



The *copBL* operon protects *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 (*copB* and *copL*) encoded within the *Staphylococcus aureus* arginine-catabolic mobile element (ACME) that we hypothesized function in Cu homeostasis. Supporting this hypothesis, mutational inactivation of *copB* or *copL* increased copper sensitivity. We found that *copBL* are co-transcribed and that their transcription is increased during copper stress and in a strain in which *csrR*, encoding a Cu-responsive transcriptional repressor, was mutated. Moreover, *copB* displayed genetic synergy with *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 *S. aureus* clone USA300 is a membrane-bound and surface-exposed lipoprotein that binds up to four Cu⁺ ions. Solution NMR structures of the homologous *Bacillus subtilis* CopL, together with phylogenetic analysis and chemical-shift perturbation experiments, identified conserved residues potentially involved in Cu⁺ coordination. The solution NMR structure also revealed a novel Cu-binding architecture. Of note, a CopL variant with defective Cu⁺ binding did not protect against Cu toxicity *in vivo*. Taken together, these findings indicate that the ACME-encoded CopB and CopL proteins are additional factors utilized by the highly successful *S. aureus* USA300 clone to suppress copper toxicity.

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This article contains Figs. S1–S19, Tables S1–S4, and supporting Refs. 1–18.

The atomic coordinates and structure factors (code 2KY9) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

The NMR chemical shift data of this paper are available from the Biological Magnetic Resonance Data Bank under BMRB accession numbers 16942 and 27741.

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Because of its ability to cycle between its reduced (Cu¹⁺) and oxidized (Cu²⁺) states, copper (Cu)⁷ has catalytic roles in metalloenzymes such as dioxygen reductases (1), superoxide dismutases (2), laccases (3), and several proteins involved in denitrification (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 Cu¹⁺ 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

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

CopBL and copper homeostasis

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-deiminase 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 P_{1B-1}-type ATPase Cu exporter (31), and CopZ is a cytoplasmic Atx1-like Cu-binding chaperon (32). Some *S. aureus* strains have an additional predicted P_{1B-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

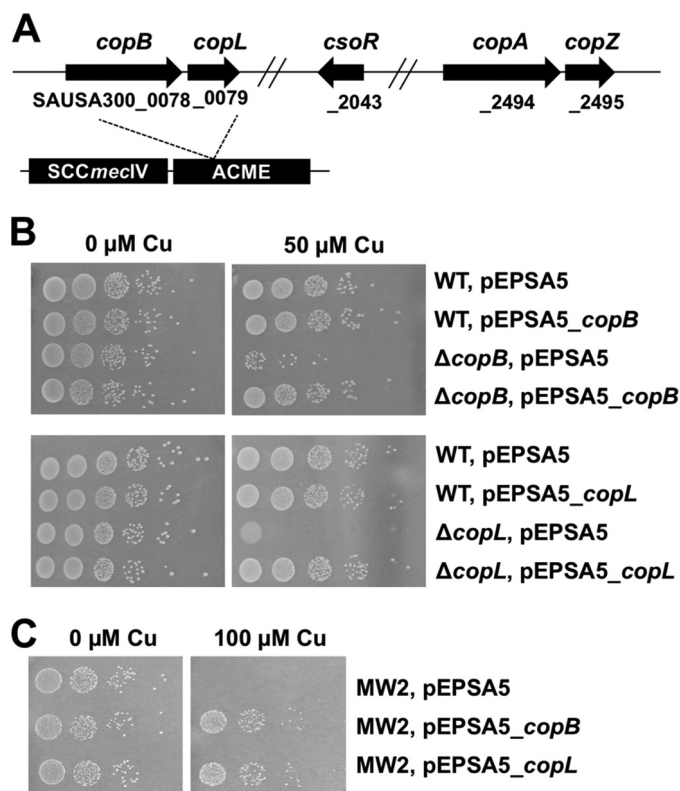


Figure 1. CopB and CopL protect against Cu toxicity in *S. aureus*. A, chromosomal location of genes involved in Cu homeostasis in *S. aureus* USA300_FPR3757. The *copB* and *copL* genes are located in the ACME, adjacent to the SCCmecIV genetic element. Locus tags are provided below the genes. B, *copA*, *copB*, and *copL* gene products are required to protect against Cu toxicity. Top, WT (JMB1100) and Δ*copB* (JMB7900) strains containing pEPSA5 or pEPSA5_ *copB* are shown. Bottom, WT and Δ*copL* (JMB7711) strains containing pEPSA5 or pEPSA5_ *copL* are shown. Strains were serially diluted and spot-plated on chemically defined media with or without 50 μM Cu. C, overexpression of *copB* or *copL* results in increased Cu resistance in the *S. aureus* USA400 strain MW2. The *S. aureus* USA400 clone MW2, which lacks genome-encoded *copB* and *copL*, containing pEPSA5, pEPSA5_ *copB*, or pEPSA5_ *copL* is shown. Strains were serially diluted and spot-plated on chemically defined media without or with 100 μM Cu.

most of the conserved structural elements of P_{1B}-ATPases (Fig. S1), including a phosphatase domain (TGES), a conserved CPX metal-binding sequence, and an ATP-binding domain (MXGDGXNDXP) (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 $\Delta copB$ and $\Delta copL$ 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, $\Delta copB$, and $\Delta copL$ strains were spot-plated on chemically defined media containing varying concentrations of Cu. The $\Delta copB$ and $\Delta copL$ 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 $\Delta copB$ mutants had decreased growth on solid media containing $>50 \mu\text{M}$ Cu, but no sensitivity was observed at lower ($10 \mu\text{M}$) Cu concentrations (Fig. 2A). The phenotypes associated with the *copA::Tn* $\Delta copB$ mutations were synergistic for Cu sensitivity (Fig. 2A). Similarly, the *copA::Tn* $\Delta copL$ double-mutant strain was more sensitive to Cu than the *copA::Tn* and $\Delta copL$ single mutants (Fig. 2B). The *copA::Tn* $\Delta copB$ $\Delta copL$ triple mutant was more sensitive to Cu than the *copA::Tn* $\Delta copB$ 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* $\Delta copB$ and *copZ::Tn* $\Delta copL$ strains displayed Cu sensitivity phenotypes similar to the $\Delta copB$ and $\Delta copL$ 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.

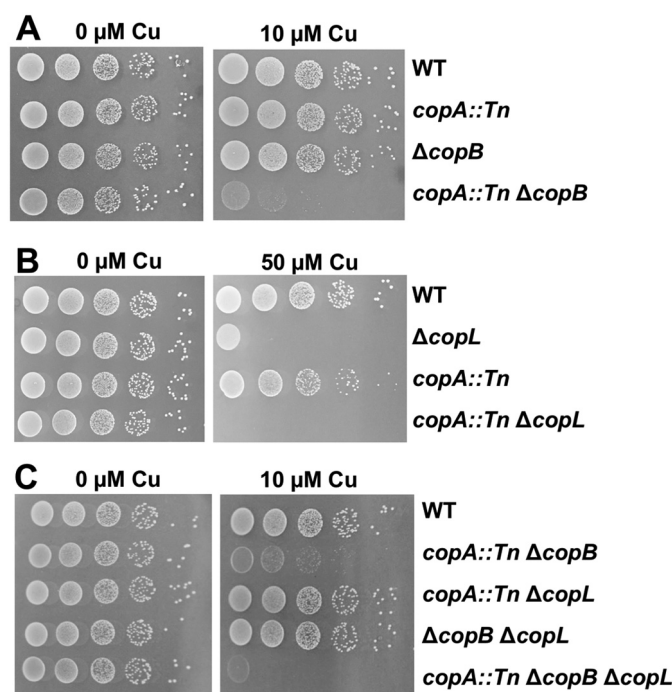


Figure 2. Intracellular Cu accumulation exacerbates the phenotypes of the *copB* and *copL* mutants and CopA, CopB, and CopL function independently. A, phenotypes associated with *copA* and *copB* mutations are synergistic. The WT (JMB1100), *copA::Tn* (JMB4084), $\Delta copB$ (JMB7900), and *copA::Tn* $\Delta copB$ (JMB8009) strains were serially diluted and spot-plated on chemically defined media without or with $50 \mu\text{M}$ Cu. B, phenotypes associated with *copB* and *copL* mutations are additive. The WT, $\Delta copL$ (JMB7711), *copA::Tn*, and *copA::Tn* $\Delta copL$ (JMB7803) strains were serially diluted and spot-plated on chemically defined media without or with $50 \mu\text{M}$ Cu. C, CopA, CopB, and CopL function independently to protect against Cu toxicity. The WT, *copA::Tn* $\Delta copB$, *copA::Tn* $\Delta copL$, $\Delta copB$ $\Delta copL$ (JMB7901), and *copA::Tn* $\Delta copB$ $\Delta copL$ (JMB7972) strains were serially diluted and spot-plated on chemically defined media without or with $10 \mu\text{M}$ Cu.

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 *copB* and *copL*, 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 Mn^{2+} , Fe^{2+} , Zn^{2+} , or Co^{2+} (Fig. 3D). In addition, the $\Delta copB$ and $\Delta copL$ mutant strains did not display increased

