained and contained bound copper that presumably originated from the LB medium. As expression in rich media always yielded OprC with ~1 equivalent copper as judged by ICP-MS, various attempts to lower the copper content were made. Removal of bound copper from purified protein with combinations of denaturants (up to 4.0 M urea) and EDTA were not successful. Expression in chelex-treated minimal medium reduced, in the best cases, the metal content of the wild type to ~45 % equivalency as assessed by ICP-MS (Fig. 3a). Subsequent incubation of OprC for 30 min in the presence of either 3 or 10 equivalents Cu(II) followed by size exclusion chromatography (SEC) and ICP-MS analysis of the peak fractions leads to co-elution and occupancy of 1 equivalent copper (Fig. 3a). However, co-incubation with 1 mM EDTA (~100-fold excess) does not result in copper loading, suggesting that EDTA effectively withholds copper from OprC (Supplementary Fig. 2). As-purified OprC does not contain zinc, a common contaminant in metal-binding proteins, indicating that OprC is highly specific. Indeed, incubation of purified OprC in the presence of 3 or 10 equivalents Zn does not result in zinc co-elution (Supplementary Fig. 2). To obtain copper-free OprC, we constructed a variant in which the binding site residues Cys143 and Met147 were both mutated to alanines, generating copper-free protein (OprC_{AA}) after purification from rich media (Fig. 3a; AA). Even after equilibration of OprC_{AA} for 30 min with 3 or 10 equivalents Cu(II) no co-elution with metal is observed (Fig. 3a), indicating that high-affinity copper binding is completely abolished. It should be noted that OprC_{AA} contains a His-tag, demonstrating that Cu(II) does not bind to the His-tag of OprC with an affinity high enough to survive SEC, possibly due to the presence of 0.5 mM EDTA in the SEC buffer.

The fact that it is not possible to obtain copper-free wild type protein, even after taking extensive precautions, suggests that copper binds to OprC with very high affinity. To explore this further, we performed a copper extraction assay with a large excess of bathocuproinedisulfonic acid (BCS) under reducing conditions (Methods). BCS is a high-affinity Cu(I) chelator ($\log\beta_2$ 20.8) and forms a 2:1 complex with Cu(I), namely $[\text{Cu}(\text{BCS})_2]^{-3}$, with a molar extinction coefficient of $13,300 \text{ cm}^{-1} \text{ M}^{-1}$ at 483nm, enabling quantitation of Cu(I).

For copper-loaded OprC_{WT}, only 20% copper was removed after 24 hrs at room temperature, and the temperature had to be increased to 60 °C to obtain near-quantitative extraction of copper (~90% after 24 hours) (Fig. 3b). For reasons that are unclear, the orange-coloured [Cu(BCS)₂]⁻³ complex was hard to separate from OprC, and BCS-treated OprC did not bind copper anymore, suggesting an irreversible change in the protein due to the harsh incubation conditions. These results demonstrate that copper is kinetically trapped inside OprC and is, for all intents and purposes, irreversibly bound. This is compatible with the consensus transport mechanism of TBBDTs, in which the interaction with TonB is thought to result in the disruption of the binding site and subsequent release of the substrate³¹.

Figure 3. OprC binds to up 1 equivalent copper near-irreversibly. **a**, Copper content of wild type OprC and binding site mutant proteins before (blue) and after aerobic incubation with either 3 (red) or 10 equivalents Cu(II) (green) for 30 min followed by analytical SEC and quantitation of the copper content of the OprC peak by ICP-MS. "As purified" metal contents were obtained from MM-grown *E. coli* cells for WT OprC and from LB-grown cells for all mutant proteins. All proteins except where stated contain a C-terminal hexa-histidine tag. The increase in copper content in "as purified" tagless WT protein is most likely due to the use of a second IMAC column after tag cleavage. Reported values are averages $\pm$ s.d. (n = 3) **b**, Copper is kinetically trapped in OprC. Time course of copper extraction experiments showing % bound copper for OprC WT (blue), OprC_{AA} (green) and OprC M147A (red), at room temperature (open symbols) and 60 °C (filled symbols). The inset shows % bound copper in the first few minutes after starting the experiment. OprC_{AA} served as a control. Reported values are averages $\pm$ s.d. (n = 3)

Conformational changes upon copper binding.

The OprC_{AA} structure was solved by molecular replacement using Cu-bound OprC as the search model (Fig.1b, d, f; Fig. 2c). The binding site residues of both structures occupy very similar positions, indicating that the introduced mutations abolish copper binding without generating gross changes in the binding site. Superposition of the structures (Figs. 4 a,b) shows that for the remainder of the transporter, structural changes upon copper binding are confined to the vicinity of the copper binding site, with parts far removed virtually unchanged

(overall $C\alpha$ r.m.s.d ~ 1.0 Å). The largest change is observed for loop L11, which undergoes an inward-directed motion of ~ 8.0 Å upon copper binding (Figs. 4 d,e and Supplementary Fig. 3). A similar inward-directed but smaller change occurs for loop L8. Some loop tips (e.g. L4, L5, L6) in OprC_{AA} lack electron density for a limited number of residues, suggesting increased mobility. However, given that the proteins were crystallised in different space groups (OprC, C222₁; OprC_{AA} P22₁2₁), such differences could also be the result of different crystal packing. Overall, the conformational changes of the loops decrease the accessibility of the copper binding site. However, the main reason why the bound copper is inaccessible to solvent is because the binding site residues Met147 and Met325, together with Asn145, effectively form a lid on the copper ion. In the double mutant, copper becomes solvent accessible due to the absence of the Met147 side chain (Fig. 4d and Supplementary Fig. 3).

Figure 4. OprC structural changes upon copper binding. a,b Cartoon superposition from the OM plane (a) and extracellular environment (b) of OprC (coloured green) and OprC_{AA} (blue), indicating locations of loops L5, L8 and L11; copper is represented as a magenta sphere. The plug domains of OprC and OprC_{AA} are coloured light green and light blue, respectively. c, N-terminal plug domains indicating the location of the TonB box, which is invisible in Cu-bound OprC. Arrows indicate the missing density for Glu88-Pro94 in OprC. d, Surface slab representations from the OM plane, showing the presence of a solvent pocket in

OprC_{AA} that is generated by the absence of Met147 (arrow). For orientation purposes, the location of OprC-bound copper is shown in both structures. **e**, Side surface views showing the conformational changes of L8 and L11 (coloured red) as a result of copper binding. As in **(d)**, the bound copper of OprC is shown in both proteins.

The current consensus mechanism for TonB-dependent transport postulates that ligand binding on the extracellular side generates conformational changes that are propagated to the periplasmic side of the plug and increase the periplasmic accessibility of the Ton box for subsequent interaction with TonB³². In OprC_{AA}, N-terminal density is visible up to Leu66 (*i.e.*, the first 10 residues of the mature protein are disordered) including the Ton box (⁶⁸PSVVTGV⁷⁵), which is tucked away against the plug domain and the barrel wall. In Cu-bound OprC, the density between Glu88 and Pro94 is hard to interpret and, more importantly, no density is observed before Pro79, including the Ton box (Figs. 1e, f and 4c). Thus, the structures of OprC in the absence and presence of ligand conform to the consensus transport mechanism, in the sense that the TonB box is accessible for potential interaction with periplasmic TonB only in the substrate-loaded protein. This would prevent non-productive interactions with transporters that do not have substrate bound³².

The OprC methionine track binds Cu(I) but not Cu(II).

Which redox state of the copper ion is present in OprC? Since it is challenging to maintain copper in its +1 state during crystallisation, we used silver (Ag⁺) as a proxy for Cu(I) and determined the co-crystal structure of WT OprC in the presence of 2 mM AgNO₃ (Methods). Data was collected at 8000 eV, at which energy the anomalous signal of copper is very small (0.6 e⁻, compared to 4.2 e⁻ for Ag). Strikingly, and in sharp contrast to copper (Supplementary Fig. 1), the anomalous map of OprC WT crystallised in the presence of silver shows not one but three anomalous peaks. The first, strong peak (Ag1; 23σ) is located at the same site as in OprC crystallised with copper, and is coordinated by the same residues (Cys143, Met147, His323 and Met325; Fig. 5). The other two sites have lower occupancy (Ag2, ~10σ and Ag3, ~10σ) and are each coordinated by two methionines in the methionine track (Met286 and Met339 for Ag2; Met341 and Met343 for Ag3). This structure therefore suggests that the methionine track provides several low-affinity binding sites for Cu(I) ions, and that the high affinity CxxxM-HxM site binds Cu(I).

