The \textit{copBL} operon protects \textit{Staphylococcus aureus} from copper toxicity: CopL is an extracellular membrane–associated copper-binding protein

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As complications associated with antibiotic resistance have intensified, copper (Cu) is attracting attention as an antimicrobial agent. Recent studies have shown that copper surfaces decrease microbial burden, and host macrophages use Cu to increase bacterial killing. Not surprisingly, microbes have evolved mechanisms to tightly control intracellular Cu pools and protect against Cu toxicity. Here, we identified two genes (\textit{copB} and \textit{copL}) encoded within the \textit{Staphylococcus aureus} arginine-catabolic mobile element (ACME) that we hypothesized function in Cu homeostasis. Supporting this hypothesis, mutational inactivation of \textit{copB} or \textit{copL} increased copper sensitivity. We found that \textit{copBL} are co-transcribed and that their transcription is increased during copper stress and in a strain in which \textit{csoR}, encoding a Cu-responsive transcriptional repressor, was mutated. Moreover, \textit{copB} displayed genetic synergy with \textit{copA}, suggesting that CopB functions in Cu export. We further observed that CopL functions independently of CopB or CopA in Cu toxicity protection and that CopL from the \textit{S. aureus} clone USA300 is a membrane-bound and surface-exposed lipoprotein that binds up to four Cu\textsuperscript{2+} ions. Solution NMR structures of the homologous \textit{Bacillus subtilis} CopL, together with phylogenetic analysis and chemical-shift perturbation experiments, identified conserved residues potentially involved in Cu\textsuperscript{2+} coordination. The solution NMR structure also revealed a novel Cu-binding architecture. Of note, a CopL variant with defective Cu\textsuperscript{2+} binding did not protect against Cu toxicity \textit{in vivo}. Taken together, these findings indicate that the ACME-encoded CopB and CopL proteins are additional factors utilized by the highly successful \textit{S. aureus} USA300 clone to suppress copper toxicity.

Because of its ability to cycle between its reduced (Cu\textsuperscript{1+}) and oxidized (Cu\textsuperscript{2+}) states, copper (Cu)\textsuperscript{7} has catalytic roles in metalloenzymes such as dioxygen reductases (1), superoxide dismutases (2), laccases (3), and several proteins involved in denitrisification (4). Cu can also have nonredox structural roles, such as in transcription factors (5). Despite its necessity, intracellular Cu accumulation is toxic due to, in part, its ability to compete with other transition metals. Cu can displace iron from iron–sulfur (FeS) clusters leading to cluster destruction and protein inactivation, as well as inhibit the assembly of FeS clusters by binding to assembly proteins that would typically bind FeS clusters (6–9). Cu poisoning may also occur as a result of Fenton-type chemistry, in which Cu\textsuperscript{1+} reacts with hydrogen peroxide leading to the formation of hydroxyl radicals (10), which in turn can damage proteins, membrane lipids, and DNA.

Copper has been used to sterilize wounds and drinking water and recently by hospitals to reduce microbial burden on touch surfaces (11, 12). The human innate immune system uses Cu to

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\textsuperscript{7}The abbreviations used are: Cu, copper; MRSA, methicillin-resistant \textit{S. aureus}; CA-MRSA, community-associated-MRSA; PDB, Protein Data Bank; TEV, tobacco etch virus; RDC, residual dipolar coupling; BCS, bathocuproine disulfonate; HSOQC, 2-dimensional heteronuclear single-quantum correlation spectroscopy; Amp, ampicillin; CM, chloramphenicol; Tet, tetracycline; IMAC, immobilized metal affinity chromatography.

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kill invading microorganisms. Upon challenge with bacteria, macrophages accumulate Cu within phagosomes, where it may synergize with reactive oxygen species produced by NADPH oxidase to increase killing (13, 14).

*Staphylococcus aureus* is a public health concern worldwide. *S. aureus* causes numerous infection types ranging from skin and soft tissue infections to more severe and life-threatening diseases, such as pneumonia, osteomyelitis, and bacteremia (15, 16). Methicillin-resistant *S. aureus* (MRSA) infections have become more prevalent in community settings, and this epidemic is widely attributed to the spread of the USA300 clone (17, 18).

The genome of the USA300 clone has various mobile genetic elements, including the arginine-catabolic mobile element (ACME) (19), which occupies a 31-kb region located adjacent to the SCCmecIV genetic element. Genes encoded within ACME provide an increased fitness advantage facilitating colonization and persistence of *S. aureus* on human skin. The ACME-encoded arginine-deaminase system (*arc*) improves survival in acidic conditions (20), and the *speG* gene product provides resistance to high levels of host-derived polyamines (20, 21).

Like other pathogens, *S. aureus* must employ strategies to tightly control intracellular Cu levels and avoid Cu toxicity. Membrane-spanning Cu exporters (22, 23), Cu chaperons (24), and intracellular metallothioneins (25) are the most common defense mechanisms employed by microorganisms. The low-molecular weight thiols bacillithiol and GSH can bind to Cu with relatively high affinity (26, 27), and mutant strains lacking these compounds display sensitivity to copper suggesting potential role(s) for low-molecular weight thiols in Cu buffering (28, 29).

In *S. aureus*, the Cu-sensitive operon repressor (CsoR) binds intracellular Cu leading to derepression of the *copAZ* operon (30). CopA is a transmembrane P1B-1-type ATPase Cu exporter (31), and CopZ is a cytoplasmic Axtl-like Cu-binding chaperon (32). Some *S. aureus* strains have an additional predicted P1B-3 subtype Cu transporter (CopB) and a Cu-dependent multicopper oxidase (Mco) (33). The described *copB* and *mco* genes are co-localized and located on mobile DNA in *S. aureus* (34). The *copBmco* genes provide increased Cu resistance and, like the *copAZ* operon, their transcription is regulated by CsoR (33, 34). The function of Mco is currently unknown (35).

In this work, we characterized the *copBL* operon located within the ACME region of the *S. aureus* USA300 clone, which encodes an additional Cu homeostatic mechanism. *copL* encodes for a membrane-bound, surface-exposed Cu-binding lipoprotein, and genetic evidence suggests that *copB* encodes a Cu exporter. The solution NMR structure of a *B. subtilis* CopL revealed that CopL has a unique Cu-binding architecture.

**Results**

**Analyses of *S. aureus* genes involved in copper homeostasis**

We analyzed the genome of the community-associated (CA)-MRSA strain USA300_FPR3757 (19) for genes involved in Cu homeostasis. We noted the presence of *csoR, copA*, and *copZ*. Further analysis identified the SAUSA300_0078 locus (CopB; Fig. 1A), which shows 36% identity with CopA. CopB contains most of the conserved structural elements of P1B-ATPases (Fig. S1), including a phoshatase domain (TGES), a conserved CPX metal-binding sequence, and an ATP-binding domain (MIXGDGXNDXP) (36). Unlike CopA, CopB lacks the N-terminal metal-binding CXXC motifs, but instead contains a His-rich N terminus (Fig. S2). The CopB described herein shares 85 and 56% identity to the previously described *S. aureus* CopB (33) and *Enterococcus hirae* CopB (37), respectively. The N-terminal metal-binding domain of the CopB described herein is 52 and 42% identical to those of the N-terminal metal-binding domains of the previously described *S. aureus* CopB (33) and *Enterococcus hirae* CopB (37), respectively. A sequence alignment of the N-terminal metal-binding domains of these proteins found that they vary in the number of amino acids and in histidine content (Fig. S2).

A second open reading frame (ORF) is located 17 bp downstream of *copB*. This ORF, which we and others named *copL* (copper-binding lipoprotein), encodes a putative lipoprotein containing two DUF1541 domains (38). The *copBL* genes are located within the ACME region.
We identified ~200 CopL-like proteins in other microorganisms with the majority belonging to the Actinobacteria and Firmicutes phyla. The genomes containing copL also encoded for at least one additional Cu detoxification protein (CopA, CopZ, or CopB) (Table S1). The copL homologues are often co-localized near genes or within apparent operons encoding for genes involved in Cu homeostasis. copL is also located adjacent to copB in other staphylococci (Fig. S3).