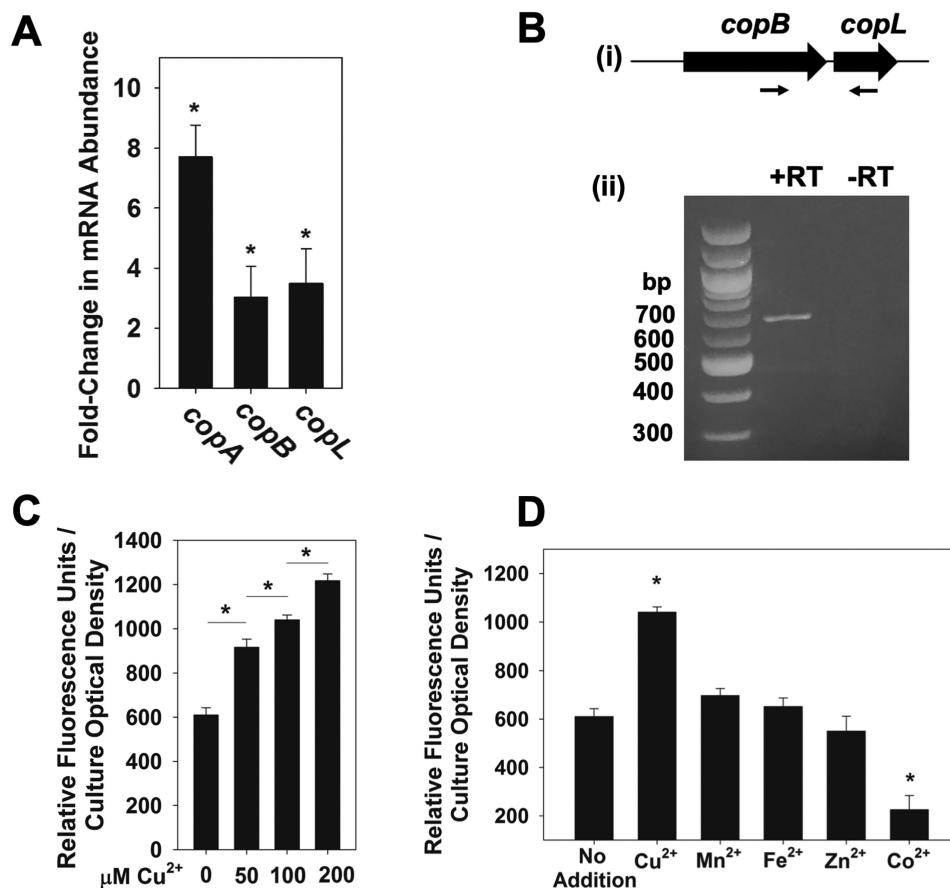


Figure 3. *copBL* operon is up-regulated under copper stress. *A*, *copA*, *copB*, and *copL* genes are induced by Cu. The *S. aureus* WT (JMB1100) was cultured in chemically defined media containing 0 or 100 μM Cu. RNA was isolated; cDNA was generated, and the abundance of the *copA*, *copB*, and *copL* transcripts was quantified using qPCR. Data show fold-induction of genes of interest upon addition of Cu with respect to no Cu addition. Data represent the average of biological triplicates with errors presented as standard deviations. *B*, *copB* and *copL* genes are co-transcribed. RNA was isolated from the WT grown in chemically defined medium with 100 μM Cu and cDNA libraries generated. *Panel i*, schematic showing the primer pair (ZRT21 and ZRT24) used to detect the *copBL* transcript; expected size: 643 bp. *Panel ii*, agarose gel electrophoresis was used to detect the *copBL* amplicon generated using cDNA libraries as template DNA. A reaction without reverse transcriptase ($-RT$) was included as a control for genomic DNA contamination. *C*, transcriptional activity of *copB* increases in synchrony with Cu concentration. Transcriptional activity of the *copB* reporter was monitored in the *S. aureus* USA300 WT grown in chemically defined media containing 0, 50, 100, or 200 μM Cu. *D*, transcriptional activity of *copB* is specific to copper stress. Transcriptional activity of *copB* was monitored in the WT grown in chemically defined media containing 100 μM Cu^{2+} , 100 μM Mn^{2+} , 100 μM Fe^{2+} , 100 μM Zn^{2+} , or 50 μM Co^{2+} . *C* and *D*, fluorescence data were standardized to culture optical density ($A_{590\text{nm}}$), and data represent the average of biological triplicates with errors presented as standard deviations. Student's *t* test were performed on the data, and * indicates $p \leq 0.05$.

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 (TACCNNNNNGGGGGTA) (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 (*xyIR0*) (pEPSA5_ copL-FLAG). The *copL*-FLAG allele genetically complemented the Cu sensitivity phenotype of the ΔcopL mutant strain, verifying the functionality of the fusion (data not shown). Cultures of the ΔcopL 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 detected in whole-cell extracts and membrane fractions, but not in cytosolic fractions (Fig. 5A). CopL-FLAG was also detected in noninduced samples, albeit at a much lower inten-

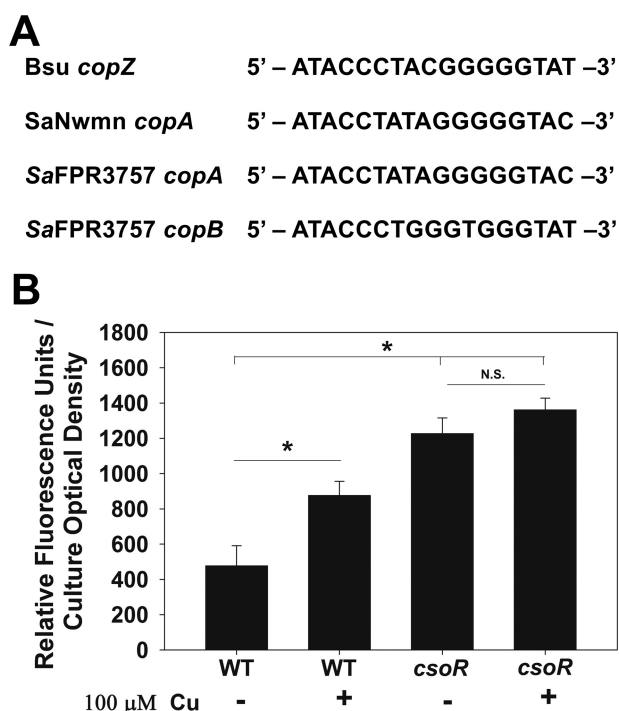


Figure 4. Transcription of the *copBL* operon is regulated by CsoR. A, proposed CsoR-binding sites are shown for the *S. aureus* USA300_FPR3757 *copA* and *copB* promoter regions, as well as the *B. subtilis* *copZ* and *S. aureus* Newman *copA* promoter regions. B, transcriptional activity of *copB* is increased in a *csoR* mutant. Transcriptional activity of *copB* was monitored in the WT (JMB1100) and *csoR::Tn* (JMB6807) strains grown in chemically defined media containing 0 or 100 μM Cu. Fluorescence data were standardized to culture optical density ($A_{590\text{ nm}}$). 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 $p \leq 0.05$, whereas N.S. denotes not significant.

sity, which is likely the result of leaky expression. CopL-FLAG was not detected in cells containing only empty vector.

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 LipoP 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.

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^{1+} binding was examined using ultraviolet (UV)-visible absorption spectroscopy. Titrating Cu^{1+} into apo-saCopL(T) resulted in gradual increases of the absorbance in the UV region ($A_{243\text{ nm}}$). The change of A_{243} was plotted as a function of the Cu^{1+} /saCopL(T) ratio (Fig. 6A). The formation of Cu^{1+} -saCopL(T) was linear and reached saturation after the addition of ~ 4 molar eq of Cu^{1+} .

Competition experiments were conducted to determine the Cu^{1+} -binding affinity of saCopL(T). Bathocuprione disulfonate (BCS) forms a complex with Cu^{1+} in a 2:1 ratio $\text{Cu}^{1+}/(\text{BCS})_2$ that has an absorption maximum at $A_{483\text{ nm}}$ (47). To determine the effective Cu^{1+} -binding affinity of saCopL(T), Cu^{1+} 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^{1+} -saCopL(T) was determined by monitoring the decrease in absorbance ($A_{483\text{ nm}}$) 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 β_2 , where β_2 is the association constant between copper and BCS. We used $\beta_2 = 10^{19.8}$ (47), and the resulting K_d value was $4.98 \pm 0.20 \times 10^{-18}$ 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 $\sim 63\%$ 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 β -barrels joined by two shared β -strands, and includes two short turns with a 3_{10} -helix geometry (Figs. 7A and Fig. S10). The β -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 β -barrel is composed of polypeptide segments from both DUF1541 domains. An unusual structural feature of

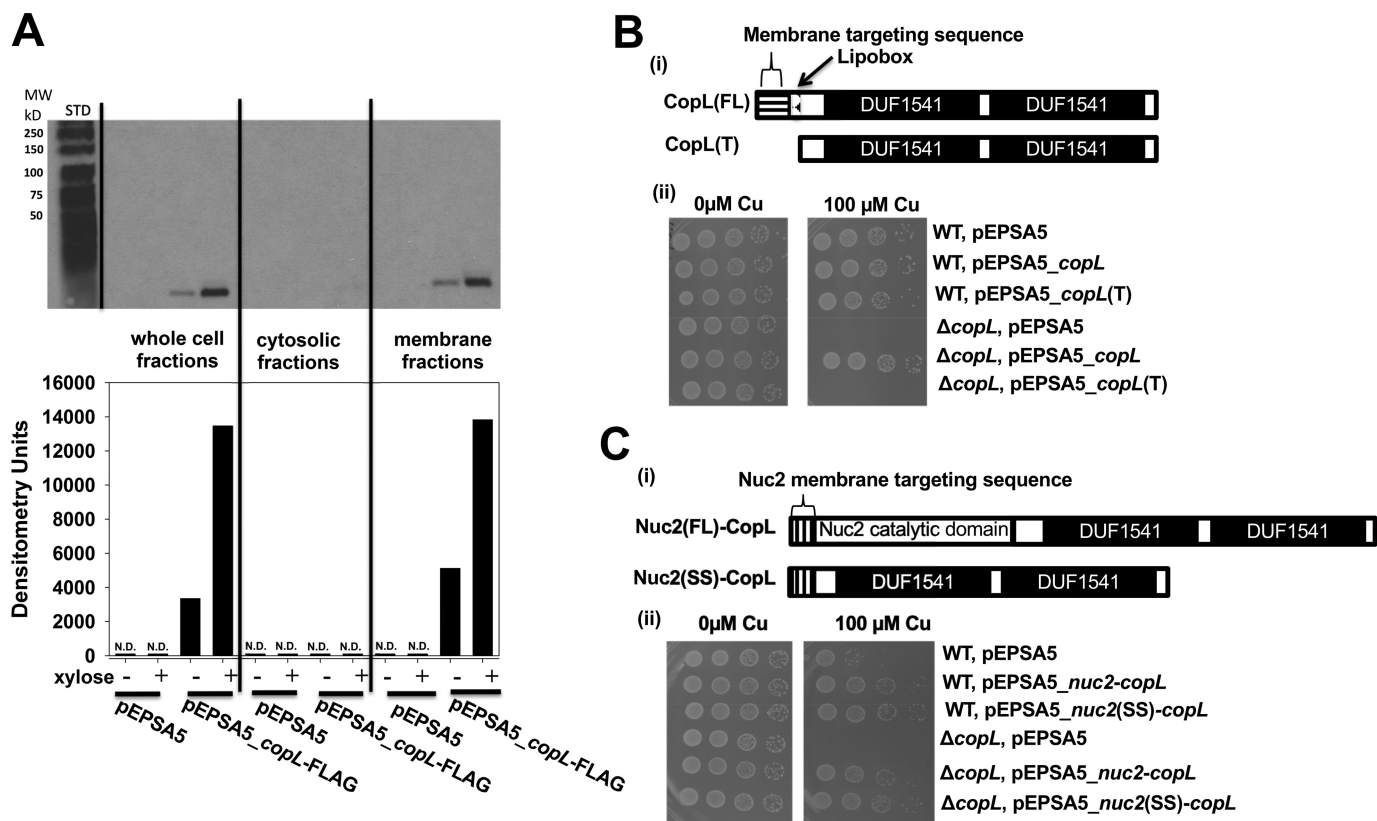


Figure 5. CopL is membrane-associated and surface-exposed. A, monitoring CopL abundance in *S. aureus* whole-cell extracts, cytosolic fractions, and membrane fractions. The $\Delta copL$ (JMB7711) mutant containing the pEPSA or pEPSA5_copL-FLAG was cultured in the absence and presence of 0.2% xylose 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_copL encoded saCopL and pEPSA5_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, pEPSA5_copL, or pEPSA5_copL(T) were serially diluted and spot-plated on chemically defined media without and with 100 μ 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 μ M Cu.