To obtain more information on the individual residue contribution to copper binding, we next generated the complete set of single alanine mutants of the principal binding site residues (C143A, M147A, H323A and M325A), and determined copper binding via analytical SEC and ICP-MS. For all single mutants, the copper content after purification from LB was below 10%, except for M147A (~20 %) (Fig. 3a). Upon incubation with 3 or 10 equivalents Cu(II), various

occupancies were obtained. C143A has no bound copper even after incubation with 10 equivalents Cu (II), suggesting this residue has a crucial role. H323A (~30%) and in particular

Figure 5 OprC binds Cu(II). **a-e**, Anomalous difference maps of **(a)** OprC wild type, **(b,d)** C143A and **(c,e)** H323A variants crystallised in the presence of **(a,d,e)** Ag or **(b,c)** Cu(II), and collected at different energies. The inset to **(a)** shows a close-up of the anomalous difference peaks (magenta) near the principal binding site in wild type OprC, with binding residues labelled and represented as stick models. Sulphurs are coloured yellow. For clarity, the metal used in co-crystallisation and the energy used for data collection are shown underneath each panel. The OprC plug domain is coloured blue.

M147A (~60%) have relatively high occupancies after copper incubation and SEC, indicating that these residues contribute less towards binding. Of the four ligands, the M147 thioether is the furthest away from the copper in the crystal structure (Fig. 2b), which may explain why it contributes the least to ligand binding. Interestingly, removal of bound copper is much faster in the M147A mutant compared to OprC_{WT} (Fig. 3b), suggesting that solvent exclusion by the M147 side chain (Fig. 4d) is the main reason why copper is kinetically trapped in the wild type protein.

Cu(II) is reduced by the principal binding site cysteine.

The fact that wild type OprC and most single alanine mutants can be loaded via Cu(II) incubation (Fig. 3a) suggests that Cu(II) is also a substrate for the transporter. However, the complete lack of copper in the C143A mutant is intriguing (Fig. 3a) and could be explained in two ways. If one assumes that OprC can bind Cu(II), removal of the cysteine could lead to much lower affinity for Cu(II), so that after SEC nothing remains bound. Alternatively, if OprC only binds Cu(I), the lack of the cysteine could prevent reduction of added Cu(II). To distinguish between these two possibilities, we determined the crystal structures of the OprC C143A and H323A mutants in the presence of either Cu(II) or Ag(I). For each crystal, two datasets were collected at different energies. The first energy (8800 eV) is below the copper K-edge (8979 eV), while the second one is above the edge (9175 eV), giving small and large anomalous signals for copper (0.5 and 3.7 e⁻ respectively). For silver, substantial anomalous signal is present at both energies (~3.6 e⁻). Thus, bound copper is expected to give a strong anomalous peak only at 9175 eV, while silver will give comparable peaks at both energies. For C143A co-crystallised with copper, no anomalous peaks are visible at both energies (Fig. 5b), showing a lack of bound copper. Crucially, in the presence of silver, the same three anomalous peaks are visible as for wild type OprC (Figs. 5 a, d), strongly suggesting that the C143A mutant does still bind Cu(I). The H323A mutant, on the other hand, still binds copper supplied as Cu(II), and also binds silver (Figs. 5 c, e). Together, these data therefore suggest that (i) Cys143 reduces Cu(II) to Cu(I) and (ii) OprC does not bind Cu(II) specifically. To support the notion that the principal OprC binding site is a reducing environment, we performed quantum chemistry calculations for a 70-atom system consisting of the OprC binding site, containing either Cu(I) or Cu(II) (Methods). The absolute energies show that the system with Cu(I) is 221 and 133 kcal/mol more stable compared to Cu(II) for the hydrogenated and dehydrogenated forms of Cys respectively (Supplementary Table 4). Energy Decomposition Analysis (EDA) of the Cu(I) interaction with Cys143, Met147, Met325 and His323, revealed the total interaction energy of the four residues with Cu(I) to be 96 kcal/mol more stable when cysteine is dehydrogenated (oxidised). The metal protein stabilisation interactions are dominated by

electrostatic interactions, with polarization and charge-transfer playing only a minor role. Additional details are provided in the Supplementary Information.

To shed additional light on the redox state of the bound copper, continuous wave EPR (cw-EPR) spectra were recorded on OprC_{WT} and single/double mutants. As-purified OprC WT containing 0.6 equivalents copper was EPR silent (Supplementary Fig. 4; WT-1), confirming the bound species is Cu(I). All other analysed variants were EPR silent as well. Subsequently, proteins were incubated with 0.25 equivalents of CuSO₄ and EPR spectra were recorded. The EPR spectra of all the OprC variants showed the appearance of the typical EPR signals associated with a Type(II) copper centre. These EPR signals are significantly different from the standard CuSO₄, Cu(II) EPR signals, as inferred from the visual inspection and also from the spectral simulations. The EPR spectra of the OprC variants show nicely resolved ^{63,65}Cu(II) hyperfine coupling along the parallel region, due to the interaction of an unpaired electron spin ($S = \frac{1}{2}$) of Cu(II) with the nuclear spin of ($I = 3/2$) of ^{63,65}Cu nuclei, as indicated by the magenta goal-post in Supplementary Fig. 4. The intense EPR signal observed at ~3300 G shows super-hyperfine splittings for the OprC_{AA} double mutant and OprC-M147A, arising plausibly from ¹H/¹⁴N nuclei in close proximity of the Cu(II) ion. The intensities of the EPR signals increased upon addition of further Cu(II) equivalents (Supplementary Fig. 4 and Supplementary Table 5). The additional Cu(II) loading caused changes in EPR line widths, which results in broadening and/or disappearance of the super-hyperfine splittings for OprC M147A. The analysis of the EPR spectra of OprC_{WT} and OprC-M147A shows the presence of two type(II) Cu(II) EPR active species ($S = \frac{1}{2}$ species), which are not clearly visible by the naked eye in the case of the M147A variant. By contrast, OprC_{AA} and M147H yielded EPR signals arising from single contributing EPR active species. The spectra suggest that Cu(II) does bind to OprC, but not at the principal binding site, as judged from the facts that (i) the spectra resembled that of a Type II copper center^{33,34,35} and (ii) Cu(II) also binds to OprC_{AA}. The EPR spectra were successfully simulated (Supplementary Fig. 5) using the spin-Hamiltonian parameters provided in Supplementary Table 5. Interestingly, the EPR signals for both wild type OprC and the M147A variant decrease slowly upon prolonged incubation (Supplementary Fig. 4). Collectively, the EPR data suggest that Cu(II) binds to OprC at one or more sites distinct from the principal binding site, and, via unknown routes, eventually ends up in the principal binding site, where it is reduced to Cu(I). It is possible, and perhaps even likely, that the Cu(II) binding sites as inferred by EPR are adventitious due to the fact that the protein is present in detergent and has much more surface (e.g. the entire periplasmic face) available for binding compared to the physiological situation, in which OprC is embedded in the OM and accepts copper from the extracellular environment. The addition of 1-10 equivalents of hydrogen peroxide oxidant directly to the 'as purified' OprC_{WT} has no significant effect on the electronic structure of the

bound Cu(I) ion as reflected by the lack of changes in the observed EPR spectra. This is consistent with the solvent inaccessibility of the principal copper binding site in OprC_{WT}.

To confirm that OprC binds the Cu(II) redox state *in vitro*, M147A and OprC_{AA} samples were analysed by recording intrinsic tryptophan fluorescence upon Cu(II) addition. OprC_{AA} was used as a control for which high-affinity copper binding is abolished. After excitation at 300 nm, an emission peak at 340nm was observed in both variants. Subsequent titrations with Cu(II) solution showed very similar reductions in the 340 nm emission peak in both OprC M147A and OprC_{AA} (Supplementary Fig. 6). In both cases, saturation is not reached at 3 equivalents copper, suggesting that Cu(II) binds non-specifically to OprC, consistent with the position of this metal at the top of the Irving-Williams series. Indeed, at high concentrations (~0.1 mM), OprC precipitates in the presence of millimolar Cu(II). Since OprC has 15 tryptophan residues it is not possible to define the locations of these binding sites.

OprC is abundant in *P. aeruginosa* during infection.