**S. aureus strains lacking CopB or CopL have increased sensitivity to Cu**

We tested the hypothesis that CopB and CopL function in Cu homeostasis. We constructed ΔcopB and ΔcopL mutants in the *S. aureus* strain USA300_LAC (WT) (Fig. S4), which differs from the *S. aureus* strain USA300_FPR3757 by a few single-nucleotide polymorphisms (39). The WT, ΔcopB, and ΔcopL strains were spot-plated on chemically defined media containing varying concentrations of Cu. The ΔcopB and ΔcopL strains displayed decreased survival when cultured in the presence of Cu, but no growth abnormalities in the absence of Cu (Fig. 1B). Genetic complementation verified that mutational inactivation of copB or copL was resulting in the observed growth defects (Fig. 1B).

The *S. aureus* USA400 strain MW2 lacks copB and copL. We mobilized copB and copL to the MW2 strain via plasmid and examined Cu sensitivity. The MW2 strain containing copB or copL displayed increased resistance to Cu (Fig. 1C). Expression of copL in the *S. aureus* strains Newman, COL, and RN4220 also resulted in increased Cu resistance (Fig. S5).

**Strains lacking CopB or CopL display exacerbated phenotypes in cells unable to export cytoplasmic Cu via CopA**

We investigated whether CopB and CopL had a functional overlap with other genes involved in Cu homeostasis. Genes encoding proteins with functional overlap often display synergistic phenotypes when the gene products are absent or non-functional (40). An *S. aureus* copA mutant accumulates intracellular Cu and displays sensitivity to Cu (31). The copA::Tn and ΔcopB mutants had decreased growth on solid media containing >50 µM Cu, but no sensitivity was observed at lower (10 µM) Cu concentrations (Fig. 2A). The phenotypes associated with the copA::Tn ΔcopB mutations were synergistic for Cu sensitivity (Fig. 2A). Similarly, the copA::Tn ΔcopL double–mutant strain was more sensitive to Cu than the copA::Tn and ΔcopL single mutants (Fig. 2B). The copA::Tn ΔcopB ΔcopL triple mutant was more sensitive to Cu than the copA::Tn ΔcopB double–mutant strain (Fig. 2C).

The *S. aureus* copZ::Tn mutant did not display a Cu sensitivity phenotype on solid media (data not shown). The copZ::Tn ΔcopB and copZ::Tn ΔcopL strains displayed Cu sensitivity phenotypes similar to the ΔcopB and ΔcopL single mutants (data not shown).

Collectively, these results suggest the following: (a) CopA and CopB have a functional overlap and serve as Cu exporters; (b) CopL functions in Cu homeostasis independently of CopA and CopB; and (c) CopZ is not required to protect against Cu toxicity under the conditions examined.

copBL transcription increases in the presence of Cu

We tested the hypothesis that transcription of copB and copL increases in response to Cu. The abundances of the copB and copL transcripts were monitored in the WT strain grown with and without Cu. The *S. aureus* copA is induced during Cu stress (31, 33); therefore, we quantified abundance of the copA transcript as a control. The transcripts of copB and copL were increased ~4-fold upon Cu stress, whereas the copA transcript was increased ~8-fold (Fig. 3A).

We tested the hypothesis that copB and copL are co-transcribed. RNA was isolated from WT cultures grown in the presence of Cu, and cDNA libraries were generated. Using cDNA as a PCR template, and primers nested within the copB and copL genes, we were able to obtain an amplicon that spanned copB and copL (Fig. 3B). No PCR products were obtained from control reactions lacking reverse transcriptase (−RT), confirming that the amplicon was not the result of genomic DNA contamination.

We created a transcriptional reporter to further analyze the regulation of copB. Transcriptional activity of copB increased in synchrony with the concentration of Cu added to the growth medium verifying the functionality of the reporter (Fig. 3C). Transcriptional activity of copB did not increase upon challenge with Mn²⁺, Fe³⁺, Zn²⁺, or Co²⁺ (Fig. 3D). In addition, the ΔcopB and ΔcopL mutant strains did not display increased.
sensitivity to these metals at concentrations that decreased the survival of the WT strain (data not shown).

**CsoR regulates the copBL operon**

We tested the hypothesis that CsoR regulates the copBL operon. CsoR-binding sites have been identified in the promoters of copZA in *Bacillus subtilis* (41) and copAZ in *S. aureus* strain Newman (30), which are characterized by a pseudo-inverted repeat (TACCNNNNGGGGGTA) (Fig. 4A). We analyzed the promoter region of the copBL in strain FPR3757 and found that it contains a putative CsoR-binding site 100 bp upstream of the translational start site.

We monitored the transcriptional activity of copB in the WT and csoR::Tn strains. The transcriptional activity of copB was increased in the csoR::Tn strain, suggesting that transcription of the copBL operon is repressed by CsoR (Fig. 4B). Addition of Cu led to increased copB transcriptional activity in the WT strain but not in the csoR::Tn strain (Fig. 4B). The *S. aureus* strain Newman lacks the copBL operon; however, the transcriptional activity of copB was also increased in an *S. aureus* Newman ΔcsoR mutant when compared with the parent strain (Fig. S6).

**CopL is membrane-associated and surface-exposed**

We tested the hypothesis that CopL is a surface-exposed lipoprotein. We conducted cell fractionation experiments to verify the cellular location of CopL. We utilized a C-terminal FLAG–tagged copL that was under the transcriptional control of a xylose-inducible promoter (xylRO) (pEPSA5_copL–FLAG). The copL–FLAG allele genetically complemented the Cu sensitivity phenotype of the csoR::Tn mutant strain, verifying the functionality of the fusion (data not shown). Cultures of the csoR::Tn strain harboring either pEPSA5 or pEPSA5_copL–FLAG were grown in the presence and absence of xylose. Cells were fractionated into cytosolic and membrane fractions. CopL–FLAG was also detected in noninduced samples, albeit at a much lower inten-
CopL and copper homeostasis

S. aureus CopL (saCopL) binds copper in vitro

We tested the hypothesis that saCopL is a Cu-binding protein. Recombinant soluble saCopL(T) was purified from Escherichia coli (Fig. S7), and Cu\(^{2+}\) binding was examined using ultraviolet (UV)-visible absorption spectroscopy. Titrating Cu\(^{2+}\) into apo-saCopL(T) resulted in gradual increases of the absorbance in the UV region (\(A_{243}\) nm). The change of \(A_{243}\) was plotted as a function of the Cu\(^{2+}\)/saCopL(T) ratio (Fig. 6A). The formation of Cu\(^{2+}\)-saCopL(T) was linear and reached saturation after the addition of ~4 molar eq of Cu\(^{2+}\).

Competition experiments were conducted to determine the Cu\(^{2+}\)-binding affinity of saCopL(T). Bathocuprione disulfonate (BCS) forms a complex with Cu\(^{2+}\) in a 2:1 ratio Cu\(^{2+}\)/BCS, that has an absorption maximum at \(A_{483}\) nm (47). To determine the effective Cu\(^{2+}\)-binding affinity of saCopL(T), Cu\(^{2+}\) and BCS were combined anaerobically. A fixed concentration of saCopL(T) was then titrated into the sample, and absorption spectra were recorded. The formation of Cu\(^{2+}\)-saCopL(T) was determined by monitoring the decrease in absorbance (\(A_{483}\) nm) after each addition (Fig. 6B).

At each of the saCopL(T) concentrations examined, Equation 6 of Xiao et al. (48) enabled calculation of the \(K_d\) for the protein–copper complex. The calculation yields the product of \(K_d\) and \(\beta_2\), where \(\beta_2\) is the association constant between copper and BCS. We used \(\beta_2 = 10^{19.8}\) (47), and the resulting \(K_d\) value was 4.98 ± 0.20 \(\times 10^{-18}\) M.

Structure of B. subtilis CopL (bsCopL) reveals a novel Cu-binding motif

B. subtilis ydhK (bsCopL) encodes a homolog of S. aureus CopL, with sequence identity of ~63% in the C-terminal region (Fig. S8). A. B. subtilis \(\Delta\)copL mutant was more sensitive to growth in the presence of Cu than the parent strain (Fig. S9). As the unpublished 3D solution NMR structure of this homologous protein was already available from our National Institutes of Health Protein Structure Initiative (49) structural bioinformatics efforts, we used these NMR assignments and structure to validate that CopL does in fact bind Cu and predict Cu-binding ligands.