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 α –H α 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 β -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 π -cation interactions (50, 51). A search for conserved residues of this type identified residues His-94, Met-95, Met-98, His-129, His-130, His-158, Met-159, Met-162, and His-194 (Fig. 7A) as the most likely candidates. This is also consistent with the neutral ϵ 1-protonated tautomer states of His-94, His-130, His-158, and His-194 side chains (Fig. S14). Additional potential contributions to Cu-binding may come from the backbone carbonyl oxygen atoms of Lys-131 and

Lys-195, which are surface-exposed, and do not form intramolecular hydrogen bonds. These conserved residues do not appear to match any known type of protein copper center (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 ≥ 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 α atom r.m.s.d. between 1.3 and 3.8 Å. Here, the matching segments corresponded to only a single β -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 α atom r.m.s.d. was quite high at 5.0 and 5.3 Å, due to a significantly different relative orientation of the β -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.

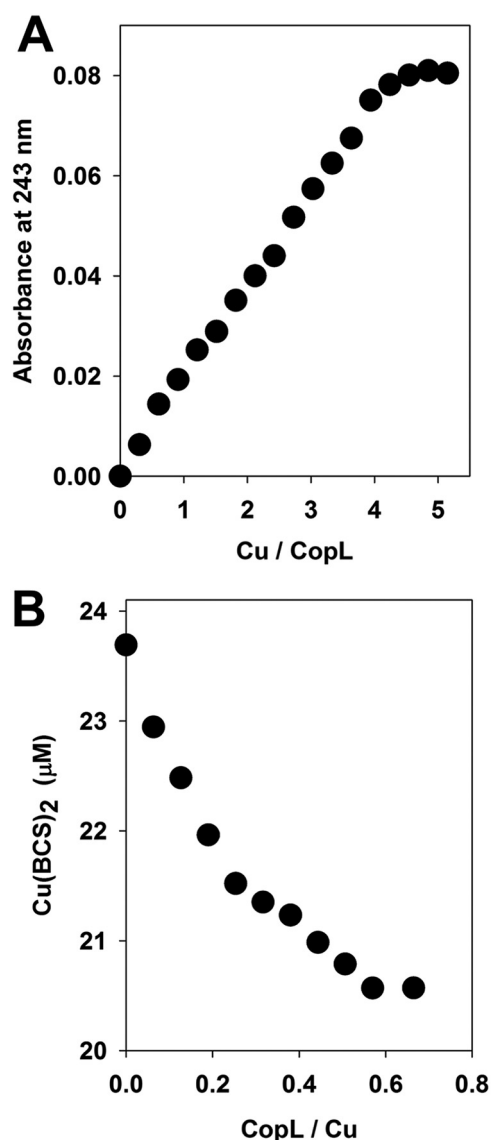


Figure 6. *S. aureus* CopL (saCopL) binds Cu¹⁺ *in vitro*. A, saCopL binds ~4 molar eq of Cu¹⁺. Apo-CopL (12.5 μM) was anaerobically titrated with Cu¹⁺, and binding was monitored by measuring absorbance at 243 nm using UV absorption spectroscopy. B, affinity of saCopL for Cu¹⁺ was monitored by BCS displacement. Solutions containing 0.5 mM BCS and 20 μM Cu¹⁺ were titrated with apo-saCopL(T), and BCS displacement was monitored by measuring absorbance at 483 nm using visible absorption spectroscopy.

Backbone ¹H, ¹⁵N chemical shift perturbation study of bsCopL interaction with Cu¹⁺

For Cu¹⁺-binding studies, the His₆ purification tag was removed by TEV protease cleavage. The resulting protein includes three extra residues (SHM) at the N-terminal end of the bsCopL sequence. The 2D ¹⁵N,¹H heteronuclear single-quantum correlation (HSQC) spectrum of bsCopL (residues 83–205) is shown in Fig. S15, with backbone and side-chain resonance assignments (deposited in the Biological Magnetic Resonance Bank (BMRB ID 16942)). Similar spectra were obtained upon addition of 1 mM EDTA (data not shown), demonstrating that under the conditions used for these NMR studies, no Cu or other related residual metal ions are associated with apo-bsCopL protein samples. The ¹⁵N,¹H HSQC spectra of the apo-bsCopL with the C-terminal hexa-His tag and apo-

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 Cu¹⁺ binding sites, 8 mol/mol eq of Cu¹⁺ were added to the apo-bsCopL protein, and the buffer was exchanged to remove adventitiously-associated Cu¹⁺ 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 ¹⁵N,¹H HSQC spectra of apo-bsCopL and holo-bsCopL (Fig. 8) revealed that Cu¹⁺ addition results in specific chemical shift perturbations. The numerous spectral changes induced upon copper addition unambiguously demonstrate Cu¹⁺ 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 β-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 Cu¹⁺ chelator BCS was added to the holo-bsCopL protein sample followed by recording a ¹⁵N,¹H 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, and 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 Cu¹⁺ ligation. The copL* allele was unable to correct the Cu-dependent growth defects of the *S. aureus* ΔcopL mutant (Fig. 10A).

We purified saCopL(T)* (*viz.* H70A, M71A, H134A, 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 Cu¹⁺ into saCopL(T)* and monitored spectral changes using UV absorption spectroscopy. Cu¹⁺ titration resulted in a linear increase in absorbance at 243 nm, and the absorbance plateaued after approximately 2 eq of Cu¹⁺ per saCopL(T)* (Fig. 10B). These data indicate that the saCopL(T)* variant binds less Cu¹⁺ than saCopL(T). We next titrated saCopL(T)* into a solution of BCS and Cu¹⁺ 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 Cu¹⁺ with a *K_d* of $1.35 \pm 0.17 \times 10^{-17}$ M, which is ~3-fold lower than the affinity of saCopL(T) for Cu¹⁺.

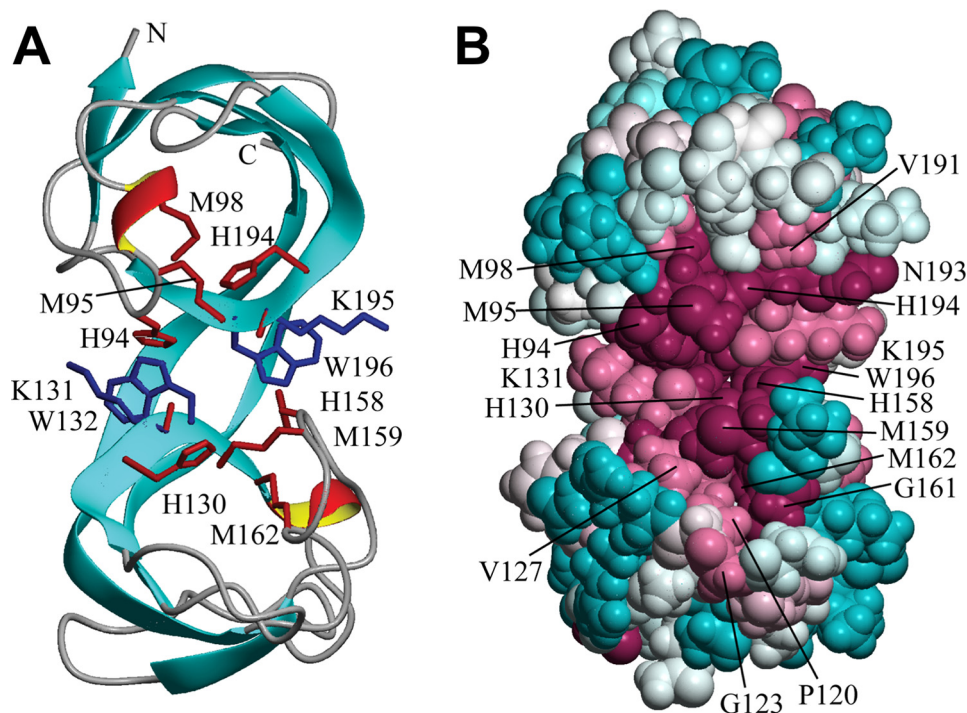


Figure 7. Solution NMR structure of *B. subtilis* CopL. A, ribbon diagram of the lowest-energy conformer of *B. subtilis* CopL: backbone of β -strands shown in cyan, short 3_1 -helical segments in red/yellow, and other polypeptide segments in gray; the terminal Lys-83 and Lys-205 are labeled as "N" and "C." Side chains of Lys-131–Trp-132 and Lys-195–Trp-196 residue pairs connected by unusual *cis*-peptide bonds are shown in blue. Moieties presumed to be involved in coordinating Cu, side chains of His-94, Met-95, Met-98, His-130, His-158, Met-159, Met-162, and His-194, as well as carbonyls of Lys-131 and Lys-195, are shown in red. B, space-filling representation of the same conformer showing the degree of residue conservation calculated with ConSurf. Conservation scores are arranged into nine groups with the corresponding colors ranging from cyan (most variable) to burgundy (most conserved). The numbering shown here is that of the UniProt entry BSU05790.

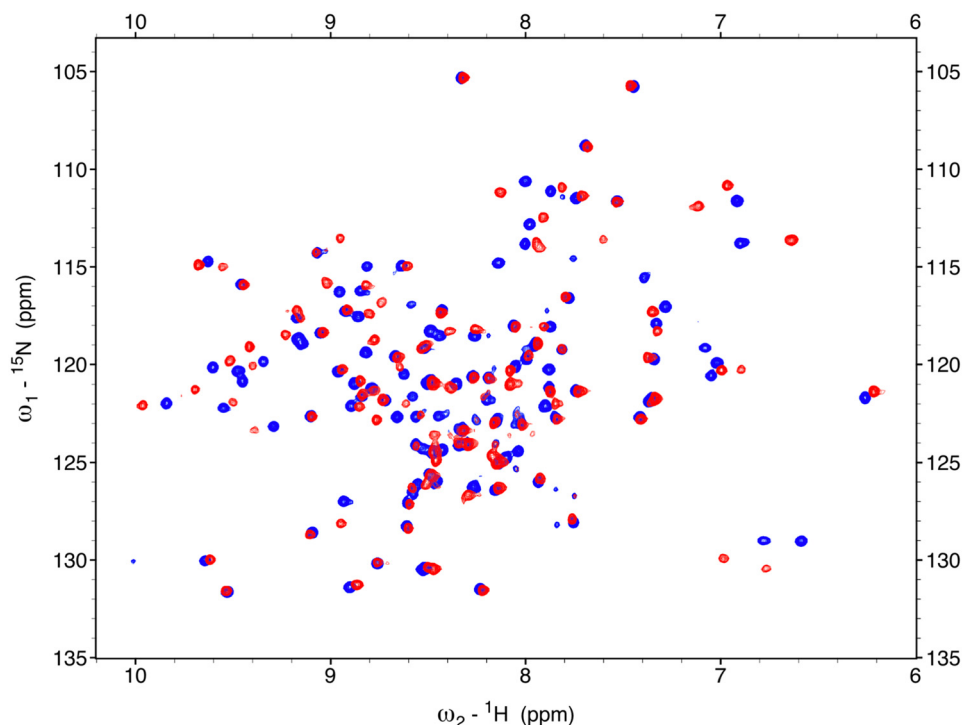


Figure 8. Chemical shift perturbations of *B. subtilis* CopL due to Cu^{2+} binding. Overlay of 600 MHz ^{15}N , ^1H HSQC spectra of 0.3 mM apo-bsCopL-N (blue) and 0.3 mM holo-bsCopL-N with 6 mol/mol eq of Cu^{2+} (red) is shown. The sequence-specific ^{15}N , ^1H HSQC resonance assignments for apo-bsCopL-N are shown in Fig. S15.