To determine the abundance of OprC in *Pseudomonas aeruginosa* UCBPP-PA14 or *Acinetobacter baumannii* ATCC 19606 in infected lung tissues, we employed a sensitive targeted proteomics approach with parallel reaction monitoring, on a high resolution and accurate mass instrument with absolute quantification using heavy-isotope labeled reference peptides (Methods). The results showed that in both mouse and rat pneumonia models, OprC was present at 1,000 to 10,000 molecules per *P. aeruginosa* cell, making it one of the five most abundant TonB-dependent transporters. As a comparison, the most abundant TonB-dependent transporter, FpvA, had 8,000 to 33,000 molecules per cell. We also assessed OprC abundance in *A. baumannii*, due to the facts that both proteins are highly similar (50% sequence identity, Supplementary Fig. 8) and that both bacteria are important human pathogens with a similar, low-permeability OM. In *A. baumannii*, OprC was less abundant in mouse and rat pneumonia models (40 to 400 molecules per cell), while the most abundant TonB-dependent transporters BfnH and BauA were present at 500 to 3'000 molecules per cell.

OprC does not confer copper toxicity in *P. aeruginosa* *in vitro*.

To demonstrate that OprC imports copper, we initially performed growth experiments in an *E. coli* $\Delta copA$ strain that should be more susceptible to copper stress. Cells were grown at various external Cu(II) concentrations (0-6 mM) while expressing plasmid-based wild type OprC or OprC_{AA}. Toxicity was observed in all three strains above 1 mM CuSO₄, but no differences were observed between the strains. However, the presence of abundant porins in *E. coli*, together with the high copper concentrations, make porin-mediated copper influx likely, and this would mask any contribution from OprC. In addition, it is also not clear whether *E. coli* TonB

couples to *P. aeruginosa* OprC. Given that OprC is highly abundant and is expressed during anaerobiosis^{26, 36, 37}, we next investigated whether OprC causes *in vitro* copper or silver toxicity in *P. aeruginosa* under anaerobic conditions. In rich media, excessive copper concentrations (mM) are required to cause toxicity, and no differences are observed for PA14 wild type and $\Delta oprC$ strains (Supplementary Fig. 7), in line with recent data showing that *oprC* is repressed in the presence of mM external copper under aerobic conditions²⁷. We next assessed minimal media, in which toxicity was observed at μ M concentrations of copper (Fig. 6). However, PA14 wild type and $\Delta oprC$ strains still had similar phenotypes, and only occasionally did we observe better growth for the $\Delta oprC$ strain over a very narrow range of copper concentrations (Supplementary Fig. 7c, d). Silver causes toxicity at 10-fold lower concentrations compared to copper, but again, no clear differences in growth were observed between wild type PA14 and the $\Delta oprC$ strain (Fig. 6). One reason for the lack of robust OprC-mediated toxicity is that *oprC* might also be repressed by external copper under anaerobic conditions.

Figure 6. Lack of OprC does not alleviate copper toxicity. *P. aeruginosa* PA14 growth under anaerobic conditions was monitored during copper and silver stress in minimal media. Growth curves of (a,c) WT and (b,d) $\Delta oprC$ in LR media supplemented with 100 mM sodium nitrate at increasing concentrations of added Cu(II) (a,b) or Ag (c,d).

Discussion

Our combined data define OprC as a specific importer of Cu(I), which is coordinated by a very unusual CxxxM-HxM binding motif in which the cysteine provides a reducing environment. The presence of a methionine binding track in OprC and the involvement of two methionine residues in the principal binding site are consistent with the preference of Cu(I) for S-containing ligands. Methionines also coordinate Cu(I) in other known copper transporters, such as the bacterial CusABC and CopA exporters and the eukaryotic Ctr1 copper importer^{16, 18, 38-40}. These transporters have been shown to also transport silver, but structures with silver have been determined only for the Cus system^{16, 38}. Our structural data strongly suggest that OprC can also import silver. With regards to Cu(II), the picture that emerges from our *in vitro* data with detergent-solubilised protein, is that Cu(II) binds non-specifically to OprC and subsequently migrates to the principal binding site via an unknown mechanism, where it is reduced to Cu(I). The Cu(II) affinity of 2.6 μ M reported by an early study²⁶ most likely resulted from non-specific binding, since the rich media used⁴¹ to culture *P. aeruginosa* would have generated OprC with 100% copper occupancy in the principal binding site. Whether Cu(II) is a substrate for OprC *in vivo* remains an open question. A recent study in *P. aeruginosa* proposed a novel copper uptake mechanism in copper-limited conditions which involves secretion of the copper binding protein azurin by a CueR-regulated Type VI secretion system. The secreted azurin would scavenge Cu(II) from the environment and load it onto OprC via a direct interaction, conferring a competitive advantage under copper-limiting conditions²⁷. While this model requires further corroboration, our data show that the imported species will be Cu(I).

The copper site most similar to that in OprC occurs in class I Type I copper proteins like cytochrome c oxidase, pseudoazurin and plastocyanin, electron transfer proteins that coordinate Cu(I) or Cu(II) via a 2Cys+1Met+1His site/2His+1Cys+1Met site. Interestingly, substitution of either of the two OprC binding site methionines with histidine generates M147H and M325H transporters that bind copper with similar efficiency as wild type OprC and, at least in the case of M325H, has a pseudoazurin-like active site with tetrahedral geometry (Supplementary Fig. 8). However, both proteins are still colourless and the spectrum of the Cu-loaded M147H variant is EPR-silent (Supplementary Fig. 4), suggesting the principal binding sites in these variants also comprise a reducing environment and bind Cu(I).

Given that copper, and in particular the more toxic Cu(I), is a known antimicrobial, the question arises why bacteria have proteins dedicated to Cu(I) acquisition from the environment. Indeed, in contrast to e.g. iron that is withheld from a pathogen by the host during infection, host-derived copper is a "nutritional immunity" antimicrobial response. Thus, bacterial virulence is generally attenuated by mutations, particularly in transporters, that cause copper sensitivity⁴². No data have yet been reported on the role of OprC in *P. aeruginosa* virulence, but a recent study reported decreased virulence of an *A. baumannii* OprC knockout⁴³, which is the opposite of what one might expect from the copper nutritional immunity model⁴². In this context, our proteomics data showing that OprC is very abundant in a *P. aeruginosa* mouse infection model are particularly intriguing. Considering the near-irreversible copper binding, we speculate that OprC could also play a role as a copper reservoir on the cell surface. As a TBDT, Cu-loaded OprC depends on TonB for transport, and it is possible that (hypothetical) regulation of the TonB-OprC interaction could "titrate" copper to the periplasmic space as required, when external copper is low. Conversely, in the presence of high concentrations of external copper, OprC would be loaded with copper, but this would not lead to import in the absence of an interaction with TonB. This model could provide an alternative explanation for the lack of a robust OprC-dependent growth phenotype in *P. aeruginosa* under copper-replete conditions (Fig. 6). Metallo-proteomes in bacteria are not well characterized, making it challenging to estimate copper requirements⁴⁴. Total cell quantitation suggests a copper concentration of ~40 μM when cells are grown in metal-restricted medium⁴⁵. Assuming a cellular volume of ~1 μm^3 (10^{-15} l), this would correspond to ~25,000 molecules per cell. Thus, a pool of 5,000-10,000 Cu-loaded OprC molecules could provide a substantial part of the total copper content of the cell, most of which will be present as an essential cofactor in enzymes and bound to chaperones and storage proteins. Given this need for copper on the one hand and its toxicity on the other, it is clear that regulation of any copper import protein is crucial, possibly both at the gene and protein level. Unfortunately, and in contrast to the many copper stress genes that, as part of the CopR or CueR regulons, are upregulated under aerobic conditions during copper stress^{10, 11, 46-49}, nothing is known about how OprC is downregulated during such stress. Intriguingly, *oprC* (PA3790) is in an operon with PA3789, which encodes for an uncharacterised inner membrane protein containing PepSY domains, hinting at a peptidase function. Another protein strongly downregulated during copper stress is PA5030, which is an MFS transporter with a large number of His+Met residues (26 out of 438 residues), suggesting it could mediate copper delivery to the cytoplasm, possibly in concert with OprC and an as yet unidentified periplasmic protein⁴⁹.

Bacterial copper import via metallophores is well established. For example, methanotrophic bacteria produce methanobactins that bind copper with micromolar affinity, and are imported by MbnT²². In *E. coli*, yersiniabactin (Ybt) binds Cu(II), which is taken up by the FyuA receptor⁵⁰. Thus, OprC is the first example of a TBDT that mediates copper import without a metallophore. The TBDT with the closest substrate specificity to OprC is the ionic zinc transporter ZnuD from *Neisseria meningitidis*, the structure of which has been solved⁵¹. Large structural differences between OprC and ZnuD exist for the extracellular loops (overall C α r.m.s.d. ~5.9 Å). ZnuD has several discrete low-affinity binding sites that may guide the metal towards the high-affinity binding site⁵¹. In OprC, a distinctive "methionine track" provides low-affinity binding sites to guide copper to the high affinity site. Interestingly, while the extracellular loops between OprC and ZnuD are very different and the overall sequence identity is only 28%, the metal binding sites are located at very similar positions and only 2.8 Å apart (Supplementary Fig. 9), suggesting that the transport channel formed via TonB interaction may be similar. Inspection of the ZnuD structure shows that the zinc binding site is excluded from solvent, and we propose that the zinc ion in ZnuD is kinetically trapped, analogous to copper in OprC.