The solution NMR structure of the bsCopL fragment comprising residues 83–205, truncated to remove the signal peptide and lipobox motif, forms a well-ordered structure in solution (Fig. S10), consisting of a pair of distorted \(\beta\)-barrels joined by two shared \(\beta\)-strands, and includes two short turns with a 3\(_{10}\)-helix geometry (Figs. 7A and Fig. S10). The \(\beta\)-strands (A–H) consist of residues 87–90, 102–118, 127–134, 135–137, 141–153, 166–184, 189–198, and 202–204, whereas the 3\(_{10}\) helical turns consist of residues 96–98 and 135–137. The two homologous DUF1541 domains are found to form a single structural unit, as each \(\beta\)-barrel is composed of polypeptide segments from both DUF1541 domains. An unusual structural feature of

The TOPCONS algorithm (42) predicted that CopL contains an N-terminal membrane–targeting signal sequence (Fig. S1), which is a characteristic of proteins that are translocated across the cytoplasmic membrane (43). The LipPs server (44) identified a lipobox motif (sequence: LSAC) that is processed to provide a covalent lipid-linked membrane anchor. Lipoproteins in Gram-positive bacteria are anchored to the cytoplasmic membrane with the C termini facing the extracellular surface (45). We examined whether the functionality of the CopL protein depends on its cellular localization. We cloned a copL allele lacking the membrane translocation signal–sequence and lipobox, which we refer to as the truncated CopL (CopL(T)), into pEPSA5 (Fig. 5B). CopL(T) did not complement the Cu sensitivity phenotype of the \(\Delta\)copL strain, whereas the full-length CopL did (Fig. 5B). Cellular fractionation experiments using the \(\Delta\)copL strain containing pEPSA5_copL(T)-FLAG revealed that the CopL(T) accumulated in the soluble cytosolic fractions but not in the membrane fraction (data not shown). These results suggested that CopL does not function to protect against Cu toxicity unless localized to the membrane.

Nuc2 is a surface-exposed nuclease anchored to the membrane via its signal peptide (46). We created constructs encoding for chimeric proteins in which CopL(T) was fused to either Nuc2 (pEPSA5_nuc2_copL) or the described Nuc2 signal sequence (pEPSA5_nuc2(SS)-copL) (Fig. 5C). Both chimeric constructs genetically complemented the Cu sensitivity phenotype of the \(\Delta\)copL strain (Fig. 5C). Altogether, the data in Fig. 5 suggested that CopL is membrane-associated and surface-exposed and requires membrane association to protect against Cu toxicity.

**Figure 4.** Transcription of the copBL operon is regulated by CsoR. A, proposed CsoR-binding sites are shown for the S. aureus USA300_FPR3757 copA and copB promoter regions, as well as the B. subtilis copZ and S. aureus Newman copA promoter regions. B, transcriptional activity of copB is increased in a csoR mutant. Transcriptional activity of copB was monitored in the WT (JMB1100) and csoR::Tn (UMB6807) strains grown in chemically defined media containing 0 or 100 \(\mu\)M Cu. Fluorescence data were standardized to culture optical density (\(A_{590}\) nm). Data shown represent the average of biological triplicates with errors presented as standard deviations. Student’s t test were performed on the data, and * indicates \(p < 0.05\), whereas N.S. denotes not significant.
bsCopL is the cis conformation of the peptide bonds between two conserved residue pairs, Lys-131–Trp-132 and Lys-195–Trp-196 (Fig. 7A), which results in a stacked orientation of their side chains. The strong sequential H$_9$251–H$_9$251 NOE peaks supporting the cis conformation of these two nonproline peptide bonds are presented in Fig. S11.

Residue conservation scores mapped onto the bsCopL structure indicate that the most conserved surface region, at the interface of the two -barrels (Fig. 7B and Figs. S12 and S13), represents a likely binding site for copper atoms. Copper coordination in proteins is usually mediated by nitrogen atoms of histidine rings and sulfur atoms of cysteines and methionines; in less common cases it involves side-chain oxygen atoms of aspartic and glutamic acids, glutamine, or tyrosine, backbone carbonyl oxygens, and even indole rings of tryptophan in the form of $\pi$–cation interactions (50, 51). A search of PDB90 (nonredundant subset of the PDB database with proteins sharing less than 90% sequence identity) for similar protein structures using DALI (52) produced 27 significant protein chain hits (Z-score $\geq$5). However, 25 of those the aligned segments were only between 45 and 69 residues long, with sequence identities to bsCopL between 10 and 23% and C-atom r.m.s.d. between 1.3 and 3.8 Å. Here, the matching segments corresponded to only a single -barrel of bsCopL. For the remaining two proteins, PDB codes 2QQR and 2MAM, the aligned segments were 82 and 91 residues long, respectively. Both are tandem tudor domain proteins of human origin, the former a histone demethylase and the latter a DNA-binding protein. However, the sequence identity to bsCopL was only 13 and 19%, respectively. Global C-atom r.m.s.d. was quite high at 5.0 and 5.3 Å, due to a significantly different relative orientation of the -barrels. None of the structurally similar proteins contained the conserved histidine and methionine residues of bsCopL. Accordingly, we conclude that bsCopL features a novel Cu-binding protein architecture.

Figure 5. CopL is membrane-associated and surface-exposed. A, monitoring CopL abundance in S. aureus whole-cell extracts, cytosolic fractions, and membrane fractions. The $\Delta$copL (JMB7711) mutant containing the pEPSA or pEPSA$_5$-copL–FLAG was cultured in the absence and presence of 0.2% xylose prior to fractionation and analysis. ND, not detected. B, membrane-anchor signal sequence is necessary for CopL function. Panel i, schematic showing the CopL variants utilized. The pEPSA$_5$-copL encoded saCopL and pEPSA$_5$-copL(T) encoded for the truncated saCopL(T), which lacked the proposed N terminus export signal-sequence and lipobox. Panel ii, WT and $\Delta$copL strains harboring the pEPSA5, pEPSA$_5$-copL, or pEPSA$_5$-copL(T) were serially diluted and spot-plated on chemically defined media without and with 100 $\mu$M Cu. C, cell-surface exposure is necessary for CopL function. Panel i, schematic showing the CopL-Nuc2 chimeric variants. The truncated copL gene, lacking the proposed N-terminal export–sequence and lipobox, was fused to either the full-length nuc2 or the nuc2 membrane-anchor signal sequence and cloned into the pEPSA5 plasmid to yield the pEPSA5-nuc2(FL)-copL and pEPSA5-nuc2(SS)-copL vectors, respectively. Panel ii, WT and $\Delta$copL strains harboring pEPSA5, pEPSA5-nuc2(FL)-copL, or pEPSA5-nuc2(SS)-copL were serially diluted and spot-plated on chemically defined media containing 0 or 100 $\mu$M Cu.
bsCopL prepared with no hexa-His tag are essentially identical, aside from the small spectral differences due to the N- and C-terminal regions (Fig. S16).

To confirm the ability of bsCopL to bind copper in vitro, and to identify the Cu\(^{1+}\) binding sites, 8 mol/mol eq of Cu\(^{1+}\) were added to the apo-bsCopL protein, and the buffer was exchanged to remove adventitiously-associated Cu\(^{1+}\) ions. Sequence-specific backbone NMR resonance assignments were then determined using standard triple-resonance NMR experiments (Table S3), and deposited in the Biological Magnetic Resonance Bank (BMRB ID 27741).

Comparison of the overlay of the assigned 2D \(^{15}\)N,\(^{1}\)H HSQC spectra of apo-bsCopL and holo-bsCopL (Fig. 8) revealed that Cu\(^{1+}\) addition results in specific chemical shift perturbations. The numerous spectral changes induced upon copper addition unambiguously demonstrate Cu\(^{1+}\) association with bsCopL. A plot of chemical shift perturbations along the protein sequence is shown in Fig. S17. When mapped onto the 3D structure of B. subtilis apo-bsCopL (Fig. 9), these data demonstrate that many residues with significant chemical shift perturbations are located in the conserved surface region at the interface of the two \(\beta\)-barrels, which, as outlined above, we predict based on amino acid composition and conservation to include copper-binding sites.