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

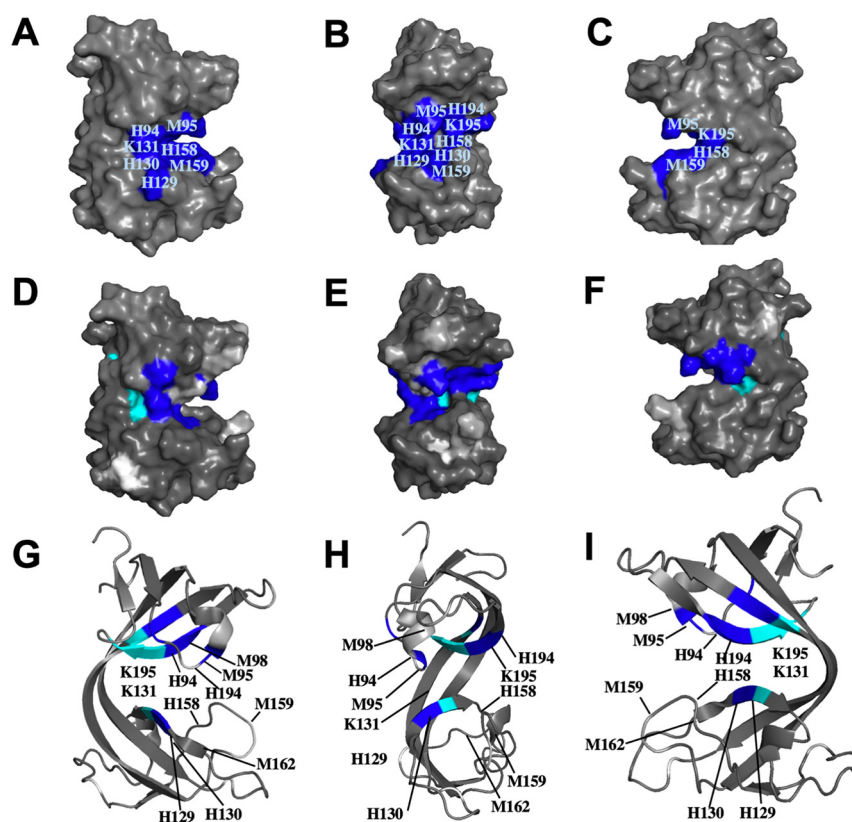


Figure 9. Cu^{2+} binding to *B. subtilis* CopL. A–C, surface molecular representation of *B. subtilis* CopL, in three orientations, showing the locations of conserved residues His-94, Met-95, Met-98 (buried), His-129, His-130, Lys-131, His-158, Met-159, Met-162 (buried), His-194, and Lys-195, presumed to be involved in coordinating Cu^{2+} . Only residues that can be seen on the surface of the 3D structure are labeled. D–F, surface representation of *B. subtilis* CopL, in same three orientations, showing chemical shift perturbations due to Cu^{2+} binding. Color code: *dark blue* ($\Delta\delta_{\text{NH}} > 0.25$ ppm, *viz.* Thr-92, Met-95, His-130, His-131, Met-179, Val-180, Asn-193, His-194, Lys-195); *cyan*, residues with H^{N} resonances that are broadened due to Cu^{2+} binding (*viz.* Trp-132, Tyr-178, Trp-196, Val-197, and Thr-198); *light gray*, residues with H^{N} resonances that are absent in the ^{15}N , ^1H HSQC spectrum of apo-CopL and not assigned (*viz.* Lys-83, His-94, Lys-96, Gly-97, Lys-160, Gly-161, and Ser-187); and *white* for proline residues that lack backbone amide protons (*viz.* Pro-120 and Pro-148). G–I, ribbon representation of *B. subtilis* CopL, in same three orientations, showing conformational perturbations due to Cu^{2+} binding using the same color code as in D–F. G–I, only the residues with H^{N} resonances that are affected by Cu^{2+} binding are labeled. The numbering shown here is that of the UniProt entry BSU05790.

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^{2+} with a dissociation constant of $4.98 \pm 0.20 \times 10^{-18}$ M. For comparison, the reported Cu^{2+} -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^{2+} K_d of $5.0 \pm 1.2 \times 10^{-15}$ 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^{2+} . This was achieved using Cu^{2+} -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^{2+} -binding networks of *S. aureus* and *B. subtilis* CopL, the basic residues in the Cu^{2+} -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^{2+} -bound form is similar to that of the apo-form because most distant residues do not exhibit significant perturbations of chemical shifts upon Cu^{2+} 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).

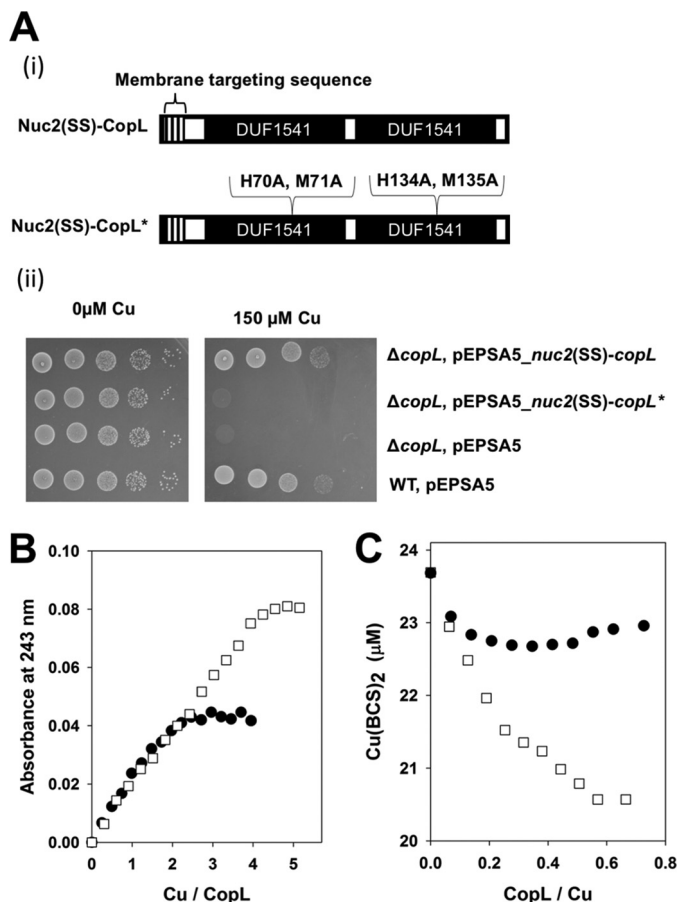


Figure 10. Effective Cu binding is necessary for CopL to protect against Cu toxicity. *A*, *copL** allele does not correct the Cu sensitivity phenotype of the *S. aureus* Δ*copL* mutant. *Panel i*, schematic of the *copL* and *copL** constructs utilized. *Panel ii*, WT and Δ*copL* strains harboring pEPSA5, pEPSA5_nuc2(SS)-*copL*, or pEPSA5_nuc2(SS)-*copL** were serially diluted and spot-plated on chemically defined media without and with 100 μM Cu. *B*, saCopL(T)* binds less Cu¹⁺ than saCopL(T). 12.5 μM apo-saCopL(T) or apo-saCopL(T)* were anaerobically titrated with Cu¹⁺. Binding was monitored by measuring absorbance at 243 nm using UV absorption spectroscopy. *C*, saCopL(T)* has a lower affinity for Cu¹⁺ than saCopL(T). Solutions containing 0.5 mM BCS and 20 μM Cu¹⁺ 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 Cu¹⁺-BCS complex. The data for the saCopL(T) are also presented in Fig. 6 and included here for comparison.

The 3D structure of bsCopL(T) contains unusual *cis* peptide bonds between two conserved residue pairs, Lys-131–Trp-132 and Lys-195–Trp-196. Nonproline *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 *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-*cis/trans* isomerases that have been shown to act on Xaa–Pro peptide bonds, but not on nonproline peptide bonds (64). Nonproline *cis* peptide bonds tend to be buried and not greatly accessible compared with Xaa–Pro *cis* peptide bonds, which suggests that they may form early during the folding process (65). They tend to be

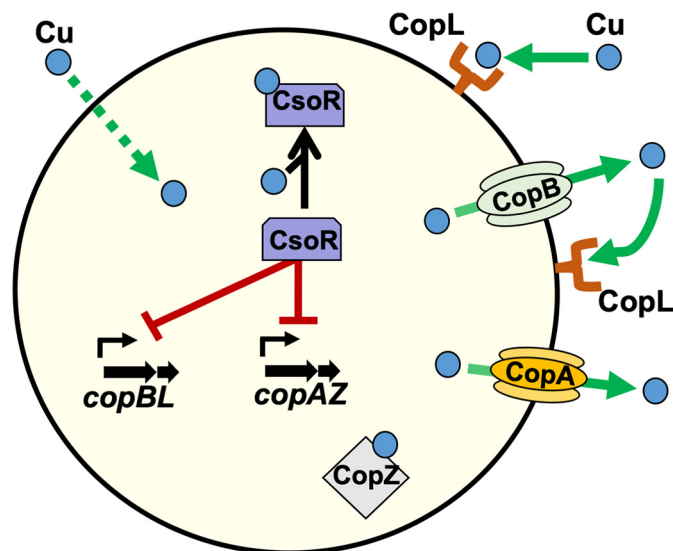


Figure 11. Working model for copper homeostasis in *S. aureus*. The CsoR transcriptional regulator binds intracellular copper, leading to derepression of the *copAZ* and *copBL* operons. CopA and CopB are transmembrane copper exporters. CopZ is a chaperon protein that binds copper and acts as a cytosolic 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.

observed near active sites indicating that they may sometimes be important for protein activity (61). The majority of the *cis* peptide bonds occur in regular secondary structures, more specifically in β-strands (65). In the case of bsCopL, the two non-Pro *cis* peptide bonds are located in β-strands β3 and β6, adjacent to the proposed Cu-binding residues His-130 and His-194, respectively. These *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 *cis* peptide bonds, such structure–function studies are difficult to carry out because mutations that prevent *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 (*arc*) improves survival in acidic conditions (20), and the ACME-encoded *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 *et al.* (54) reported that *S. aureus* strains lacking *copB* (called *copX* in their study) or *copL* are sensitive to growth in the presence of Cu. Interestingly, they were not able to genetically complement the *copL* mutant and were only able to complement in the *copB* mutant in one of the two media utilized. They found that the *copB* or *copL* mutants, but not the *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 *copAZ* and *copBL* operons. They also reported that apo-

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

Table 1
Strains and plasmids used in this study

Refs. 30, 74, 77, 79, 81, and 113 are cited in the table.