OprC shares ~60 % identity to NosA from *Pseudomonas stutzeri*, for which no structure is available. Like OprC, NosA is expressed under anaerobic conditions and repressed in the presence of μ M concentrations Cu(II)⁵²⁻⁵⁴. *P. stutzeri* NosA antibodies did not react with *P. aeruginosa*⁵², but our structure identifies NosA as an OprC ortholog, since the CxxxM-HxM copper binding motif and some of the methionine track residues are conserved (Supplementary Fig. 10). NosA is important during denitrification in *P. stutzeri* JM300 and was proposed to load copper either directly or indirectly to the periplasmic N₂O reductase NosZ⁵²⁻⁵⁴. However, a more recent report for a different *P. stutzeri* strain found no difference between NosZ activity and copper content for a *nosA* knockout⁵⁵. In addition, OprC/NosA also occurs in a number of non-denitrifying *Proteobacteria* such as *Salmonella enterica*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* (Supplementary Fig. 10), showing that NosZ maturation is not a general function of OprC. The occurrence of OprC in some (e.g. *S. enterica*) but not in other (e.g. *E. coli*) Enterobacteria is intriguing, given that the OM of these bacteria is relatively permeable to small molecules due to abundant general porins such as OmpC and OmpF.

The ability of OprC to take up Cu(I) could be important for biofilms, which are anaerobic to various degrees depending on the location inside the biofilm^{56, 57}. Oxygen tension also reduces as lung chronic disease mediated by *P. aeruginosa* progresses, turning airway mucus into an anaerobic environment in cystic fibrosis patients that will favour the availability of Cu(I)⁵⁸. *P.*

aeruginosa is capable of anaerobic respiration by using nitrate, nitrite or nitrous oxide as terminal electron acceptor⁵⁹, and OprC has been shown to be induced under anaerobic conditions³⁶. Together, our data suggest that OprC is a novel type of copper transporter for the efficient scavenging of copper under a wide range of physiological conditions, including the anaerobiosis that is a hallmark of disease caused by *P. aeruginosa* in humans.

Methods

Recombinant production of *Pseudomonas aeruginosa* OprC. The mature version of the gene coding for *oprC* of *P. aeruginosa* PAO1 (UniProt ID; PA3790)⁶⁰, starting with His56 as determined by Nakae et al.²⁶, was synthesized to include a 7 x His tag at the N-terminus (Eurofins, UK), cloned into the pB22 arabinose-inducible expression vector⁶¹ and transformed into chemically competent *Escherichia coli* DH5 α cells. After expression and processing by signal peptidase, the N-terminal sequence of this construct is NVRLQH HHHHHHLEAEEHSQH Q-. A second version of this construct was constructed in a pB22 version containing a tobacco etch virus (TEV) site after the His7-tag. Correct sequences were confirmed by DNA sequencing (Eurofins, UK) using both forward and reverse plasmid-specific primers. The OprC_{AA} mutant was produced by changing the key amino acids Cys143 and Met147 to alanine residues using the KLD Quickchange site-directed mutagenesis kit (New England Biolabs, UK) and specific primers containing both mutation sites (forward: 5'-tcgcgcggatgcaccaaccagctatattagc-3'; reverse: 5'-ttcggggcggcgccaagcatcatgc-3'). The single mutants C143A, M147A, H323A, M325A, M147H and M325H were made in similar ways.

OprC recombinant protein production and purification was performed as follows: *E. coli* C43 Δ cyo was electroporated with expression vector, recovered for 60 minutes in LB (Sigma, UK) at 37 °C, and plated on LB agar (Sigma, UK) containing 100 μ g mL⁻¹ ampicillin (Melford, UK). Transformants were cultured in LB medium or in LeMasters-Richards (LR) minimal medium with glycerol (2-3 g/l) as carbon source. All media contained 100 μ g mL⁻¹ ampicillin. For rich media, cells were grown (37 °C, 180rpm) until OD₆₀₀ ~0.6, when protein expression was induced with 0.1% arabinose for 4-5 h at 30 °C or overnight at 16 °C (150 rpm). For LR media, a small overnight pre-culture in LB was used at 1/100 v/v to inoculate an LR-medium pre-culture early in the morning (typically 1 ml preculture for 100 ml of cells was used), which was grown during the day at 37 °C. After late afternoon inoculation, large-scale cultures (typically 6-8 l) were grown overnight at 30 °C until OD 0.4-0.7, followed by induction with 0.1% arabinose at 30 °C for 6-8 hours. Cells were harvested by centrifugation (5,000 rpm, 20 minutes, 4 °C), and pellets homogenized in 20 mM Tris (Sigma), 300 mM NaCl (Fisher) pH 8.00 (TBS buffer), in the presence of 10 mM ethylene diamine tetraacetic acid (EDTA, Sigma).

Cells were broken by one pass through a cell disruptor (Constant Systems 0.75 kW operated at 23 kpsi), centrifuged at 42,000 rpm for 45 minutes at 4 °C (45Ti rotor; Beckman), and the resulting total membrane fraction was homogenized in TBS buffer containing 1.5% Lauryldimethylamine oxide (LDAO) (Sigma, UK). Membrane proteins were extracted by stirring (60 minutes, 4 °C), centrifuged (42,000 rpm in 45Ti rotor, 30 minutes, 4°C), and the membrane extract was loaded on a Chelating Sepharose Fast Flow bed resin (~10 ml; GE Healthcare, UK) previously activated with 200 mM NiCl₂ (Sigma) and equilibrated in TBS containing 0.15 % n-dodecyl-beta-D-maltopyranoside (DDM). After washing with 15 column volumes buffer with 30 mM imidazole, protein was eluted with 0.25 M imidazole buffer (Fisher), incubated with 20 mM EDTA (30 minutes, 4 °C), and loaded on a Superdex 200 16/600 size exclusion column equilibrated with 10 mM Hepes, 100 mM NaCl, 0.05% DDM, 10 mM EDTA, pH 7.5. Peak fractions were pooled and concentrated using a 50 MWCO Amicon filter (Millipore, UK), analyzed on SDS-PAGE, flash-frozen in liquid nitrogen and stored at -80°C. Typical yields of purified wild type and most mutant OprC proteins ranged between 2-5 mg per l media grown at 16 °C. All media and buffer components were made in fresh milli-Q water.

Protein preparations intended for crystal trials were pooled and buffer-exchanged to 10 mM Hepes 100 mM NaCl, 0.4% tetraethylene glycol mono-octyl ether (C₈E₄) (Anatrace, US), pH 7.5. NaNO₃ was substituted for NaCl for protein preparations intended for crystal trials with silver. Protein preparations to be used for metal analysis after removal of the His-tag underwent a slightly different protocol. The elution fraction from immobilized metal affinity chromatography (IMAC) was buffer-exchanged to 50 mM Tris, 0.5 mM EDTA, 0.2 mM TCEP, 100 mM NaCl, 0.05% DDM (Anatrace, US), pH 7.50, and submitted to TEV protease digestion (ratio 1 mg TEV: 10 mg protein, 4 °C, overnight). Samples were submitted to a second IMAC column, where flow-through and wash fractions were combined for the subsequent SEC step in 10 mM HEPES 100 mM NaCl 0.05 % DDM 0.5 mM EDTA pH 7.5. Protein concentration was determined by BCA assay (Thermo Scientific, UK) and by UV/Vis absorbance at 280nm (considering OprC E_{0.1%} = 1.6 as determined by ProtParam).

In vitro metal binding assays and Inductively Coupled Plasma Mass Spectrometry (ICP-MS). OprC samples intended for metal binding assays were exchanged into respective chelex-treated buffers without EDTA and were equilibrated with different equivalents of Cu(II) or Zn, for 30 min at room temperature (n=3). Protein concentrations used were in the range of 10 – 20 µM. Samples were loaded on an analytical Superdex 200 Increase 10/300GL (GE Healthcare) column, equilibrated in 10 mM Hepes, 100 mM NaCl, 0.05% DDM, 0.5 mM EDTA pH 7.5. Size exclusion peaks were pooled, concentrated and quantified for protein by UV absorbance at 280 nm. Protein samples were diluted 10-fold in 2.5% HNO₃. Analytical metal

standards of 0- 500 ppb were prepared by serial dilution from individual metal stocks (VWR, UK) and were matrix-matched to protein samples. Samples and standard curves were analysed by ICP-MS using a Thermo X-series instrument (Thermo Fisher Scientific), and samples were typically screened for the presence of ^{63}Cu and ^{65}Zn .