To further confirm reversible copper association, the Cu\(^{1+}\) chelator BCS was added to the holo-bsCopL protein sample followed by recording a \(^{15}\)N,\(^{1}\)H HSQC spectrum. As expected, the spectrum of the BCS-treated sample almost fully returned to match that of the apo-bsCopL (Fig. S18).

**A CopL variant with decreased Cu binding does not protect against Cu toxicity in vivo**

We tested the hypothesis that Cu binding by CopL is necessary to protect from Cu toxicity. We created a construct that encoded for the S. aureus copL mutant allele (called copL\(^{-}\)) that encoded for the following directed changes to CopL: H70A, M71A, H134A, M135A (His-94, Met-95, His-158, and Met-159 in bsCopL) (Fig. 10A). These are strictly conserved residues that NMR studies suggested may function in Cu\(^{1+}\) ligation. The copL\(^{-}\) allele was unable to correct the Cu-dependent growth defects of the S. aureus \(\Delta\)copL mutant (Fig. 10A).

We purified saCopL(T)* (viz. H70A, M71A, H134A, and M135A copL(T)) and examined the near UV CD spectra of the apo-saCopL(T) and apo-saCopL(T)* proteins. The spectra were nearly identical indicating little difference in secondary structure between the variants (Fig. S19). We next titrated with Cu\(^{1+}\) into saCopL(T)* and monitored spectral changes using UV absorption spectroscopy. Cu\(^{1+}\) titration resulted in a linear increase in absorbance at 243 nm, and the absorbance plateaued after approximately 2 eq of Cu\(^{1+}\) per saCopL(T)* (Fig. 10B). These data indicate that the saCopL(T)* variant binds less Cu\(^{1+}\) than saCopL(T). We next titrated saCopL(T)* into a solution of BCS and Cu\(^{1+}\) and monitored absorbance at 483 nm. The addition of saCopL(T)* decreased absorbance at 483 nm but not to the extent of that of saCopL(T) (Fig. 10C). saCopL(T)* binds Cu\(^{1+}\) with a \(K_d\) of \(1.35 \pm 0.17 \times 10^{-17}\) m, which is \(\sim 3\)-fold lower than the affinity of saCopL(T) for Cu\(^{1+}\).
Discussion

This study was initiated to further investigate the mechanisms of copper (Cu) homeostasis in the epidemic CA-MRSA *S. aureus* USA300 clone. The work presented has re-affirmed the roles of CopA and CsoR in Cu efflux and intracellular Cu sensing, respectively. We have also assigned roles for copB and...
CopL in Cu homeostasis. These data, as well as published work on CopA (31), CopZ (32), and CsoR (53), resulted in a working model for Cu homeostasis in the S. aureus USA300_LAC, which is illustrated in Fig. 11. Upon sensing Cu in the cytosol, CsoR derepresses transcription of the copAZ and copBL operons. The binding of apo-CsoR to the copB promoter was recently confirmed by Purves et al. (54). CopZ binds Cu and acts as an intracellular Cu buffer delivering it to CopA. The CopA and CopB proteins function to efflux Cu from the cytosol. CopL is a membrane-associated, surface-exposed protein that binds Cu on the outside of the cell preventing it from entering the cytosol and binds Cu after efflux by CopA or CopB.

CopL tightly binds Cu\(^{1+}\) with a dissociation constant of 4.98 ± 0.20 \(\times 10^{-18}\) M. For comparison, the reported Cu\(^{1+}\)-binding affinities of the S. aureus (30), B. subtilis (55), and Mycobacterium tuberculosis (53) CsoR proteins, HAH1 from Homo sapiens (56), and N-terminal metal-binding domain of CopA (57) and CopZ from B. subtilis (58) are of the same order of magnitude as CopL. The CusCFBA system protects the E. coli cytosol and periplasm from Cu (59). The metallochaperone of this system (CusF) has a Cu\(^{1+}\) \(K_d\) of 5.0 ± 1.2 \(\times 10^{-15}\) M (60).

It should be emphasized that small differences in binding affinity among the individual CopL-binding sites could exist, but this could not be confirmed by the present data. The \(K_d\) values reported here should therefore be considered weighted averages of the sites. It is clear nevertheless that the binding is tight and comparable with that of other copper-binding proteins. It is also clear (Fig. 10C) that the CopL* variant binds copper with less affinity than the WT CopL.

One aim of the work presented was to validate that CopL, from both S. aureus or B. subtilis, binds Cu\(^{1+}\). This was achieved using Cu\(^{1+}\)-binding data on S. aureus CopL and NMR studies on the B. subtilis CopL. Although there may in fact be structural differences in the Cu\(^{1+}\)-binding networks of S. aureus and B. subtilis CopL, the basic residues in the Cu\(^{1+}\)-binding site of B. subtilis CopL form a highly conserved network that is common to other homologs of this two-domain protein family, including S. aureus CopL.

The solution NMR structure of bsCopL revealed a novel copper-binding architecture. The overall structure of the Cu\(^{1+}\)-bound form is similar to that of the apo-form because most distant residues do not exhibit significant perturbations of chemical shifts upon Cu\(^{1+}\) binding. To preserve these NMR data for more comprehensive future studies, we have deposited these chemical shift data in the BioMagRes Database (BMRB ID 27741).
CopBL and copper homeostasis

Figure 10. Effective Cu binding is necessary for CopL to protect against Cu toxicity. A, copL* allele does not correct the Cu sensitivity phenotype of the \textit{S. aureus} \textit{ΔcopL} mutant. Panel i, schematic of the \textit{copl} and \textit{copL*} constructs utilized. Panel ii, WT and \textit{ΔcopL} strains harboring pEPSAS, pEPSAS\_nuc2(SS)-\textit{copL}, or pEPSAS\_nuc2(SS)-\textit{copL*} were serially diluted and spot-plated on chemically defined media without and with 100 \textmu M Cu. B, saCopL(T)* binds less Cu\textsuperscript{2+} than saCopL(T). C, saCopL(T)* was anaerobically titrated with Cu\textsuperscript{2+}. Binding was monitored by measuring absorbance at 243 nm using UV absorption spectroscopy. C, saCopL(T)* has a lower affinity for Cu\textsuperscript{2+} than saCopL(T). Solutions containing 0.5 mM BCS and 20 \textmu M Cu\textsuperscript{2+} were titrated with either apo-saCopL(T) or apo-saCopL(T*). Copper binding to CopL was examined using BCS displacement by monitoring sample absorbance at 483 nm, which is the absorption maxima for the Cu\textsuperscript{2+}-BCS complex. The data for the saCopL(T) are also presented in Fig. 6 and included here for comparison.

The 3D structure of \textit{bsCopL(T)} contains unusual \textit{cis} peptide bonds between two conserved residue pairs, Lys-131–Trp-132 and Lys-195–Trp-196. Nonproline \textit{cis} peptide bonds are rare and occur only in about 0.03% of all peptide bonds in known protein structures (61). Their occurrence is significantly more frequent in X-ray crystal structures determined at high resolution than in structures determined at medium and low resolution, suggesting that these bonds may be more abundant than generally recognized (61). The \textit{cis-trans} isomerization of peptide bonds before proline residues can play an important kinetic role in controlling protein folding (62, 63). This isomerization can be catalyzed by enzymes known as prolyl-\textit{cis-trans} isomerases that have been shown to act on Xaa–Pro peptide bonds, but not on nonproline peptide bonds (64). Nonproline \textit{cis} peptide bonds tend to be buried and not greatly accessible compared with Xaa–Pro \textit{cis} peptide bonds, which suggests that they may form early during the folding process (65). They tend to be observed near active sites indicating that they may sometimes be important for protein activity (61). The majority of the \textit{cis} peptide bonds occur in regular secondary structures, more specifically in \beta-structures (65). In the case of \textit{bsCopL(T)}, the two non-Pro \textit{cis} peptide bonds are located in \beta-structures \beta3 and \beta6, adjacent to the proposed Cu-binding residues His-130 and His-194, respectively. These \textit{cis} peptide bond conformations result in unique local side-chain structure, with stacked orientation of their side chains. Although there may be functional consequences of such non-Pro \textit{cis} peptide bonds, such structure–function studies are difficult to carry out because mutations that prevent \textit{cis} peptide bonds also tend to have structurally-disruptive effects.