Staphylococcus aureus strains			
Strain name	Genotype/Description	Background	Source / Reference
RN4220	Restriction-negative	RN1	(74)
JMB2790	<i>copA</i> ::Tn(<i>ermB</i>) (SAUSA300_2494)	JE2	(113)
JMB3488	<i>csoR</i> ::Tn(<i>ermB</i>) (SAUSA300_2043)	JE2	(113)
JMB1100	USA300 wild-type strain	LAC	A.R. Horswill
JMB4084	<i>copA</i> ::Tn(<i>ermB</i>)	LAC	This study
JMB6807	<i>csoR</i> ::Tn(<i>ter</i>)	LAC	This study
JMB7900	Δ <i>copB</i> (SAUSA300_0078)	LAC	This study
JMB7711	Δ <i>copL</i> (SAUSA300_0079)	LAC	This study
JMB7901	Δ <i>copBL</i>	LAC	This study
JMB8009	Δ <i>copB</i> , <i>copA</i> ::Tn(<i>ermB</i>)	LAC	This study
JMB7803	Δ <i>copL</i> , <i>copA</i> ::Tn(<i>ermB</i>)	LAC	This study
JMB7972	Δ <i>copBL</i> , <i>copA</i> ::Tn(<i>ermB</i>)	LAC	This study
JMB1324	parent strain	MW2	P. Schlievert
JMB1325	parent strain	COL	A. Horswill
JMB1422	parent strain	Newman	E. Skaar
JMB6338	Δ <i>csoR</i> (NWMN_1991)	Newman	(30)
Other Strains			
Name	Relevant genotype/Description	Source / Reference	
<i>Escherichia coli</i> DH5a	Molecular cloning	Protein Express	
<i>E. coli</i> BL21 DE3	Protein expression and purification	New England Biolabs	
JMB7761	<i>Bacillus subtilis</i> 168 (parent)	V. Carabetta	
JMB7762	<i>Bacillus subtilis</i> 168 Δ <i>yhkK</i> ::ery (BSU05790)	V. Carabetta	
<i>Saccharomyces cerevisiae</i> FY2	<i>ura3-52</i> yeast recombination cloning	W. Belden	
Plasmids			
Plasmid name	Locus/Function	Source / Reference	
pJB38	Mutant construction	(77)	
pTET	pJB38 with <i>tetM</i>	(77)	
pJB38_Δ <i>copB</i>	Δ <i>copB</i> chromosomal deletion	This study	
pJB38_Δ <i>copL</i>	Δ <i>copL</i> chromosomal deletion	This study	
pJB38_Δ <i>copBL</i>	Δ <i>copBL</i> chromosomal deletion	This study	
pCM11	Promoterless <i>gfp</i> for transcriptional studies	(81)	
pEPSA5	Genetic complementation; XylIRO promoter	(79)	
pCM11_Δ <i>copB</i>	<i>copB</i> transcriptional reporter	This study	
pEPSA5_Δ <i>copB</i>	<i>copB</i> complementation	This study	
pEPSA5_Δ <i>copL</i>	<i>copL</i> complementation	This study	
pEPSA5_Δ <i>copL</i> (T)	truncated <i>copL</i> complementation	This study	
pEPSA5_Δ <i>copL</i> -FLAG	<i>copL</i> containing a C-terminal FLAG tag	This study	
pEPSA5_Δ <i>nuc2</i> (FL)- <i>copL</i>	<i>nuc2</i> fused to <i>copL</i>	This study	
pEPSA5_Δ <i>copL</i> (T)-FLAG	truncated <i>copL</i> containing a C-terminal FLAG tag	This study	
pEPSA5_Δ <i>nuc2</i> (SS)- <i>copL</i>	<i>nuc2</i> signal sequence fused to truncated <i>copL</i>	This study	
pEPSA5_Δ <i>nuc2</i> (SS)- <i>copL</i> *	<i>nuc2</i> signal sequence fused to truncated <i>copL</i> *	This study	
pGEX-6P-1	gene expression for protein purification	GE Healthcare	
pGEX-6P-1_Δ <i>copL</i>	CopL expression	This study	
pGEX-6P-1_Δ <i>copL</i> *	CopL* expression	This study	

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

CopBL and copper homeostasis

of $(\text{NH}_4)_2\text{SO}_4$, 4.5 g of KH_2PO_4 , 10.5 g of K_2HPO_4 , 110 mM NaCl, 30 mM KCl, 50 μg of nicotinic acid, 50 μg of pantothenic 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 CuSO_4 ; 50–200 μM $\text{Fe}_2(\text{NH}_4)_2(\text{SO}_4)_2$; 5–200 μM CoCl_2 ; 10–300 μM MnSO_4 ; 10–300 μM ZnSO_4 . When appropriate, antibiotics were added at the following concentrations: 150 $\mu\text{g ml}^{-1}$ ampicillin (Amp), 6 or 30 $\mu\text{g ml}^{-1}$ chloramphenicol (Cm) (defined or complex media, respectively), 10 $\mu\text{g ml}^{-1}$ erythromycin, 3 $\mu\text{g ml}^{-1}$ 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* DH5 α and transformed into electrocompetent *S. aureus* RN4220 using a standard protocol (74). Phage α 80 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 SalI and ligated into similarly digested pJB38 (77). The recombinant vectors were transformed into chemically competent *E. coli* DH5 α . PCR was used to screen for *E. coli* colonies harboring the recombinant plasmids using ZRC196 and ZRC201 primers for pJB38_ ΔcopB , ZRC196 and ZRC169 for pJB38_ ΔcopL , and ZRC196 and ZRC169 for pJB38_ $\Delta\text{copBcopL}$. 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 *copR::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; *copR::Tn(ermB)* and *copR::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 SalI and ligated into similarly digested pEPSA5 to yield pEPSA5_*copB*, pEPSA5_*copL*, and pEPSA5_*copL*(T) vectors. Plasmid-containing strains were PCR-verified, using the pEPSA5upveri and ZRC141 (pEPSA5_*copB*) or the pEPSA5upveri and ZRC150 (pEPSA5_*copL* or pEPSA5_*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 NheI 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 XhoI 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 NheI. The amplicon for the pEPSA5_*nuc2*(FL)-*copL* was created using the following primer pairs: ZRC188 and ZRC189; ZRC191 and ZRC193. The amplicon for the pEPSA5_*nuc2*(SS)-*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 ($\text{OD} \sim 1$, $A_{600 \text{ nm}}$) 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 lysostaphin

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 $\Delta\Delta Ct$ 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 16sfdwRT 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 μ l), and culture optical density ($A_{590\text{ nm}}$), 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 ($A_{600\text{ nm}}$) in fresh TSB with Cm. Cultures were induced with 0 or 0.2% xylose at 1 OD ($A_{600\text{ nm}}$), incubated for an additional 2 h, and harvested by centrifugation. Cultures were resuspended and washed with PBS. Cells were lysed (PBS with 10 μ g of DNase, 10 μ g 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 \times g, 4 °C) to remove unbroken cells and debris. Supernatants (whole cell, crude lysates) were spun at 100,000 \times 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 \times 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 μ g 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 $\epsilon_{280} = 19,940\text{ M}^{-1}\text{ cm}^{-1}$. Apo-bsCopL(T) concentrations were estimated spectrophotometrically using $\epsilon_{280} = 18,450\text{ M}^{-1}\text{ cm}^{-1}$.

Recombinant saCopL(T) expression and purification

E. coli BL21 DE3 containing pGEX-6P-1_{copL} or pGEX-6P-1_{copL*} was cultured overnight in LB-Amp and used to inoculate 1 liter of 2 \times YT media (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl, pH 7.0) to 0.1 OD ($A_{600\text{ nm}}$). Cultures were grown shaking at 30 °C, induced with 1 mM isopropyl 1-thio- β -D-galactopyranoside at 0.8 OD ($A_{600\text{ nm}}$), 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 \times 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 Centrplus 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. Cu¹⁺ 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 M NaCl, and 1 mM ascorbic acid. For Cu¹⁺-binding assays, 12.5 μ M saCopL(T) and 100 μ M ascorbic acid in a total volume of 600 μ l 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- μ l 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 μ M Cu¹⁺, 0.2 mM ascorbic acid, 50 mM NaCl, 50 mM MOPS, pH 7.4, were combined in a 700- μ l sealed gas-tight cuvette. A series of additions of 80 μ M saCopL(T) in buffer R were added to the cuvette. After each addition, and a subsequent 3-min incubation, the absorbance at

CopBL 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^{1+} in a 2:1 ratio $\text{Cu}^{1+}/(\text{BCS})_2$, which can be monitored by changes in absorbance at 483 nm with an overall association constant of $\beta_2 = 10^{19.8}$ (47).