Copper extraction (demetallation) experiments. OprC_{WT}, OprC_{AA} and M147A samples were incubated with 3 equivalents copper for 30 min and then loaded onto a Superdex S-200 Increase 10/300GL column equilibrated with 10 mM HEPES 100 mM NaCl 0.05 % DDM 0.5 mM EDTA pH 7.5. Peak fractions were pooled, concentrated and quantified by UV absorbance at 280 nm. Samples were exchanged into respective chelex-treated buffer without EDTA. For demetallation experiments, 20 μM of copper-bound proteins were taken in duplicates and incubated with 100-fold excess of the copper chelator bathocuproine disulfonate (BCS) (Sigma) and 100-fold excess of the reducing agent hydroxyl amine (NH_2OH) (Sigma) at 60 °C and room temperature. The amount of extracted Cu(I) was quantified by measuring the formation of coloured copper chelate complex, using the complex molar extinction coefficient $\epsilon_{483\text{nm}} = 13,300 \text{ M}^{-1} \text{ cm}^{-1}$ ⁶².

Fluorescence spectroscopy

OprC samples in chelex-treated buffers without EDTA were diluted in 10 mM HEPES, 100 mM NaCl, 0.05% DDM (pH 7.5) to a volume of 250 μL and a concentration of 20 μM , and samples were titrated with consecutive additions of Cu(II). Fluorescence emission spectra were recorded on a Varian Cary Eclipse spectrophotometer (Agilent). The excitation wavelength was 300 nm and emission spectra were recorded between 300 – 500 nm, with excitation slit of 5 nm, at a scan rate of 600 nm/min. Data was plotted and analysed for peak changes in the region of 300-400 nm due to Cu(II) binding⁶³. Experiments were performed in triplicates under aerobic conditions, and reactions were equilibrated for 5 minutes at room temperature prior to recording each fluorescence spectrum.

Protein crystallization, data collection and structure determination. Sitting-drop crystallization trials were set up using a Mosquito crystallization robot (TTP Labtech) with commercial screens (MemGold1 and MemGold2, Molecular Dimensions) at 20 °C. To obtain the initial structure of Cu-bound OprC, the protein (~12 mg/ml) was incubated with 3 mM CuCl_2 for 1 hr at room temperature, followed by setting up crystallisation trials. A number of initial hits were obtained and were subsequently optimised by manual hanging drop vapour diffusion using larger drops (typically 1-1.5 μL protein + 1 μL reservoir). Well-diffracting crystals (~3 Å resolution at a home source) were obtained in 0.1 M NaCl/0.15 M NH_4SO_4 /0.1 M MES pH 6.5/18-22% PEG1000. Crystals were cryoprotected with mother liquor lacking CuCl_2 .

containing 10% PEG400 for ~5-10 s and flash-frozen in liquid nitrogen. Diffraction data were collected at Diamond Light Source (Didcot, UK) at beamline i02. For the best crystal, belonging to space group C222₁, 720 degrees of data were collected at an energy of 8994 eV, corresponding to the K-edge of copper (Supplementary Table 1). Data were auto processed by xia2. The structure was solved via single anomalous dispersion (SAD) via AUTOSOL in Phenix⁶⁴. Two copper sites were found, one for each OprC molecule in the asymmetric unit (Supplementary Fig. 1). The phases were of sufficient quality to allow automated model building via Phenix AUTOBUILD, generating ~60% of the structure and using data to 2.0 Å. The remainder of the structure was built manually, via iterative cycles of refinement in Phenix and model building in COOT⁶⁵. Metal coordination was analysed by the Check-my-metal server⁶⁶. The final refinement statistics are listed in Supplementary Table 1. Subsequently, crystals were also obtained without any copper supplementation of the protein. These were isomorphous to those described above and obtained under identical conditions. Molecular replacement indicated the presence of copper and an identical structure to that obtained above (data not shown). OprC_{AA} crystals (~10 mg/ml protein) were obtained and optimized by hanging drop vapor diffusion as described above, and diffraction-quality crystals were obtained in the same conditions as for Cu-OprC, *i.e.* 0.1 M sodium chloride/0.15 M ammonium sulfate/0.01 M MES sodium pH 6.5/19% (w/v) PEG1000. Interestingly however, the OprC_{AA} crystals belong to a different space group (P22₁2₁), most likely as a result of structural differences between both OprC variants. Diffraction data were collected at Diamond Light Source (Didcot, UK) at beamline I24. Diffraction data were processed in XDS⁶⁷. The structure was solved by molecular replacement (MR) using Phaser, with wildtype OprC as the search model. Model building was done in COOT and refinement in Phenix. As for Cu-OprC, the data collection and refinement statistics are shown in Supplementary Table 1. C143A and H323A proteins (~10 – 12 mg/ml protein) were incubated with 2 mM CuSO₄ at room temperature for 1 hr, followed by co-crystallisation. Diffracting crystals for both C143A and H323A in the presence of copper were obtained in 0.34 M Ammonium sulfate/0.1 M Sodium citrate pH 5.5/12 -16 % w/v PEG 4000 and were cryo-protected using mother liquor lacking CuSO₄ and with 25% ethylene glycol for ~10 s and flash-frozen in liquid nitrogen. M147H and M325H crystals were obtained in the same condition as those for wild type OprC. For co-crystallisation with silver, OprC proteins were incubated with 2 mM AgNO₃ for 1 hour at room temperature, followed by co-crystallisation. Well-diffracting OprC_{WT} crystals with silver were obtained under the same conditions as in the presence of copper. For the best OprC_{WT} crystal, belonging to space group C222₁, 999 degrees of data were collected at an energy of 8000 eV to obtain anomalous signals for Ag. C143A and H323A crystals (~10-12 mg/ml protein) with Ag were obtained from 0.2 M Choline chloride/0.1 M Tris pH 7.5/12-16 % w/v PEG 2000 MME and 0.5 M Potassium chloride/0.05 M HEPES pH 6.5/12-16 % v/v PEG 400, respectively. Crystals

were cryoprotected for 5-20 s with mother liquor lacking AgNO₃ but containing 25% ethylene glycol for C143A and 20 % PEG 400 for H323A. For C143A and M325A crystallised in the presence of copper or silver, datasets of 360 degrees each were collected at energies of 8800 and 9175 eV, using different parts of the same crystal (Supplementary Tables 2 and 3).

Electron Paramagnetic Resonance Spectroscopy.

Electron Paramagnetic Resonance (EPR) measurements were carried out using a Bruker ELEXSYS-E500 X-band EPR spectrometer operating in continuous wave mode, equipped with an Oxford variable-temperature unit and ESR900 cryostat with Super High-Q resonator. All EPR samples were prepared in quartz capillary tubes (outer diameter; 4.0 mm, inner diameter 3.0 mm) and frozen immediately in liquid N₂ until further analysis. The experimental setup and conditions were similar to reported earlier⁶⁸. The low temperature EPR spectra were acquired using the following conditions: sweep time of 84 s, microwave power of 0.2 mW, time constant of 81 ms, average microwave frequency of 9.44 GHz and modulation amplitude of 5 G, T = 20 K. The concentration of OprC_{WT} and mutants varied from 210-260 μM in 10 mM HEPES, 100 mM NaCl, 0.03 % DDM (n-dodecyl-D-maltoside), pH 7.5 and 10 mM HEPES, 100 mM NaNO₃, 0.4 % C8E4 (tetraethylene glycol monoethyl ether) buffer pH 8, respectively. Analysis and simulations of the cw-EPR spectra were performed using EasySpin toolbox (5.2.28) for the Matlab (R2017a) program package^{69, 70}.

Quantum chemistry calculations. A truncated version of the crystal structure of OprC (PDB ID 6FOK) consisting of only the copper ion, Cys143, Met147, His323 and Met2325 was used as input for single point energy calculations. The link atoms between these four amino acids and the rest of the protein were capped with hydrogen atoms, giving a total of 70 atoms. The calculations were performed on the system (i) without copper, (ii) with Cu(I) or (iii) with Cu(II). In addition, two possibilities were considered for the state of the cysteine: hydrogenated (reduced) or dehydrogenated (oxidised). The calculations were performed in linear-scaling Density Functional Theory (DFT) with the PBE exchange-correlation functional in ONETEP⁷¹. Norm-conserving pseudopotentials, an energy cutoff of 800 eV and a kernel cutoff of 1000 Bohr in open-boundary conditions were used in a cubic box with sides of 30 Å in length. Quantum chemistry calculations produce huge energies because they are the energies of all the electrons (including core electrons) interacting with the nuclei and themselves. However, the total energies are meaningless and what is chemically important are differences between different conformations and different bonding situations, which are typically much smaller as they concern re-arrangements of valence electrons. For this reason, it is crucial to converge the huge total energies very tightly in order to capture the chemically interesting energy differences.