The CA-MRSA epidemic is widely attributed to the spread of the USA300 clone (17, 18). The majority of the genetic differences between USA300 and other staphylococcal strains of clinical importance is the presence of mobile genetic elements, including ACME (19). Expression of the ACME-encoded arginine-deiminase system (\textit{arc}) improves survival in acidic conditions (20), and the ACME-encoded \textit{speG} gene provides resistance to high levels of host-derived polyamines and survival within murine abscesses (20, 21, 66).

While this manuscript was being revised, Purves \textit{et al.} (54) reported that \textit{S. aureus} strains lacking \textit{copB} (called \textit{copX} in their study) or \textit{copL} are sensitive to growth in the presence of Cu. Interestingly, they were not able to genetically complement the \textit{copL} mutant and were only able to complement in the \textit{copB} mutant in one of the two media utilized. They found that the \textit{copB} or \textit{copL} mutants, but not the \textit{copA} mutant, had slightly increased cell-associated Cu. The authors used these data to suggest a role for CopBL in Cu efflux. Similar to the findings reported herein, they found that CsoR controls transcription of the \textit{copAZ} and \textit{copBL} operons. They also reported that apo-

Figure 11. Working model for copper homeostasis in \textit{S. aureus}. The CsoR transcriptional regulator binds intracellular copper, leading to derepression of the \textit{copAZ} and \textit{copBL} operons. CopA and CopB are transmembrane copper exporters. CopZ is a chaperon protein that binds copper and acts as a cytosolic buffer. CopL is a membrane-bound, surface-exposed, and copper-binding lipoprotein. CopL likely functions in preventing copper uptake by binding extracellular Cu to prevent it from entering or re-entering the cell after export by CopB or CopA.
CsoR binds to the copB promoter suggesting that it directly controls transcription of copBL. Importantly, Purves et al. (54) showed that strains lacking CopB or CopL, but not a strain lacking CopA, had decreased survival in murine macrophages. These data highlight a potentially important roles of CopB and CopL in pathogenesis.

The human skin commensal *Staphylococcus epidermidis* contains the copB, mco, and copL genes in an apparent operon, as well as two additional Cu efflux proteins (67). Phylogenetic analyses suggest that the genes comprising ACME were assembled into a single genetic locus in *S. epidermidis* before they were transferred to *S. aureus*, which occurred prior to the epidemic expansion of the USA300 clone (66). Our bioinformatics analysis (Table S1) revealed that *Staphylococcus xylosus*, *Staphylococcus capitis*, and *Staphylococcus haemolyticus*, which are skin commensals, also have copB and copL. The acquisition of genetic elements from species that share the same niche is a strategy employed by *S. aureus* to adapt to new environments (68, 69). It is tempting to speculate that additional Cu detoxification mechanisms provided by the ACME-encoded copBL operon further promote survival on skin; however, this speculation awaits further examination.

A USA300 Latin American variant (USA300-LV) has become one of the most prevalent clones associated with MRSA infections in community settings in South America. Most of the genomic differences between USA300-LV and USA300 can be attributed to mobile genetic elements and, specifically, to the absence of ACME in USA300-LV (70). Despite this difference, the USA300-LV genome contains copBL. Similar to the location of copBL in USA300, copBL in USA300-LV are located adjacent to SCCmec on a mobile genetic element, which has been designated as the copper and mercury resistance mobile element (COMER) (70).

Mobile genetic elements containing copper detoxification islands have been discovered in other organisms (71). Copper-resistance mechanisms acquired via transposable elements include copper transporters, multicopper oxidases, or as reported in this study, membrane-bound lipoproteins. These studies provide additional evidence that copper exposure (immune system, healthcare settings, and/or diet) may not only promote the dissemination of genetic elements that result in the development of metal-resistant microorganisms but also strains that are hypervirulent and resistant to antimicrobials.

In summary, the work presented here describes an additional strategy by which *S. aureus* protects against copper toxicity. The ACME-encoded copBL may contribute to the high success of USA300, by providing protection from Cu-dependent killing. Moreover, the fact that copBL is encoded on mobile DNA alongside virulence factors suggests that the maintenance of Cu homeostasis could be a selective pressure for mobilization of alternative virulence factors or antibiotic resistance genes.

**Experimental procedures**

**Reagents**

Restriction enzymes, quick DNA ligase kit, deoxynucleoside triphosphates, and Phusion DNA polymerase were purchased from New England Biolabs. Primers were obtained from Integrated DNA Technologies and listed in Table S2. Plasmid mini-prep and gel extraction kits were purchased from Qiagen. Lysostaphin was purchased from Ambi Products. Tryptic Soy Broth (TSB) was purchased from MP Biomedicals. The ELC chemiluminescent detection kit was purchased from Pierce. Pierce protease and phosphatase inhibitor mini tablets were purchased from ThermoFisher Scientific. GSTrap 4B columns and PreScission Protease were purchased from GE Healthcare. Unless specified, all other chemicals were purchased from Sigma and were of the highest purity available.

**Bacterial strains and growth conditions**

Bacterial strains used in this work are listed in Table 1. Unless otherwise noted, the *S. aureus* strains used in this study are derived from the community-associated MRSA USA300 LAC strain that was cured of the pUSA03 plasmid, which confers erythromycin resistance (72). *S. aureus* strains were cultured in TSB or a defined medium, and *E. coli* strains were grown in Luria Broth (LB). Unless otherwise specified, all bacterial strains were cultured at 37 °C. The chemically defined medium was previously described (73) and contained the following: 1 g from New England Biolabs. Primers were obtained from Integrated DNA Technologies and listed in Table S2. Plasmid mini-prep and gel extraction kits were purchased from Qiagen. Lysostaphin was purchased from Ambi Products. Tryptic Soy Broth (TSB) was purchased from MP Biomedicals. The ELC chemiluminescent detection kit was purchased from Pierce. Pierce protease and phosphatase inhibitor mini tablets were purchased from ThermoFisher Scientific. GSTrap 4B columns and PreScission Protease were purchased from GE Healthcare. Unless specified, all other chemicals were purchased from Sigma and were of the highest purity available.

**Table 1**

**Strains and plasmids used in this study**

| Strains and plasmids used in this study | Refs. | 30, 77, 79, 81, and 113 are cited in the table. |

**Table 2**

**Strains and plasmids used in this study**

| Strain name | Genotype/Description | Background | Source / Reference |
|-------------|----------------------|------------|------------------|
| RN4220      | Restriction-negative  | RN1        | (74)             |
| JMB2799     | copB::Tn917(SAUSA300_2994) | JE2       | (113)            |
| JMB4388     | csoR::Tn917(SAUSA300_2943) | JE2       | (115)            |
| JMB1100     | USA300 wild-type strain | LAC       | A. R. Horvill    |
| JMB4084     | copL::Tn917(SAUSA300_2994) | LAC       | This study       |
| JMB6807     | csoR::Tn917(SAUSA300_2994) | LAC       | This study       |
| JMB9000     | ΔcopB(SAUSA300_0078) | LAC       | This study       |
| JMB7711     | ΔcopL(SAUSA300_0079) | LAC       | This study       |
| JMB2901     | ΔcopB | LAC       | This study       |
| JMB4009     | ΔcopB, csoR::Tn917(SAUSA300_2994) | LAC       | This study       |
| JMB7083     | ΔcopB, ΔcopL::Tn917(SAUSA300_2994) | LAC       | This study       |
| JMB1324     | parent strain | MW2       | P. Schievlev     |
| JMB1325     | parent strain | COL       | A. Horvill       |
| JMB1422     | parent strain | Newman    | E. Skar          |
| JMB4338     | ΔcsoR(NWMN 1991) | Newman    | (30)             |

**Other Strains**

| Strain name | Relevant genotype/Description | Source / Reference |
|-------------|--------------------------------|
| 30, 74, 77, 79, 81, and 113 are cited in the table. |

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**ASBMB**
of (NH₄)₂SO₄, 4.5 g of KH₂PO₄, 10.5 g of K₂HPO₄, 110 mM NaCl, 30 mM KCl, 50 μg of nicotinic acid, 50 μg of pantethic acid, 50 μg of thiamine, 0.3 μg of biotin, and 2.5 mg of each of the 20 amino acids per 100 ml. When supplemented to the media, chemicals were added at the following concentrations: 10–300 μM CuSO₄; 50–200 μM Fe₂(NH₄)₂(SO₄)₂; 5–200 μM CoCl; 10–300 μM MnSO₄; 10–300 μM ZnSO₄. When appropriate, antibiotics were added at the following concentrations: 150 μg ml⁻¹ ampicillin (Amp), 6 or 30 μg ml⁻¹ chloramphenicol (Cm) (defined or complex media, respectively), 10 μg ml⁻¹ erythromycin, 3 μg ml⁻¹ tetracycline (Tet), and 150 ng ml⁻¹ anhydrotetracycline. Overnight cultures were grown in 7-ml culture tubes containing 2 ml of TSB shaken at 200 rpm. When growing overnight cultures of strains containing pEPSA-derived plasmids, 2% xylose (w/v) was added to the media.