Recombinant expression and purification of bsCopL(T)

B. subtilis bsCopL(T) (NESG Target ID SR518) was cloned, expressed, and purified following the standard NESG protocols (87). Briefly, the construct SR518-83-205-21.8, containing the 83–205-residue fragment with a C-terminal affinity purification tag LEHHHHHH 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 MJ9 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 AKTApur (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_2O , 100 mM NaCl, 5 mM DTT, 0.02% NaN_3 , and proteinase 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 a 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_2O , 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 - ^1H RDCs, additional anisotropic NMR samples, CopL-NC5-PEG 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 His₆-fusion protein was expressed and purified as described above, with uniform ^{15}N -enrichment. The His₆ 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 was prepared anaerobically in 10 mM HCl, 1 M NaCl, and 1 mM ascorbic acid. 2 mol/mol eq of CuCl 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 Cu^{1+}). Subsequently, the buffer of the sample was exchanged to remove any excess or adventitiously associated Cu^{1+} . The final sample was adjusted to contain 5% D_2O in 500 μl (~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 VNMRJ 4.2A (Agilent Technologies) and integrated over the ^1H range 10.0–8.5 ppm. ^{15}N relaxation times $t_1 = 740 \pm 30$ ms and $t_2 = 85 \pm 9$ ms were extracted by fitting exponential decay curves. Rotational correlation time (τ_c) was calculated using the “slow molecular tumbling” approximation of the complete relaxation Equation 1 (90),

$$\tau_c = \frac{1}{4\pi\nu_N} \sqrt{6\frac{T_1}{T_2} - 7} \quad (\text{Eq. 1})$$

where ν_N represents the ^{15}N resonance frequency. The resulting τ_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 CARRA (93), XEASY (94), and Sparky (95). Chemical shifts of ^1H spins were referenced to internal 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), whereas ^{13}C and ^{15}N chemical shift were referenced indirectly via their gyromagnetic ratios according to IUPAC recommendations (96). Sequence-specific assignments of backbone ($^1\text{H}^N$, ^{15}N , ^{13}CO , and $^{13}\text{C}^\alpha$) 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 CARRA using HBHA(CO)NH, (H)CCH-COSY, and (H)CCH-TOCSY, and ^{15}N , ^{13}C -resolved ^1H , ^1H -NOESY (mixing time 70 ms) spectra 2D ^{13}C , ^1H 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 ^{15}N , ^1H HSQC (99) revealing that His-129 is positively charged, whereas His-94, His-130, His-158, and His-194 are $\text{N}^{\epsilon 2}$ -protonated. Chemical shifts, NOE peak lists, ^{15}N - ^1H RDCs, and time-domain NOESY data were deposited in the BioMagResBank (100) under accession number 16942.

^1H - ^1H upper distance constraints for structure determination were obtained from 3D ^{15}N , ^{13}C -resolved ^1H , ^1H -NOESY. Constraints for φ and ψ 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 - ^1H 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 ϕ and ψ based on chemical shift data, and an additional 164 ^{15}N - ^1H residual dipolar coupling measurements. These data include 15.4 distance restraints 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 NOESY 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 NOESY 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 ($^1\text{H}^{\text{N}}$, ^{15}N , ^{13}CO , and $^{13}\text{C}^{\alpha}$) and $^{13}\text{C}^{\beta}$ resonances were obtained for Cu^{1+} -loaded holo-CopL by manual interactive analysis of HNCO, HNCA, HN(CO)CA, CBCA(CO)NH, and HNCACB spectra. Chemical shift perturbations (absolute value) of ^1H 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),

$$|\Delta\delta_{\text{HN}}| = ((\Delta\delta_{\text{H}})^2 + (\Delta\delta_{\text{N}}/6)^2)^{1/2} \quad (\text{Eq. 2})$$

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

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References

- Sousa, F. L., Alves, R. J., Ribeiro, M. A., Pereira-Leal, J. B., Teixeira, M., and Pereira, M. M. (2012) The superfamily of heme-copper oxygen reductases: types and evolutionary considerations. *Biochim. Biophys. Acta* **1817**, 629–637 [CrossRef Medline](#)
- Osman, D., Patterson, C. J., Bailey, K., Fisher, K., Robinson, N. J., Rigby, S. E., and Cavet, J. S. (2013) The copper supply pathway to a Salmonella Cu,Zn-superoxide dismutase (SodCII) involves P(1B)-type ATPase copper efflux and periplasmic CueP. *Mol. Microbiol.* **87**, 466–477 [CrossRef Medline](#)
- Claus, H. (2003) Laccases and their occurrence in prokaryotes. *Arch. Microbiol.* **179**, 145–150 [CrossRef Medline](#)
- Tavares, P., Pereira, A. S., Moura, J. J., and Moura, I. (2006) Metalloenzymes of the denitrification pathway. *J. Inorg. Biochem.* **100**, 2087–2100 [CrossRef Medline](#)
- Rutherford, J. C., and Bird, A. J. (2004) Metal-responsive transcription factors that regulate iron, zinc, and copper homeostasis in eukaryotic cells. *Eukaryot. Cell* **3**, 1–13 [CrossRef Medline](#)
- Macomber, L., and Imlay, J. A. (2009) The iron–sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 8344–8349 [CrossRef Medline](#)
- Chillappagari, S., Seubert, A., Trip, H., Kuipers, O. P., Marahiel, M. A., and Miethke, M. (2010) Copper stress affects iron homeostasis by destabilizing iron–sulfur cluster formation in *Bacillus subtilis*. *J. Bacteriol.* **192**, 2512–2524 [CrossRef Medline](#)
- Fung, D. K., Lau, W. Y., Chan, W. T., and Yan, A. (2013) Copper efflux is induced during anaerobic amino acid limitation in *Escherichia coli* to protect iron–sulfur cluster enzymes and biogenesis. *J. Bacteriol.* **195**, 4556–4568 [CrossRef Medline](#)
- Tan, G., Cheng, Z., Pang, Y., Landry, A. P., Li, J., Lu, J., and Ding, H. (2014) Copper binding in IscA inhibits iron-sulphur cluster assembly in *Escherichia coli*. *Mol. Microbiol.* **93**, 629–644 [CrossRef Medline](#)

10. Gunther, M. R., Hanna, P. M., Mason, R. P., and Cohen, M. S. (1995) Hydroxyl radical formation from cuprous ion and hydrogen peroxide: a spin-trapping study. *Arch. Biochem. Biophys.* **316**, 515–522 [CrossRef Medline](#)
11. Mikolay, A., Huggett, S., Tikana, L., Grass, G., Braun, J., and Nies, D. H. (2010) Survival of bacteria on metallic copper surfaces in a hospital trial. *Appl. Microbiol. Biotechnol.* **87**, 1875–1879 [CrossRef Medline](#)
12. Schmidt, M. G., Attaway, H. H., Sharpe, P. A., John, J., Jr., Sepkowitz, K. A., Morgan, A., Fairey, S. E., Singh, S., Steed, L. L., Cantey, J. R., Freeman, K. D., Michels, H. T., and Salgado, C. D. (2012) Sustained reduction of microbial burden on common hospital surfaces through introduction of copper. *J. Clin. Microbiol.* **50**, 2217–2223 [CrossRef Medline](#)
13. White, C., Lee, J., Kambe, T., Fritsche, K., and Petris, M. J. (2009) A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. *J. Biol. Chem.* **284**, 33949–33956 [CrossRef Medline](#)
14. Achard, M. E., Stafford, S. L., Bokil, N. J., Chartres, J., Bernhardt, P. V., Schembri, M. A., Sweet, M. J., and McEwan, A. G. (2012) Copper redistribution in murine macrophages in response to *Salmonella* infection. *Biochem. J.* **444**, 51–57 [CrossRef Medline](#)
15. Klevens, R. M., Morrison, M. A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L. H., Lynfield, R., Dumyati, G., Townes, J. M., Craig, A. S., Zell, E. R., Fosheim, G. E., McDougal, L. K., Carey, R. B., et al. (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA* **298**, 1763–1771 [CrossRef Medline](#)
16. Otto, M. (2010) Basis of virulence in community-associated methicillin-resistant *Staphylococcus aureus*. *Annu. Rev. Microbiol.* **64**, 143–162 [CrossRef Medline](#)
17. Tenover, F. C., McDougal, L. K., Goering, R. V., Killgore, G., Projan, S. J., Patel, J. B., and Dunman, P. M. (2006) Characterization of a strain of community-associated methicillin-resistant *Staphylococcus aureus* widely disseminated in the United States. *J. Clin. Microbiol.* **44**, 108–118 [CrossRef Medline](#)
18. Talan, D. A., Krishnadasan, A., Gorwitz, R. J., Fosheim, G. E., Limbago, B., Albrecht, V., Moran, G. J., and EMERGENCY ID Net Study Group. (2011) Comparison of *Staphylococcus aureus* from skin and soft-tissue infections in US emergency department patients, 2004 and 2008. *Clin. Infect. Dis.* **53**, 144–149 [CrossRef Medline](#)
19. Diep, B. A., Gill, S. R., Chang, R. F., Phan, T. H., Chen, J. H., Davidson, M. G., Lin, F., Lin, J., Carleton, H. A., Mongodin, E. F., Sensabaugh, G. F., and Perdreau-Remington, F. (2006) Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* **367**, 731–739 [CrossRef Medline](#)
20. Thurlow, L. R., Joshi, G. S., Clark, J. R., Spontak, J. S., Neely, C. J., Maile, R., and Richardson, A. R. (2013) Functional modularity of the arginine-catabolic mobile element contributes to the success of USA300 methicillin-resistant *Staphylococcus aureus*. *Cell Host Microbe* **13**, 100–107 [CrossRef Medline](#)
21. Joshi, G. S., Spontak, J. S., Klapper, D. G., and Richardson, A. R. (2011) Arginine-catabolic mobile element encoded speG abrogates the unique hypersensitivity of *Staphylococcus aureus* to exogenous polyamines. *Mol. Microbiol.* **82**, 9–20 [CrossRef Medline](#)
22. Argüello, J. M., González-Guerrero, M., and Raimunda, D. (2011) Bacterial transition metal P(1B)-ATPases: transport mechanism and roles in virulence. *Biochemistry* **50**, 9940–9949 [CrossRef Medline](#)
23. Argüello, J. M., Patel, S. J., and Quintana, J. (2016) Bacterial Cu⁺-ATPases: models for molecular structure–function studies. *Metallomics* **8**, 906–914 [CrossRef Medline](#)
24. Singleton, C., and Le Brun, N. E. (2007) Atx1-like chaperones and their cognate P-type ATPases: copper-binding and transfer. *Biometals* **20**, 275–289 [CrossRef Medline](#)
25. Ding, C., Festa, R. A., Chen, Y. L., Espart, A., Palacios, Ò., Espín, J., Capdevila, M., Atrian, S., Heitman, J., and Thiele, D. J. (2013) *Cryptococcus neoformans* copper detoxification machinery is critical for fungal virulence. *Cell Host Microbe* **13**, 265–276 [CrossRef Medline](#)
26. Kay, K. L., Hamilton, C. J., and Le Brun, N. E. (2016) Mass spectrometry of *B. subtilis* CopZ: Cu(i)-binding and interactions with bacillithiol. *Metallomics* **8**, 709–719 [CrossRef Medline](#)
27. Osterberg, R., Ligaarden, R., and Persson, D. (1979) Copper(I) complexes of penicillamine and glutathione. *J. Inorg. Biochem.* **10**, 341–355 [CrossRef Medline](#)
28. Rajkarnikar, A., Strankman, A., Duran, S., Vargas, D., Roberts, A. A., Barretto, K., Upton, H., Hamilton, C. J., and Rawat, M. (2013) Analysis of mutants disrupted in bacillithiol metabolism in *Staphylococcus aureus*. *Biochem. Biophys. Res. Commun.* **436**, 128–133 [CrossRef Medline](#)
29. Helbig, K., Bleuel, C., Krauss, G. J., and Nies, D. H. (2008) Glutathione and transition-metal homeostasis in *Escherichia coli*. *J. Bacteriol.* **190**, 5431–5438 [CrossRef Medline](#)
30. Grossoehme, N., Kehl-Fie, T. E., Ma, Z., Adams, K. W., Cowart, D. M., Scott, R. A., Skaar, E. P., and Giedroc, D. P. (2011) Control of copper resistance and inorganic sulfur metabolism by paralogous regulators in *Staphylococcus aureus*. *J. Biol. Chem.* **286**, 13522–13531 [CrossRef Medline](#)
31. Sittthisak, S., Knutsson, L., Webb, J. W., and Jayaswal, R. K. (2007) Molecular characterization of the copper transport system in *Staphylococcus aureus*. *Microbiology* **153**, 4274–4283 [CrossRef Medline](#)
32. Radford, D. S., Kihlken, M. A., Borrelly, G. P., Harwood, C. R., Le Brun, N. E., and Cavet, J. S. (2003) CopZ from *Bacillus subtilis* interacts *in vivo* with a copper exporting CPx-type ATPase CopA. *FEMS Microbiol. Lett.* **220**, 105–112 [CrossRef Medline](#)
33. Baker, J., Sengupta, M., Jayaswal, R. K., and Morrissey, J. A. (2011) The *Staphylococcus aureus* CsoR regulates both chromosomal and plasmid-encoded copper resistance mechanisms. *Environ. Microbiol.* **13**, 2495–2507 [CrossRef Medline](#)
34. Zapotoczna, M., Riboldi, G. P., Moustafa, A. M., Dickson, E., Narechania, A., Morrissey, J. A., Planet, P. J., Holden, M. T. G., Waldron, K. J., and Geoghegan, J. A. (2018) Mobile-genetic-element-encoded hypertolerance to copper protects *Staphylococcus aureus* from killing by host phagocytes. *MBio* **9**, e00550 [Medline](#)
35. Sittthisak, S., Howieson, K., Amezola, C., and Jayaswal, R. K. (2005) Characterization of a multicopper oxidase gene from *Staphylococcus aureus*. *Appl. Environ. Microbiol.* **71**, 5650–5653 [CrossRef Medline](#)
36. Argüello, J. M., Eren, E., and González-Guerrero, M. (2007) The structure and function of heavy metal transport P1B-ATPases. *Biometals* **20**, 233–248 [CrossRef Medline](#)
37. Odermatt, A., Suter, H., Krapf, R., and Solioz, M. (1993) Primary structure of two P-type ATPases involved in copper homeostasis in *Enterococcus hirae*. *J. Biol. Chem.* **268**, 12775–12779 [Medline](#)
38. Marchler-Bauer, A., Derbyshire, M. K., Gonzales, N. R., Lu, S., Chitsaz, F., Geer, L. Y., Geer, R. C., He, J., Gwadz, M., Hurwitz, D. I., Lanczycki, C. J., Lu, F., Marchler, G. H., Song, J. S., Thanki, N., et al. (2015) CDD: NCBI's conserved domain database. *Nucleic Acids Res.* **43**, D222–D226 [CrossRef Medline](#)
39. Li, M., Diep, B. A., Villaruz, A. E., Braughton, K. R., Jiang, X., DeLeo, F. R., Chambers, H. F., Lu, Y., and Otto, M. (2009) Evolution of virulence in epidemic community-associated methicillin-resistant *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 5883–5888 [CrossRef Medline](#)
40. Pérez-Pérez, J. M., Candela, H., and Micol, J. L. (2009) Understanding synergy in genetic interactions. *Trends Genet.* **25**, 368–376 [CrossRef Medline](#)
41. Smaldone, G. T., and Helmann, J. D. (2007) CsoR regulates the copper efflux operon copZA in *Bacillus subtilis*. *Microbiology* **153**, 4123–4128 [CrossRef Medline](#)
42. Bernsel, A., Viklund, H., Hennerdal, A., and Elofsson, A. (2009) TOPCONS: consensus prediction of membrane protein topology. *Nucleic Acids Res.* **37**, W465–W468 [CrossRef Medline](#)
43. Hutchings, M. I., Palmer, T., Harrington, D. J., and Sutcliffe, I. C. (2009) Lipoprotein biogenesis in Gram-positive bacteria: knowing when to hold 'em, knowing when to fold 'em. *Trends Microbiol.* **17**, 13–21 [CrossRef Medline](#)
44. Rahman, O., Cummings, S. P., Harrington, D. J., and Sutcliffe, I. C. (2008) Methods for the bioinformatic identification of bacterial lipoproteins encoded in the genomes of Gram-positive bacteria. *World J. Microbiol. Biotechnol.* **24**, 2377–2382 [CrossRef](#)
45. Navarre, W. W., Daefler, S., and Schneewind, O. (1996) Cell wall sorting of lipoproteins in *Staphylococcus aureus*. *J. Bacteriol.* **178**, 441–446 [CrossRef Medline](#)