***In vivo* metal toxicity assays**

For metal toxicity assays in *E. coli*, JW0473-3 ($\Delta copA$)⁷² were transformed with plasmids containing OprC_{WT}, OprC_{AA} or empty cloning vector (pB22). The copper exporter *copA* mutant was obtained from the Keio collection; Growth assays were set up in flat-bottomed 96-well plates (Thermo Scientific) containing LB-ampicillin supplemented with 0.01% arabinose. Different concentrations (0-6mM) of Cu(II) (CuSO₄) were tested in order to assess growth phenotypes under metal excess conditions. Cultures in triplicates were inoculated with 1:100 dilution of overnight pre-inoculum from isolated colonies. Assays were performed in a plate reader (Tecan Infinite M200), with 37C incubation and regular orbital shaking of 30 seconds at every 5 minutes, for a total of 25 hours. Time points were collected with 30 mins intervals. For metal toxicity assays in *P. aeruginosa*, PA14 WT and PA14 $\Delta oprc$ strains were used and assays were performed in anaerobic conditions. Pre- cultures were grown under anaerobiosis in rich LB or LR minimal media supplemented with 100 mM sodium nitrate. The Cu(II) (CuSO₄) range tested varied from 0 – 7 mM in rich media to 0 – 8 μ M in LR media. Cultures in triplicates were inoculated with precultures grown in respective media under anaerobic conditions without any excess metal added. Plates were set up and sealed inside an anaerobic chamber (Don Whitley Scientific). For silver toxicity, 0 – 1 μ M Ag (AgNO₃) was used and assays were performed as described for Cu (II) in LR minimal media supplemented with 100 mM NaNO₃. Experiments were performed in triplicates for all tested conditions.

Animal infection models.

Intra-tracheal instillation model: specific pathogen free (SPF) immunocompetent male Sprague-Dawley rats weighing 100 - 120 g or male CD-1 mice weighing 20 - 25 g were infected by depositing an agar bead containing around 10⁷ colony-forming units *Acinetobacter baumannii* ATCC 19606 and *Pseudomonas aeruginosa* UCBPP-PA14, deep into the lung via nonsurgical intra-tracheal intubation⁷³. In brief, animals were anesthetized with isoflurane (5%) and oxygen (1.5 L/min) utilizing an anesthesia machine. Depth of anesthesia was evaluated by absence of gag reflex; if the reflex was present, the animal was placed back under anesthesia until the reflex disappeared. No animals were utilized until they were fully anesthetized. Animals were infected via intra-bronchial instillation of molten agar suspension (rats- 100 μ l) (mice- 20 μ l) via intra-tracheal intubation, and then allowed to recover. Animals were returned in their home cages and observed until recovered from anesthesia. At 24 h post infection, animals were sacrificed and lung was homogenized in sterile saline using a lab blender. All procedures are in accordance with protocols approved by the GSK Institutional Animal Care and Use Committee (IACUC), and meet or exceed the standards of the American

Association for the Accreditation of Laboratory Animal Care (AAALAC), the United States Department of Health and Human Services and all local and federal animal welfare laws.

Sample workup for proteomics

The sample workup protocol was optimized to deplete host material while maintaining *A. baumannii* and *P. aeruginosa* viability until lysis. All buffers and equipment were used at 0 to 4 °C to minimize proteome changes during sample workup. The sample volume (maximum of 1 ml) was estimated and an equal volume of 1% Tergitol in PBS was added followed by vigorous vortexing for 30 s. After centrifugation at 500 x g for 5 min, the supernatant was transferred to a fresh tube, and the pellet was extracted again with 2 ml 0.5% Tergitol in PBS. The supernatant was combined with the first supernatant and centrifuged at 18'000 x g for 5 min. The pellet was washed with 2 ml and again centrifuged at 18'000 x g for 5 min. The supernatant was removed, and the pellet was resuspended in 100 µL 5% sodium deoxycholate, 5 mM Tris (2-carboxyethyl) phosphine hydrochloride, 100 mM NH₄HCO₃. The sample was incubated at 90°C for 1 min. and then stored at -80 °C. Samples were thawed and sonicated for 2 x 20 s (1 s interval, 100% power). Proteins were alkylated with 10 mM iodoacetamide for 30 min in the dark at room temperature. Samples were diluted with 0.1M ammonium bicarbonate solution to a final concentration of 1% sodium deoxycholate before digestion with trypsin (Promega) at 37°C overnight (protein to trypsin ratio: 50:1). After digestion, the samples were supplemented with TFA to a final concentration of 0.5% and HCl to a final concentration of 50 mM. Precipitated sodium deoxycholate was removed by centrifugation at 4°C and 14'000 rpm for 15 min. Peptides in the supernatant were desalted on C18 reversed phase spin columns according to the manufacturer's instructions (Macrospin, Harvard Apparatus), dried under vacuum, and stored at -80°C until further processing.

Parallel reaction monitoring

Heavy proteotypic peptides (JPT Peptide Technologies GmbH) were chemically synthesized for *A. baumannii* and *P. aeruginosa* outer membrane proteins. Peptides were chosen dependent on their highest detection probability and their length ranged between 7 and 20 amino acids. Heavy proteotypic peptides were spiked into each sample as reference peptides at a concentration of 20 fmol of heavy reference peptides per 1 µg of total endogenous protein mass. For spectrum library generation, we generated parallel reaction-monitoring (PRM)⁷⁴ assays from a mixture containing 500 fmol of each reference peptide. The setup of the µRPLC-MS system was as described previously⁷⁵. Chromatographic separation of peptides was carried out using an EASY nano-LC 1000 system (Thermo Fisher Scientific) equipped with a heated RP-HPLC column (75 µm x 37 cm) packed in-house with 1.9 µm C18 resin (Reprosil-AQ Pur, Dr. Maisch). Peptides were separated using a linear gradient ranging from 97%

solvent A (0.15% formic acid, 2% acetonitrile) and 3% solvent B (98% acetonitrile, 2% water, 0.15% formic acid) to 30% solvent B over 60 minutes at a flow rate of 200 nl/min. Mass spectrometry analysis was performed on Q-Exactive HF mass spectrometer equipped with a nanoelectrospray ion source (both Thermo Fisher Scientific). Each MS1 scan was followed by high-collision-dissociation (HCD) of the 10 most abundant precursor ions with dynamic exclusion for 20 seconds. Total cycle time was approximately 1 s. For MS1, 3e6 ions were accumulated in the Orbitrap cell over a maximum time of 100 ms and scanned at a resolution of 120,000 FWHM (at 200 m/z). MS2 scans were acquired at a target setting of 1e5 ions, accumulation time of 50 ms and a resolution of 30,000 FWHM (at 200 m/z). Singly charged ions and ions with unassigned charge state were excluded from triggering MS2 events. The normalized collision energy was set to 35%, the mass isolation window was set to 1.1 m/z and one microscan was acquired for each spectrum.

The acquired raw-files were converted to the mascot generic file (mgf) format using the msconvert tool (part of ProteoWizard, version 3.0.4624 (2013-6-3)). Converted files (mgf format) were searched by MASCOT (Matrix Sciences) against normal and reverse sequences (target decoy strategy) of the UniProt database of *Acinetobacter baumannii* strains ATCC 19606 and ATCC 17978, and *Pseudomonas aeruginosa* UCBPP-PA14, as well as commonly observed contaminants. The precursor ion tolerance was set to 20 ppm and fragment ion tolerance was set to 0.02 Da. Full tryptic specificity was required (cleavage after lysine or arginine residues unless followed by proline), three missed cleavages were allowed, carbamidomethylation of cysteins (+57 Da) was set as fixed modification and arginine (+10 Da), lysine (+8 Da) and oxidation of methionine (+16 Da) were set as variable modifications. For quantitative PRM experiments the resolution of the orbitrap was set to 30,000 FWHM (at 200 m/z) and the fill time was set to 50 ms to reach a target value of 1e6 ions. Ion isolation window was set to 0.7 Th (isolation width) and the first mass was fixed to 100 Th. Each condition was analyzed in biological triplicates. All raw-files were imported into Spectrodrive (Biognosys AG) for protein and peptide quantification.