**Construction of mutant strains and plasmids**

Chromosomal DNA from JMB1100 was used as the template for PCRs used in the construction of plasmids. All plasmids were isolated from E. coli DH5α and transformed into electrocompetent S. aureus RN4220 using a standard protocol (74). Phase a80 was used for plasmid and chromosomal transductions (75). All bacterial strains and DNA constructs were verified by PCR, genetic complementation, or DNA sequencing prior to use. DNA sequencing was conducted by Genewiz (South Plainfield, NJ).

Mutational inactivation of the S. aureus copB and copL genes was achieved by chromosomal deletion to yield the ΔcopB, ΔcopL, and ΔcopBL mutant strains as described previously (76). For the ΔcopB mutant, upstream and downstream regions of the copB gene (SAUSA300_0078) were PCR-amplified using the following primers: ZRC199 and ZRC200; ZRC164 and ZRC201. PCR products were gel-purified and fused by PCR using the ZRC199 and ZRC201 primers. For the ΔcopL mutant, upstream and downstream regions of copL (SAUSA300_0079) were PCR-amplified using the following primers: ZRC166 and ZRC167; ZRC168 and ZRC169. PCR products were gel-purified and fused by PCR using the ZRC166 and ZRC169 primers. For the ΔcopBL double mutant, upstream and downstream regions of the copBL operon were PCR-amplified using the following primers: ZRC185 and ZRC186; ZRC168 and ZRC169. PCR products were gel-purified and fused by PCR using the ZRC185 and ZRC169 primers. The ΔcopB, ΔcopL, and ΔcopBL PCR products were digested with EcoRI and Sall and ligated into similarly digested pBl38 (77). The recombinant vectors were transformed into chemically competent E. coli DH5α. PCR was used to screen for E. coli colonies harboring the recombinant plasmids using ZRC196 and ZRC201 primers for pBl38 ΔcopB, ZRC196 and ZRC169 for pBl38 ΔcopL, and ZRC196 and ZRC169 for pBl38 ΔcopBcopL. The plasmids were isolated and mobilized into RN4220 and subsequently JMB1100. Colonies that were Cm-sensitive were screened using PCR for the double-recombination event. The ΔcopB mutant strain was verified using the ZRC139 and ZRC201 primers. The ΔcopL and ΔcopBL mutants were verified using the ZRC139 and ZRC169 primers.

The pTET plasmid was used to construct the copA::Tn(tet) and csoR::Tn(tet) in the USA300 LAC background by allelic exchange as described previously (78). Mutational inactivation was confirmed using PCR with the following primers: copA::Tn(ermB), copA::Tn(tet), and copZ::Tn(ermB) with ZRC133 and ZRC134; csoK::Tn(ermB) and csoK::Tn(tet) with ZRC153 and ZRC155.

For complementation and expression studies, genes were cloned into pEPSA5 (79). The ZRC146 and ZRC141 primers were used to PCR amplify the copB gene. The ZRC149 and ZRC150 primers were used to PCR amplify the full-length copL. The ZRC184 and ZRC150 primers were used to PCR amplify the truncated copL. PCR products were digested with BamHI and Sall and ligated into similarly digested pEPSA5 to yield pEPSA5 copB, pEPSA5 copL, and pEPSA copL(T) vectors. Plasmid-containing strains were PCR-verified, using the pEPSA5upveri and ZRC141 (pEPSA_copB) or the pEPSA5upveri and ZRC150 (pEPSA copL or pEPSA copL(T)).

The pEPSA5 copL−FLAG and pEPSA5 copL(T)-FLAG vectors were constructed by using the ZRC149−ZRC181 and ZRC184−ZRC181, respectively. The inserts were digested with BamHI and Nhel and then ligated into similarly digested pEPSA5_CitB-FLAG (80). Strains containing the pEPSA5 copL−FLAG plasmid were PCR-verified using the pEPSA5upveri and ZRC181 primers. The pGEX-6p-1 copL was constructed using the ZRC198 and ZRC178 primers. The PCR product was digested with BamHI and Xhol and then ligated into similarly digested pGEX-6p-1(GE Healthcare). The pGEX-6p-1 copL* was created using the pEPSA5_nuc2(SS)-copL* as a PCR template for copL*. The copB transcriptional reporter was created by amplifying the copB promoter using the ZRC139 and ZRC140 primers. The PCR product was digested with HindIII and KpnI and ligated into similarly digested pCM11 (81).

The pEPSA5_nuc2-copL and pEPSA5_nuc2(SS)-copL vectors were created by using yeast homologous recombination cloning in Saccharomyces cerevisiae FY2 as described previously (82, 83). The pEPSA5_CitB-FLAG vector was linearized with Nhel. The amplon for the pEPSA5_nuc2(FL)-copL was created using the following primer pairs: ZRC188 and ZRC189; ZRC191 and ZRC193. The amplon for the pEPSA5_nuc2(FL)-copL was created using the following primer pairs: ZRC188 and ZRC190; ZRC192 and ZRC193. Strains containing the pEPSA5_nuc2-copL and pEPSA5_nuc2(SS)-copL plasmids were PCR-verified using the ZRC188 and ZRC193 primers. The pEPSA5_nuc2(SS)-copL* was created using YCC cloning as outlined above using the additional following primers: CblSDM1for, CblSDM1rev, CblSDM2for, and CblSDM2rev.

**qRT-PCR**

RNA isolation and quantitative real-time PCR were performed as described previously with a few modifications (84). The WT (JMB1100) strain was cultured overnight in TSB in biological triplicates. Cells were pelleted by centrifugation and resuspended in PBS before diluting 1:100 into chemically defined media without and with 100 μM Cu. Cells were harvested 6 h post-inoculation (OD_600 nm) by centrifugation, treated with RNAProtect (Qiagen) for 10 min at room temperature, and stored at −80 °C. Pellets were thawed and washed twice with 0.5 ml of lysis buffer (50 mM RNase-free Tris, pH 8). Cells were lysed with 20 μg of DNase and 20 μg of lysozatinm
for 30 min at 37 °C. RNAs were isolated using TRIzol reagent (Ambion) as per the manufacturer’s protocol. DNA was digested with the TURBO DNA-free kit (Ambion, Life Technologies, Inc.) and RNA-quantified using a Nanodrop (ND-1000) spectrophotometer. cDNA libraries were constructed using isolated RNA as a template with a high capacity RNA-to-cDNA kit (Applied Biosystems). Power SYBR Green PCR Master Mix (Applied Biosystems) was used to perform qRT-PCR in an Applied Biosystems StepOnePlus thermocycler. Data were analyzed using the ΔΔCt method. CopARTfwd and CopARTrev primers were used to detect copA transcripts; CopBRTfwd and CopBRTrev primers were used to detect copB transcripts; CblRTfwd and CblRTrev primers were used to detect copL transcripts. Transcripts corresponding to the 16S rRNA gene were detected with 16sfwdRT and 16srevRT primers. RT primers were designed using the Primer Express 3.0 software from Applied Biosystems.