46. Kiedrowski, M. R., Crosby, H. A., Hernandez, F. J., Malone, C. L., McNamara, J. O., 2nd, and Horswill, A. R. (2014) *Staphylococcus aureus* Nuc2 is a functional, surface-attached extracellular nuclease. *PLoS ONE* **9**, e95574 [CrossRef Medline](#)
47. Xiao, Z., Loughlin, F., George, G. N., Howlett, G. J., and Wedd, A. G. (2004) C-terminal domain of the membrane copper transporter Ctr1 from *Saccharomyces cerevisiae* binds four Cu(I) ions as a cuprous-thiolate polynuclear cluster: sub-femtomolar Cu(I) affinity of three proteins involved in copper trafficking. *J. Am. Chem. Soc.* **126**, 3081–3090 [CrossRef Medline](#)
48. Xiao, Z., Gottschlich, L., van der Meulen, R., Udagedara, S. R., and Wedd, A. G. (2013) Evaluation of quantitative probes for weaker Cu(i) binding sites completes a set of four capable of detecting Cu(i) affinities from nanomolar to attomolar. *Metallomics* **5**, 501–513 [CrossRef Medline](#)
49. Montelione, G. T. (2012) The protein structure initiative: achievements and visions for the future. *F1000 Biol. Rep.* **4**, 7 [Medline](#)
50. Bertini, I., Cavallaro, G., and McGreevy, K. S. (2010) Cellular copper management—a draft user's guide. *Coord. Chem. Rev.* **254**, 506–524 [CrossRef](#)
51. Rubino, J. T., and Franz, K. J. (2012) Coordination chemistry of copper proteins: how nature handles a toxic cargo for essential function. *J. Inorg. Biochem.* **107**, 129–143 [CrossRef Medline](#)
52. Holm, L., and Laakso, L. M. (2016) Dali server update. *Nucleic Acids Res.* **44**, W351–W355 [CrossRef Medline](#)
53. Ma, Z., Cowart, D. M., Ward, B. P., Arnold, R. J., DiMarchi, R. D., Zhang, L., George, G. N., Scott, R. A., and Giedroc, D. P. (2009) Unnatural amino acid substitution as a probe of the allosteric coupling pathway in a mycobacterial Cu(I) sensor. *J. Am. Chem. Soc.* **131**, 18044–18045 [CrossRef Medline](#)
54. Purves, J., Thomas, J., Riboldi, G. P., Zapotoczna, M., Tarrant, E., Andrew, P. W., Londoño, A., Planet, P. J., Geoghegan, J. A., Waldron, K. J., and Morrissey, J. A. (2018) A horizontally gene transferred copper resistance locus confers hyper-resistance to antibacterial copper toxicity and enables survival of community acquired methicillin-resistant *Staphylococcus aureus* USA300 in macrophages. *Environ. Microbiol.* **20**, 1576–1589 [CrossRef Medline](#)
55. Ma, Z., Cowart, D. M., Scott, R. A., and Giedroc, D. P. (2009) Molecular insights into the metal selectivity of the copper(I)-sensing repressor CsoR from *Bacillus subtilis*. *Biochemistry* **48**, 3325–3334 [CrossRef Medline](#)
56. Badarau, A., and Dennison, C. (2011) Copper trafficking mechanism of CXXC-containing domains: insight from the pH-dependence of their Cu(I) affinities. *J. Am. Chem. Soc.* **133**, 2983–2988 [CrossRef Medline](#)
57. Singleton, C., and Le Brun, N. E. (2009) The N-terminal soluble domains of *Bacillus subtilis* CopA exhibit a high affinity and capacity for Cu(I) ions. *Dalton Trans.* **2009**, 688–696 [Medline](#)
58. Singleton, C., Hearnshaw, S., Zhou, L., Le Brun, N. E., and Hemmings, A. M. (2009) Mechanistic insights into Cu(I) cluster transfer between the chaperone CopZ and its cognate Cu(I)-transporting P-type ATPase, CopA. *Biochem. J.* **424**, 347–356 [CrossRef Medline](#)
59. Delmar, J. A., Su, C. C., and Yu, E. W. (2013) Structural mechanisms of heavy-metal extrusion by the Cus efflux system. *Biomaterials* **26**, 593–607 [CrossRef Medline](#)
60. Bagchi, P., Morgan, M. T., Bacsá, J., and Fahrni, C. J. (2013) Robust affinity standards for Cu(I) biochemistry. *J. Am. Chem. Soc.* **135**, 18549–18559 [CrossRef Medline](#)
61. Jabs, A., Weiss, M. S., and Hilgenfeld, R. (1999) Non-proline cis peptide bonds in proteins. *J. Mol. Biol.* **286**, 291–304 [CrossRef Medline](#)
62. Brandts, J. F., Halvorson, H. R., and Brennan, M. (1975) Consideration of the Possibility that the slow step in protein denaturation reactions is due to cis-trans isomerism of proline residues. *Biochemistry* **14**, 4953–4963 [CrossRef Medline](#)
63. Schmidpeter, P. A., and Schmid, F. X. (2015) Prolyl isomerization and its catalysis in protein folding and protein function. *J. Mol. Biol.* **427**, 1609–1631 [CrossRef Medline](#)
64. Scholz, C., Scherer, G., Mayr, L. M., Schindler, T., Fischer, G., and Schmid, F. X. (1998) Prolyl isomerases do not catalyze isomerization of non-prolyl peptide bonds. *Biol. Chem.* **379**, 361–365 [Medline](#)
65. Pal, D., and Chakrabarti, P. (1999) Cis peptide bonds in proteins: residues involved, their conformations, interactions and locations. *J. Mol. Biol.* **294**, 271–288 [CrossRef Medline](#)
66. Planet, P. J., LaRussa, S. J., Dana, A., Smith, H., Xu, A., Ryan, C., Uhlmann, A. C., Boundy, S., Goldberg, J., Narechania, A., Kulkarni, R., Ratner, A. J., Geoghegan, J. A., Kolokotronis, S. O., and Prince, A. (2013) Emergence of the epidemic methicillin-resistant *Staphylococcus aureus* strain USA300 coincides with horizontal transfer of the arginine-catabolic mobile element and speG-mediated adaptations for survival on skin. *MBio* **4**, e00889-13 [Medline](#)
67. Zhang, Y. Q., Ren, S. X., Li, H. L., Wang, Y. X., Fu, G., Yang, J., Qin, Z. Q., Miao, Y. G., Wang, W. Y., Chen, R. S., Shen, Y., Chen, Z., Yuan, Z. H., Zhao, G. P., Qu, D., et al. (2003) Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228). *Mol. Microbiol.* **49**, 1577–1593 [CrossRef Medline](#)
68. Lowder, B. V., Guinane, C. M., Ben Zakour, N. L., Weinert, L. A., Conway-Morris, A., Cartwright, R. A., Simpson, A. J., Rambaut, A., Nübel, U., and Fitzgerald, J. R. (2009) Recent human-to-poultry host jump, adaptation, and pandemic spread of *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 19545–19550 [CrossRef Medline](#)
69. Resch, G., François, P., Morisset, D., Stojanov, M., Bonetti, E. J., Schrenzel, J., Sakwinska, O., and Moreillon, P. (2013) Human-to-bovine jump of *Staphylococcus aureus* CC8 is associated with the loss of a β -hemolysin converting prophage and the acquisition of a new staphylococcal cassette chromosome. *PLoS ONE* **8**, e58187 [CrossRef Medline](#)
70. Planet, P. J., Diaz, L., Kolokotronis, S. O., Narechania, A., Reyes, J., Xing, G., Rincon, S., Smith, H., Panesso, D., Ryan, C., Smith, D. P., Guzman, M., Zurita, J., Sebra, R., Deikus, G., et al. (2015) Parallel epidemics of community-associated methicillin-resistant *Staphylococcus aureus* USA300 infection in North and South America. *J. Infect. Dis.* **212**, 1874–1882 [CrossRef Medline](#)
71. Hao, X., Lüthje, F. L., Qin, Y., McDevitt, S. F., Lutay, N., Hobman, J. L., Asiani, K., Soncini, F. C., German, N., Zhang, S., Zhu, Y. G., and Rensing, C. (2015) Survival in amoeba—a major selection pressure on the presence of bacterial copper and zinc resistance determinants? Identification of a “copper pathogenicity island”. *Appl. Microbiol. Biotechnol.* **99**, 5817–5824 [CrossRef Medline](#)
72. Pang, Y. Y., Schwartz, J., Bloomberg, S., Boyd, J. M., Horswill, A. R., and Nauseef, W. M. (2014) Methionine sulfoxide reductases protect against oxidative stress in *Staphylococcus aureus* encountering exogenous oxidants and human neutrophils. *J. Innate Immun.* **6**, 353–364 [Medline](#)
73. Mashruwala, A. A., Bhatt, S., Poudel, S., Boyd, E. S., and Boyd, J. M. (2016) The DUF59 containing protein SufT is involved in the maturation of iron-sulfur (FeS) proteins during conditions of high FeS cofactor demand in *Staphylococcus aureus*. *PLoS Genet.* **12**, e1006233 [CrossRef Medline](#)
74. Kreiswirth, B. N., Löfdahl, S., Betley, M. J., O'Reilly, M., Schlievert, P. M., Bergdoll, M. S., and Novick, R. P. (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* **305**, 709–712 [CrossRef Medline](#)
75. Novick, R. P. (1991) Genetic systems in *Staphylococci*. *Methods Enzymol.* **204**, 587–636 [CrossRef Medline](#)
76. Rosario-Cruz, Z., Chahal, H. K., Mike, L. A., Skaar, E. P., and Boyd, J. M. (2015) Bacillithiol has a role in Fe-S cluster biogenesis in *Staphylococcus aureus*. *Mol. Microbiol.* **98**, 218–242 [CrossRef Medline](#)
77. Bose, J. L., Fey, P. D., and Bayles, K. W. (2013) Genetic tools to enhance the study of gene function and regulation in *Staphylococcus aureus*. *Appl. Environ. Microbiol.* **79**, 2218–2224 [CrossRef Medline](#)
78. Mashruwala, A. A., Guchte, A. V., and Boyd, J. M. (2017) Impaired respiration elicits SrrAB-dependent programmed cell lysis and biofilm formation in *Staphylococcus aureus*. *Elife* **6**, e23845 [CrossRef Medline](#)
79. Forsyth, R. A., Haselbeck, R. J., Ohlsen, K. L., Yamamoto, R. T., Xu, H., Trawick, J. D., Wall, D., Wang, L., Brown-Driver, V., Froelich, J. M., C, K. G., King, P., McCarthy, M., Malone, C., Misiner, B., Robbins, D., et al. (2002) A genome-wide strategy for the identification of essential genes in *Staphylococcus aureus*. *Mol. Microbiol.* **43**, 1387–1400 [CrossRef Medline](#)
80. Mashruwala, A. A., Pang, Y. Y., Rosario-Cruz, Z., Chahal, H. K., Benson, M. A., Mike, L. A., Skaar, E. P., Torres, V. J., Nauseef, W. M., and Boyd,