Author contributions

BvdB designed the study. SPB and BvdB expressed, purified, and crystallized proteins. AB collected the diffraction data. SPB and BvdB analysed the diffraction data and refined the structures. SPB performed metal binding and *in vivo* growth experiments. TRY performed ICP-MS analyses. MS performed cw-EPR measurements, simulations and interpreted the data. BB performed quantum energy calculations, supervised by SK. SH performed proteomics experiments, supervised by DB. BvdB and SPB wrote the paper.

Acknowledgements

We would like to thank Scott Sucoloski, Jennifer Hoover, Josh West (Glaxo Smith Kline) for providing proteomics samples. We would also like to thank Kevin Waldron (Newcastle University) for useful discussions and for carrying out initial ICP-MS analyses. We also thank Deenah Osman and Nigel Robinson (Durham University) for elemental analyses by ICP-MS and helpful discussions, supported by awards BB/L009226/1 and BB/R002118/1 from the Biotechnology and Biological Sciences Research Council, BBSRC, UK. We are indebted to the Diamond Light Source for beam time (proposals mx9948, mx13587 and mx18598) and beamline assistance. MS acknowledges the EPSRC National (UK) EPR Research Facility and Service for use of the EPR spectrometers. MS and BvdB thank Luisa Ciano for the useful discussions at an early stage. The research leading to these results was in part conducted as part of the Translocation consortium (www.translocation.eu) and has received support from the Innovative Medicines Initiatives Joint Undertaking under Grant Agreement No. 115525, resources that are composed of financial contributions from the European Union's seventh framework programme (FP7/2007–2013) and European Federation of Pharmaceutical Industries and Associations companies in-kind contribution. BvdB would also like to acknowledge the Royal Society for salary support.

Accession codes

Coordinates and structure factors have been deposited in the Protein Data Bank (<http://www.ebi.ac.uk/pdbe/>) with accession codes 6FOK (OprC_{WT}), 6FOM (OprC_{AA}), 6Z8Q (OprC_{WT} Ag 8000 eV), 6Z9I (OprC_{C143A} Ag 8800 eV), 6Z99 (OprC_{C143A} Ag 9175 eV), 6Z8Y (OprC_{C143A} Cu 8800 eV), 6Z8Z (OprC_{C143A} Cu 9175 eV), 6Z8T (OprC_{H323A} Ag 8800 eV), 6Z8U (OprC_{H323A} Ag 9175 eV), 6Z8R (OprC_{M147H}), 6Z8S (OprC_{M325H}), 6Z9N (OprC_{H323A} Cu 9175 eV), 6Z9Y (OprC_{H323A} Cu 8800 eV).

References

1. Hodgkinson, V. & Petris, M.J. Copper homeostasis at the host-pathogen interface. *The Journal of biological chemistry* **287**, 13549-13555 (2012).
2. Harrison, J.J., Ceri, H. & Turner, R.J. Multimetal resistance and tolerance in microbial biofilms. *Nature reviews. Microbiology* **5**, 928-938 (2007).
3. Lemire, J.A., Harrison, J.J. & Turner, R.J. Antimicrobial activity of metals: mechanisms, molecular targets and applications. *Nature reviews. Microbiology* **11**, 371-384 (2013).
4. Braymer, J.J. & Giedroc, D.P. Recent developments in copper and zinc homeostasis in bacterial pathogens. *Current opinion in chemical biology* **19**, 59-66 (2014).

5. Robinson, N.J. & Winge, D.R. Copper metallochaperones. *Annual review of biochemistry* **79**, 537-562 (2010).
6. Hernandez-Montes, G., Arguello, J.M. & Valderrama, B. Evolution and diversity of periplasmic proteins involved in copper homeostasis in gamma proteobacteria. *BMC microbiology* **12**, 249 (2012).
7. Zimmermann, M. et al. PcoE--a metal sponge expressed to the periplasm of copper resistance *Escherichia coli*. Implication of its function role in copper resistance. *Journal of inorganic biochemistry* **115**, 186-197 (2012).
8. Vita, N. et al. A four-helix bundle stores copper for methane oxidation. *Nature* **525**, 140-143 (2015).
9. Arguello, J.M., Raimunda, D. & Padilla-Benavides, T. Mechanisms of copper homeostasis in bacteria. *Frontiers in cellular and infection microbiology* **3**, 73 (2013).
10. Zhang, X.X. & Rainey, P.B. Regulation of copper homeostasis in *Pseudomonas fluorescens* SBW25. *Environmental microbiology* **10**, 3284-3294 (2008).
11. Hu, Y.H., Wang, H.L., Zhang, M. & Sun, L. Molecular analysis of the copper-responsive CopRSCD of a pathogenic *Pseudomonas fluorescens* strain. *Journal of microbiology* **47**, 277-286 (2009).
12. Rubino, J.T. & Franz, K.J. Coordination chemistry of copper proteins: how nature handles a toxic cargo for essential function. *Journal of inorganic biochemistry* **107**, 129-143 (2012).
13. Boal, A.K. & Rosenzweig, A.C. Structural biology of copper trafficking. *Chemical reviews* **109**, 4760-4779 (2009).
14. Ma, Z., Jacobsen, F.E. & Giedroc, D.P. Coordination chemistry of bacterial metal transport and sensing. *Chemical reviews* **109**, 4644-4681 (2009).
15. Gonzalez-Guerrero, M. & Arguello, J.M. Mechanism of Cu⁺-transporting ATPases: soluble Cu⁺ chaperones directly transfer Cu⁺ to transmembrane transport sites. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 5992-5997 (2008).
16. Su, C.C. et al. Crystal structure of the CusBA heavy-metal efflux complex of *Escherichia coli*. *Nature* **470**, 558-562 (2011).
17. Outten, F.W., Huffman, D.L., Hale, J.A. & O'Halloran, T.V. The independent cue and cus systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*. *The Journal of biological chemistry* **276**, 30670-30677 (2001).
18. Kulathila, R., Kulathila, R., Indic, M. & van den Berg, B. Crystal structure of *Escherichia coli* CusC, the outer membrane component of a heavy metal efflux pump. *PloS one* **6**, e15610 (2011).

19. Khalfaoui-Hassani, B. et al. Widespread Distribution and Functional Specificity of the Copper Importer CcoA: Distinct Cu Uptake Routes for Bacterial Cytochrome c Oxidases. *mBio* **9** (2018).
20. Ekici, S., Yang, H., Koch, H.G. & Daldal, F. Novel transporter required for biogenesis of cbb3-type cytochrome c oxidase in *Rhodobacter capsulatus*. *mBio* **3** (2012).
21. De Feo, C.J., Aller, S.G., Siluvai, G.S., Blackburn, N.J. & Unger, V.M. Three-dimensional structure of the human copper transporter hCTR1. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 4237-4242 (2009).
22. Dassama, L.M., Kenney, G.E., Ro, S.Y., Zielazinski, E.L. & Rosenzweig, A.C. Methanobactin transport machinery. *Proceedings of the National Academy of Sciences of the United States of America* **113**, 13027-13032 (2016).
23. Govan, J.R. & Deretic, V. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiological reviews* **60**, 539-574 (1996).
24. Lyczak, J.B., Cannon, C.L. & Pier, G.B. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes and infection* **2**, 1051-1060 (2000).
25. Chevalier, S. et al. Structure, function and regulation of *Pseudomonas aeruginosa* porins. *FEMS microbiology reviews* **41**, 698-722 (2017).
26. Yoneyama, H. & Nakae, T. Protein C (OprC) of the outer membrane of *Pseudomonas aeruginosa* is a copper-regulated channel protein. *Microbiology* **142** (Pt 8), 2137-2144 (1996).
27. Han, Y. et al. A *Pseudomonas aeruginosa* type VI secretion system regulated by CueR facilitates copper acquisition. *PLoS pathogens* **15**, e1008198 (2019).
28. Tsukihara, T. et al. The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 Å. *Science* **272**, 1136-1144 (1996).
29. Robinson, H. et al. Structural basis of electron transfer modulation in the purple CuA center. *Biochemistry* **38**, 5677-5683 (1999).
30. Shibata, N. et al. Novel insight into the copper-ligand geometry in the crystal structure of *Ulva pertusa* plastocyanin at 1.6-Å resolution. Structural basis for regulation of the copper site by residue 88. *The Journal of biological chemistry* **274**, 4225-4230 (1999).
31. Celia, H. et al. Structural insight into the role of the Ton complex in energy transduction. *Nature* **538**, 60-65 (2016).
32. Noinaj, N., Guillier, M., Barnard, T.J. & Buchanan, S.K. TonB-dependent transporters: regulation, structure, and function. *Annual review of microbiology* **64**, 43-60 (2010).
33. Frandsen, K.E. et al. The molecular basis of polysaccharide cleavage by lytic polysaccharide monooxygenases. *Nature chemical biology* **12**, 298-303 (2016).