Transcriptional reporter assays

Strains containing the pCM11-derived reporters were grown overnight in TSB-Erm. The overnight cultures (≥16 h) were pelleted and resuspended in PBS. Washed cells were subcultured into 5 ml of fresh chemically defined media (1:100) in 30-ml culture tubes with and without copper. Culture aliquots were periodically removed (200 μl), and culture optical density (A_{590 nm}), and fluorescence was monitored using a PerkinElmer Life Sciences HTS 7000 Plus Bio Assay Reader. GFP was excited at 485 nm, and emission was read at 535 nm. Relative fluorescence units were normalized with respect to the culture optical density at each time point.

Cell fractionation

Overnight cultures were diluted to 0.1 OD (A_{600 nm}) in fresh TSB with Cm. Cultures were induced with 0 or 0.2% xylose and harvested by centrifugation. Cultures were resuspended and washed with PBS. Cells were lysed (PBS with 10 μg of DNase, 10 μg of lyso-staphin, protease, and phosphatase inhibitor mini tablets) at 37 °C for ~45 min. The cell fractionation protocol was followed as described previously with modifications (85). Cell lysates were spun for 10 min at (12,000 × g, 4 °C) to remove unbroken cells and debris. Supernatants (whole cell, crude lysates) were spun at 100,000 × g for 2 h at 4 °C in Beckman polyallomer centrifuge tubes using a Beckman Optima TLX ultracentrifuge and TLA 120.2 rotor. The resulting supernatant was saved as the cytoplasmic fraction. The pellet (crude membrane fraction) was resuspended in membrane buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 10% glycerol, 0.1% SDS) to solubilize membrane proteins and spun at 100,000 × g for 2 h at 4 °C to remove the detergent-insoluble material. Supernatants were saved as the membrane-soluble fractions.

Western blot analysis

A total of 40 μg of total protein per sample were separated using SDS-polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride membrane and incubated with mouse monoclonal anti-FLAG primary antibody (Sigma) (1:4000 dilution) and subsequently horseradish peroxidase–conjugated secondary antibody (Bio-Rad) (1:12,000 dilution). The blots were developed using chemiluminescent detection (Pierce) and scanned as TIFF images.

Protein concentration determination

Protein concentration was determined using a bicinchoninic acid assay modified for a 96-well plate (86) with BSA as a protein standard. Apo-saCopL(T) concentrations were also estimated spectrophotometrically using ε_{280} = 19,940 M⁻¹ cm⁻¹. Apo-bsCopL(T) concentrations were estimated spectrophotometrically using ε_{280} = 18,450 M⁻¹ cm⁻¹.

Recombinant saCopL(T) expression and purification

E. coli BL21 DE3 containing pGEX-6P-1_copL or pGEX-6P-1_copL*T was cultured overnight in LB-Amp and used to inoculate 1 liter of 2× YT media (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl, pH 7.0) to 0.1 OD (A_{600 nm}). Cultures were grown shaking at 30 °C, induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 0.8 OD (A_{600 nm}), and incubated for additional 4 h. Cultures were harvested by centrifugation, resuspended in cold PBS, and stored at ~80 °C. For lysis, thawed cell pastes were passed through a French press three times, and cell lysates were clarified by centrifugation (15,000 × g for 30 min at 4 °C). Cell extracts were loaded onto GSTrap 4B columns pre-equilibrated with binding buffer (PBS, pH 7.4) and then washed with 30 column volumes of binding buffer. The column was washed with PreScission cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5) and then incubated overnight at 4 °C with the PreScission Protease before eluting the recombinant protein with PreScission cleavage buffer. Fractions were analyzed for purity by SDS-PAGE. Protein was concentrated using YM-3 Centriplus Centrifugal Concentrators (Millipore). CD spectra were recorded using an AVIV model 62A (Aviv Associates, Lakewood, NJ) spectropolarimeter.

Copper binding and BCS competition assays

All biochemical assays were conducted under strict anaerobic conditions, either in a Coy anaerobic chamber (Grass Lake, MI) or using sealed cuvettes and Hamilton gas-tight syringes. Cu²⁺ binding was conducted as described previously (55). After purification, apo-saCopL(T) was transferred to the anaerobic chamber and buffer-exchanged (buffer R: 50 mM MOPS, 50 mM NaCl, pH 7.4) using a PD-10 column (GE Healthcare), concentrated, and allowed to incubate overnight to establish anaerobiosis. A 0.5 mM CuCl stock was prepared anaerobically in 10 mM HCl, 1 mM NaCl, and 1 mM ascorbic acid. For Cu¹⁺-binding assays, 12.5 μM saCopL(T) and 100 μM ascorbic acid in a total volume of 600 μl buffer R was placed in an anaerobic cuvette. The absorbance of the sample at 243 nm was determined using a Beckman-Coulter DU800 spectrophotometer after each 4-μl addition of 0.5 mM Cu¹⁺.

Competition assays with the Cu¹⁺-specific chelator BCS were conducted as described previously (55). Anaerobic solutions containing 0.5 mM BCS, 20 μM Cu¹⁺, 0.2 mM ascorbic acid, 50 mM NaCl, 50 mM MOPS, pH 7.4, were combined in a 700-μl sealed gas-tight cuvette. A series of additions of 80 μM saCopL(T) in buffer R were added to the cuvette. After each addition, and a subsequent 3-min incubation, the absorbance at
CopL and copper homeostasis

483 nm was recorded using a Beckman-Coulter DU800 spectrophotometer. For dilution correction, experiments were also performed in an identical manner using only buffer R. BCS forms a complex with Cu\(^{+2}\) in a 2:1 ratio Cu\(^{+2}\)/(BCS)\(_2\), which can be monitored by changes in absorbance at 483 nm with an overall association constant of \(\beta_1 = 10^{19.8} (47)\).

Recombinant expression and purification of bsCopL(T)

B. subtilis bsCopL(T) (NESG Target ID SR518) was cloned, expressed, and purified following the standard NSEG protocols (87). Briefly, the construct SR518-83-205-21.8, containing the 83–205 residue fragment with a C-terminal affinity purification tag LEHHHHHHH and an N-terminal methionine, was cloned into a pET21 (Novagen) derivative vector. E. coli BL21 (DE3) pMGK cells, a rare codon-enhanced strain, were transformed with SR518-83-205-21.8 and cultured in M9 minimal media (92) with [\(^{15}\)N]ammonium sulfate and/or [\(^{13}\)C]glucose as sole nitrogen and carbon sources. Uniformly \(^{13}\)C,\(^{15}\)N-labeled CopL (CopL-NC) was purified using an AKTApex (GE Healthcare)-based two-step protocol consisting of IMAC (HisTrap HP) and gel-filtration (HiLoad 26/60 Superdex 75) chromatography. The final yield of purified CopL-NC (>95% homogeneous by SDS-PAGE; 15.4 kDa by MALDI-TOF MS) was about 73 mg/liter. The final sample of CopL-NC for structure determination with NMR spectroscopy was prepared at a concentration of 0.4 mM in 10 mM Tris-HCl buffer solution, pH 7.5, containing 5% D\(_2\)O, 100 mM NaCl, 5 mM DTT, 0.02% NaN\(_3\), and protease inhibitors (Roche Applied Science). A uniformly \(^{15}\)N-labeled and 5% biosynthetically-directed fractionally \(^{13}\)C-labeled sample CopL-NC5 was prepared in the same manner using a mixture of 95% natural abundance and 5% \([^{13}\)C\]glucose as carbon source yielding the final concentration of 0.7 mM. NMR samples were prepared by exchange via spin columns into 10 mM Tris-HCl buffer at pH 7.5, containing 100 mM NaCl and 5% v/v D\(_2\)O, and concentrating to about 0.12 mM.

This material was used to produce anisotropic NMR samples, CopL-NC5-PEG and CopL-NC5-Pf1, by adding 4% (w/v) C\(_{12}\)E\(_5\)-PEG/hexanol at 1:1 molar ratio or 12.5 mg/ml Pf1 phage, respectively. For measurement of \(^{15}\)N-\(^1\)H RDCs, additional anisotropic NMR samples, CopL-NC5-PFEG and CopL-NC5-Pf1, were prepared from CopL-NC5 material by adding 4% (w/v) C\(_{12}\)E\(_5\)-PEG/hexanol at 1:1 molar ratio or 12.5 mg/ml Pf1 phage (ASLA Biotech), respectively.

For Cu-binding studies the same gene fragment (residues 83–205) was cloned into a pET15 (Novagen) derivative vector, pET15TEV_NESG (88), yielding the construct SR518-83-205-TEV. The corresponding N-terminal His\(_8\)-fusion protein was expressed and purified as described above, with uniform \(^{15}\)N-enrichment. The His\(_8\) purification tag was then removed by TEV protease cleavage, followed by IMAC (HisTrap HP) purification and gel-filtration chromatography, as described elsewhere (89). The resulting protein includes three extra residues (SHM) at the N-terminal end of the bsCopL sequence.

To prepare the holo-bsCopL-N protein sample, CuCl\(_2\) was prepared anaerobically in 10 mM HCl, 1 mM NaCl, and 1 mM ascorbic acid. 2 mol/mol eq of CuCl\(_2\) was added to 1.4 ml of uniformly \(^{15}\)N-enriched apo-bsCopL (0.11 mM), to prevent protein precipitation. The sample was then concentrated to ~0.1 ml and then diluted to 1.4 ml with 10 mM Tris-HCl buffer at pH 7.5, containing 100 mM NaCl and 1 mM ascorbic acid. This was repeated three times (8 eq of CuCl\(_2\)). Subsequently, the buffer of the sample was exchanged to remove any excess or adventitiously associated Cu\(^{+2}\). The final sample was adjusted to contain 5% D\(_2\)O in 500 \(\mu\)l (~0.25 mM). The BCS-treated sample holo-bsCopL-N-BCS was prepared by adding 1 mM anaerobically prepared BCS to the holo-bsCopL-N protein sample. All the above procedures were carried out at room temperature and under aerobic conditions using sealed NMR tubes (Wilmad Glass).

NMR spectroscopy and structure determination of bsCopL

NMR data acquisition was carried out on Bruker AVANCE 600, 700, 800, and 900 MHz as well as Varian INOVA 600 and 750 MHz spectrometers, all equipped with cryogenic inverse-detected triple resonance probes. All NMR experiments were carried out at 25 °C and are summarized in Table S3. 1D \(^{15}\)N relaxation HSQC spectra were processed with VNMRFIT 4.2A (Agilent Technologies) and integrated over the \(^1\)H range 10.0–8.5 ppm. \(^{15}\)N relaxation times \(t_1 = 740 \pm 30\) ms and \(t_2 = 85\) ± 4 ms were extracted by fitting exponential decay curves. Rotational correlation time \((\tau_c)\) was calculated using the “slow molecular tumbling” approximation of the complete relaxation Equation 1 (90),

\[
\tau_c = \frac{1}{4\pi\nu_N} \sqrt{\frac{6}{T_2} - 7} \quad \text{(Eq. 1)}
\]

where \(\nu_N\) represents the \(^{15}\)N resonance frequency. The resulting \(\tau_c\) value of 7 ns indicates that CopL-NC is monomeric in solution. This finding was confirmed by analytical gel filtration (GE Healthcare) followed by static light scattering (Wyatt Technology).

2D and 3D NMR spectra were processed with TopSpin (Bruker BioSpin), NMRPipe (91), or PROSA (92). Visualization and analysis of NMR spectra were performed with the programs CARA (93), XEASY (94), and Sparky (95). Chemical shifts of \(^1\)H spins were referenced to internal 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), whereas \(^{13}\)C\(_\text{a}\) and \(^{13}\)N\(_\text{a}\) chemical shift were referenced indirectly via their gyromagnetic ratios according to IUPAC recommendations (96). Sequence-specific assignments of backbone \((^{1}\text{H}, ^{15}\text{N}, ^{13}\text{C}a, ^{13}\text{C}\beta)\) and \(^{13}\text{C}\beta\) resonances were obtained in a semi-automated manner by analyzing HNCO, HN(CA)CO, CBCA(CO)NH, and HNCACB peak lists with the program PINE (97). Side-chain resonance assignment was performed interactively in CARA using HBHA(CO)NH, (H)CCH-COSY, and (H)CCH-TOCSY, and \(^{15}\text{N}, ^{13}\text{C}\)-resolved \(^1\text{H}, ^{1\text{H}}\text{NOESY}\) (mixing time 70 ms) spectra 2D \(^{1}\text{N}, ^{1}\text{H}\) constant-time HSQC of CopL-NC5 was used to obtain stereospecific resonance assignments of valine and leucine methyl groups (98). Protonation and tautomeric states of His side chains were determined from long-range 2D \(^{1}\text{N}, ^{1}\text{H}\) HSQC (99) revealing that His-129 is positively charged, whereas His-94, His-130, His-158, and His-194 are \(\text{N}^2\)-protonated. Chemical shifts, NOE peak lists, \(^{15}\text{N}, ^{1}\text{H}\) RDCs, and time-domain NOESY data were deposited in the BioMagResBank (100) under accession number 16942.
$^{1}$H–$^{1}$H upper distance constraints for structure determination were obtained from 3D $^{15}$N,$^{13}$C-resolved $^{1}$H, $^{1}$H-NOESY. Constraints for $\phi$ and $\psi$ dihedral angle constraints were derived from backbone chemical shifts using TALOS+ (101). Automated iterative NOE peak assignment and structure calculation was initially performed with AutoStructure 2.2.1 (102) and CYANA 3.0 (103) using dihedral angle constraints and stereospecific assignments of Val and Leu methyl groups. The resulting consensus NOE assignments were verified and corrected by interactive spectral analysis. Subsequently, calculations were performed iteratively with CYANA, with iterations used to verify and complete resonance assignments, refine NOESY peak lists, and optimize the distance calibration constants. Backbone $^{15}$N–$^{1}$H RDCs for two alignment media were determined from $J$-modulated spectra (104) and used as orientational constraints for the folded core at the later stages of refinement. The final 20 conformers out of 100 were further refined by restrained molecular dynamics in explicit water (105) using the program CNS 1.2 (106) with the PARAM19 force field.

The bsCopL structure was determined using 1725 NOE-based conformation-restricting distance restraints, together with 130 restraints on backbone dihedral angle $\phi$ and $\psi$ based on chemical shift data, and an additional 164 $^{15}$N–$^{1}$H residual dipolar coupling measurements. These data include 15.4 distance restraint per residue, and 7.1 long-range distance restraints per residue. Structural statistics and global quality factors were computed with PSVS 1.4 (107) and are summarized in Table S4. The goodness-of-fit between the final ensemble of conformers and the NOE peak lists (108) was calculated with RPF module of AutoStructure 2.2.1 (102). The NMR-derived structures are well-converged and have structure quality scores, including model versus data metrics for back-calculated NOEY spectra and knowledge-based packing scores, typical of very high quality, accurate NMR structures. The resulting coordinates of bsCopL were deposited in the Protein Data Bank with PDB code 2KY9.

The structural presentations of the protein were produced with the programs Molmol (109) and PyMOL (110). Residue conservation analysis was performed with the ConSurf server (111). A total of 399 nonredundant protein sequences with sequence identity between 35 and 95% were selected from the UniRef90 database after three iterations of CSI-BLAST search with $E$-value cutoff of 0.0001 by using $B$. subtilis fragment 83–205 as a query and aligned with MAFFT algorithm.

For Cu$^{1+}$ studies, the construct used did not include a C-terminal hexaHis tag and incorporated two additional N-terminal residues, compared with the construct used for structure determination. Sequence-specific assignments of backbone ($^{1}$H$^{N}$, $^{15}$N, $^{13}$CO, and $^{13}$C$^{\alpha}$) and $^{13}$C$^{\delta}$ resonances were obtained for Cu$^{1+}$-loaded holo-CopL by manual interactive analysis of HNCO, HNCA, HN(CO)CA, CBCA(CO)NH, and HNCA(CB) spectra. Chemical shift perturbations (absolute value) of $^{1}$H and $^{15}$N backbone amides, $\Delta \delta_{\text{NH}}$ were then calculated relative to the same construct of apo-CopL lacking the C-terminal hexaHis tag using Equation 2 (112),

$$|\Delta \delta_{\text{NH}}| = (\frac{\Delta \delta_{\text{N}}}{6})^{2}$$

where $\Delta \delta_{\text{H}}$ and $\Delta \delta_{\text{N}}$ are the chemical shift changes in the $^{1}$H$^{N}$ and $^{15}$N dimensions, respectively.

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