34. Hedison, T.M., Heyes, D.J., Shanmugam, M., Iorgu, A.I. & Scrutton, N.S. Solvent-slaved protein motions accompany proton coupled electron transfer reactions catalysed by copper nitrite reductase. *Chemical communications* **55**, 5863-5866 (2019).
35. Simmons, T.J. et al. Structural and electronic determinants of lytic polysaccharide monooxygenase reactivity on polysaccharide substrates. *Nature communications* **8**, 1064 (2017).
36. Filiatrault, M.J. et al. Effect of anaerobiosis and nitrate on gene expression in *Pseudomonas aeruginosa*. *Infection and immunity* **73**, 3764-3772 (2005).
37. Yamano, Y., Nishikawa, T. & Komatsu, Y. Cloning and nucleotide sequence of anaerobically induced porin protein E1 (OprE) of *Pseudomonas aeruginosa* PAO1. *Molecular microbiology* **8**, 993-1004 (1993).
38. Xue, Y. et al. Cu(I) recognition via cation- π and methionine interactions in CusF. *Nature chemical biology* **4**, 107-109 (2008).
39. Ren, F. et al. X-ray structures of the high-affinity copper transporter Ctr1. *Nature communications* **10**, 1386 (2019).
40. Stoyanov, J.V., Magnani, D. & Solioz, M. Measurement of cytoplasmic copper, silver, and gold with a lux biosensor shows copper and silver, but not gold, efflux by the CopA ATPase of *Escherichia coli*. *FEBS letters* **546**, 391-394 (2003).
41. Yoshihara, E. & Nakae, T. Identification of porins in the outer membrane of *Pseudomonas aeruginosa* that form small diffusion pores. *The Journal of biological chemistry* **264**, 6297-6301 (1989).
42. Ladomersky, E. & Petris, M.J. Copper tolerance and virulence in bacteria. *Metallomics : integrated biometal science* **7**, 957-964 (2015).
43. Abdollahi, S., Rasooli, I. & Mousavi Gargari, S.L. The role of TonB-dependent copper receptor in virulence of *Acinetobacter baumannii*. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* **60**, 181-190 (2018).
44. Cvetkovic, A. et al. Microbial metalloproteomes are largely uncharacterized. *Nature* **466**, 779-782 (2010).
45. Schalk, I.J. & Cunrath, O. An overview of the biological metal uptake pathways in *Pseudomonas aeruginosa*. *Environmental microbiology* **18**, 3227-3246 (2016).
46. Thaden, J.T., Lory, S. & Gardner, T.S. Quorum-sensing regulation of a copper toxicity system in *Pseudomonas aeruginosa*. *Journal of bacteriology* **192**, 2557-2568 (2010).
47. Teitzel, G.M. et al. Survival and growth in the presence of elevated copper: transcriptional profiling of copper-stressed *Pseudomonas aeruginosa*. *Journal of bacteriology* **188**, 7242-7256 (2006).

48. Frangipani, E., Slaveykova, V.I., Reimmann, C. & Haas, D. Adaptation of aerobically growing *Pseudomonas aeruginosa* to copper starvation. *Journal of bacteriology* **190**, 6706-6717 (2008).
49. Quintana, J., Novoa-Aponte, L. & Arguello, J.M. Copper homeostasis networks in the bacterium *Pseudomonas aeruginosa*. *The Journal of biological chemistry* **292**, 15691-15704 (2017).
50. Koh, E.I., Robinson, A.E., Bandara, N., Rogers, B.E. & Henderson, J.P. Copper import in *Escherichia coli* by the yersiniabactin metallophore system. *Nature chemical biology* **13**, 1016-1021 (2017).
51. Calmettes, C. et al. The molecular mechanism of Zinc acquisition by the neisserial outer-membrane transporter ZnuD. *Nature communications* **6**, 7996 (2015).
52. Lee, H.S., Hancock, R.E. & Ingraham, J.L. Properties of a *Pseudomonas stutzeri* outer membrane channel-forming protein (NosA) required for production of copper-containing N2O reductase. *Journal of bacteriology* **171**, 2096-2100 (1989).
53. Mokhele, K., Tang, Y.J., Clark, M.A. & Ingraham, J.L. A *Pseudomonas stutzeri* outer membrane protein inserts copper into N2O reductase. *Journal of bacteriology* **169**, 5721-5726 (1987).
54. Lee, H.S., Abdelal, A.H., Clark, M.A. & Ingraham, J.L. Molecular characterization of nosA, a *Pseudomonas stutzeri* gene encoding an outer membrane protein required to make copper-containing N2O reductase. *Journal of bacteriology* **173**, 5406-5413 (1991).
55. Wunsch, P., Herb, M., Wieland, H., Schiek, U.M. & Zumft, W.G. Requirements for Cu(A) and Cu-S center assembly of nitrous oxide reductase deduced from complete periplasmic enzyme maturation in the nondenitrifier *Pseudomonas putida*. *Journal of bacteriology* **185**, 887-896 (2003).
56. Flemming, H.C. & Wingender, J. The biofilm matrix. *Nature reviews. Microbiology* **8**, 623-633 (2010).
57. Kragh, K.N. et al. Role of Multicellular Aggregates in Biofilm Formation. *mBio* **7**, e00237 (2016).
58. Alvarez-Ortega, C. & Harwood, C.S. Responses of *Pseudomonas aeruginosa* to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. *Molecular microbiology* **65**, 153-165 (2007).
59. Hassett, D.J. et al. *Pseudomonas aeruginosa* hypoxic or anaerobic biofilm infections within cystic fibrosis airways. *Trends in microbiology* **17**, 130-138 (2009).
60. Stover, C.K. et al. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959-964 (2000).

61. Guzman, L.M., Belin, D., Carson, M.J. & Beckwith, J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *Journal of bacteriology* **177**, 4121-4130 (1995).
62. Bagchi, P., Morgan, M.T., Bacsá, J. & Fahrni, C.J. Robust affinity standards for Cu(I) biochemistry. *Journal of the American Chemical Society* **135**, 18549-18559 (2013).
63. Wijekoon, C.J., Young, T.R., Wedd, A.G. & Xiao, Z. CopC protein from *Pseudomonas fluorescens* SBW25 features a conserved novel high-affinity Cu(II) binding site. *Inorganic chemistry* **54**, 2950-2959 (2015).
64. Adams, P.D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta crystallographica. Section D, Biological crystallography* **66**, 213-221 (2010).
65. Emsley, P., Lohkamp, B., Scott, W.G. & Cowtan, K. Features and development of Coot. *Acta crystallographica. Section D, Biological crystallography* **66**, 486-501 (2010).
66. Zheng, H. et al. Validation of metal-binding sites in macromolecular structures with the CheckMyMetal web server. *Nature protocols* **9**, 156-170 (2014).
67. Kabsch, W. Xds. *Acta crystallographica. Section D, Biological crystallography* **66**, 125-132 (2010).
68. Karimov, R.R. & Hartwig, J.F. Transition-Metal-Catalyzed Selective Functionalization of C(sp³) -H Bonds in Natural Products. *Angewandte Chemie* **57**, 4234-4241 (2018).
69. Stoll, S. & Schweiger, A. EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *Journal of magnetic resonance* **178**, 42-55 (2006).
70. Stoll, S. & Schweiger, A. in ESR spectroscopy in membrane biophysics, Vol. 27. (eds. M.A. Hemminga & L.J. Berliner) 299-321 (Springer, Boston, MA, 2007).
71. Prentice, J.C.A. et al. The ONETEP linear-scaling density functional theory program. *The Journal of chemical physics* **152**, 174111 (2020).
72. Baba, T. et al. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular systems biology* **2**, 2006 0008 (2006).
73. Hoover, J.L. et al. A Robust Pneumonia Model in Immunocompetent Rodents to Evaluate Antibacterial Efficacy against *S. pneumoniae*, *H. influenzae*, *K. pneumoniae*, *P. aeruginosa* or *A. baumannii*. *Journal of visualized experiments : JoVE* (2017).
74. Peterson, A.C., Russell, J.D., Bailey, D.J., Westphall, M.S. & Coon, J.J. Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. *Molecular & cellular proteomics : MCP* **11**, 1475-1488 (2012).
75. Ahrne, E. et al. Evaluation and Improvement of Quantification Accuracy in Isobaric Mass Tag-Based Protein Quantification Experiments. *Journal of proteome research* **15**, 2537-2547 (2016).