- J. M. (2015) Nfu facilitates the maturation of iron–sulfur proteins and participates in virulence in *Staphylococcus aureus*. *Mol. Microbiol.* **95**, 383–409 [CrossRef Medline](#)
81. Malone, C. L., Boles, B. R., Lauderdale, K. J., Thoendel, M., Kavanaugh, J. S., and Horswill, A. R. (2009) Fluorescent reporters for *Staphylococcus aureus*. *J. Microbiol. Methods* **77**, 251–260 [CrossRef Medline](#)
 82. Joska, T. M., Mashruwala, A., Boyd, J. M., and Belden, W. J. (2014) A universal cloning method based on yeast homologous recombination that is simple, efficient, and versatile. *J. Microbiol. Methods* **100**, 46–51 [CrossRef Medline](#)
 83. Mashruwala, A. A., and Boyd, J. M. (2016) *De novo* assembly of plasmids Using yeast recombinational cloning. *Methods Mol. Biol.* **1373**, 33–41 [CrossRef Medline](#)
 84. Roberts, C. A., Al-Tameemi, H. M., Mashruwala, A. A., Rosario-Cruz, Z., Chauhan, U., Sause, W. E., Torres, V. J., Belden, W. J., and Boyd, J. M. (2017) The Suf iron–sulfur cluster biosynthetic system is essential in *Staphylococcus aureus* and decreased Suf function results in global metabolic defects and reduced survival in human neutrophils. *Infect. Immun.* **85**, e00100–17 [Medline](#)
 85. Ranjit, D. K., Endres, J. L., and Bayles, K. W. (2011) *Staphylococcus aureus* CidA and LrgA proteins exhibit holin-like properties. *J. Bacteriol.* **193**, 2468–2476 [CrossRef Medline](#)
 86. Olson, B. J., and Markwell, J. (2007) Assays for determination of protein concentration. *Curr. Protoc. Protein Sci.* Chapter 3, Unit 3.4 [CrossRef Medline](#)
 87. Xiao, R., Anderson, S., Aramini, J., Belote, R., Buchwald, W. A., Ciccosanti, C., Conover, K., Everett, J. K., Hamilton, K., Huang, Y. J., Janjua, H., Jiang, M., Kornhaber, G. J., Lee, D. Y., Locke, J. Y., *et al.* (2010) The high-throughput protein sample production platform of the Northeast Structural Genomics Consortium. *J. Struct. Biol.* **172**, 21–33 [CrossRef Medline](#)
 88. Jansson, M., Li, Y. C., Jendeborg, L., Anderson, S., Montelione, G. T., and Nilsson, B. (1996) High-level production of uniformly ¹⁵N- and ¹³C-enriched fusion proteins in *Escherichia coli*. *J. Biomol. NMR* **7**, 131–141 [Medline](#)
 89. Acton, T. B., Xiao, R., Anderson, S., Aramini, J., Buchwald, W. A., Ciccosanti, C., Conover, K., Everett, J., Hamilton, K., Huang, Y. J., Janjua, H., Kornhaber, G., Lau, J., Lee, D. Y., Liu, G., *et al.* (2011) Preparation of protein samples for NMR structure, function, and small-molecule screening studies. *Methods Enzymol.* **493**, 21–60 [CrossRef Medline](#)
 90. Kay, L. E., Torchia, D. A., and Bax, A. (1989) Backbone dynamics of proteins as studied by ¹⁵N inverse detected heteronuclear NMR spectroscopy: application to staphylococcal nuclease. *Biochemistry* **28**, 8972–8979 [CrossRef Medline](#)
 91. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **6**, 277–293 [Medline](#)
 92. Güntert, P., Dötsch, V., Wider, G., and Wüthrich, K. (1992) Processing of multi-dimensional NMR data with the new software PROSA. *J. Biomol. NMR.* **2**, 619–629 [CrossRef](#)
 93. Keller, R. L. J. (2004) *The Computer Aided Resonance Assignment Tutorial*, pp. 1–73. CANTINA Verlag, Switzerland
 94. Bartels, C., Xia, T. H., Billeter, M., Güntert, P., and Wüthrich, K. (1995) The program XEASY for computer-supported NMR spectral analysis of biological macromolecules. *J. Biomol. NMR* **6**, 1–10 [CrossRef Medline](#)
 95. Goddard, T. D., and Knelle, D. G. (2008) SPARKY 3. University of California, San Francisco, CA
 96. Harris, R. K., Becker, E. D., Cabral de Menezes, S. M., Granger, P., Hoffman, R. E., and Zilm, K. W. (2008) Further conventions for NMR shielding and chemical shifts (IUPAC Recommendations 2008). *Pure Appl. Chem.* **80**, 59–84 [CrossRef](#)
 97. Bahrami, A., Assadi, A. H., Markley, J. L., and Eghbalnia, H. R. (2009) Probabilistic interaction network of evidence algorithm and its application to complete labeling of peak lists from protein NMR spectroscopy. *PLoS Comput. Biol.* **5**, e1000307 [CrossRef Medline](#)
 98. Neri, D., Szyperski, T., Otting, G., Senn, H., and Wüthrich, K. (1989) Stereospecific nuclear magnetic resonance assignments of the methyl groups of valine and leucine in the DNA-binding domain of the 434 repressor by biosynthetically directed fractional ¹³C labeling. *Biochemistry* **28**, 7510–7516 [CrossRef Medline](#)
 99. Pelton, J. G., Torchia, D. A., Meadow, N. D., and Roseman, S. (1993) Tautomeric states of the active-site histidines of phosphorylated and unphosphorylated IIIIGlc, a signal-transducing protein from *Escherichia coli*, using two-dimensional heteronuclear NMR techniques. *Protein Sci.* **2**, 543–558 [Medline](#)
 100. Ulrich, E. L., Akutsu, H., Doreleijers, J. F., Harano, Y., Ioannidis, Y. E., Lin, J., Livny, M., Mading, S., Maziuk, D., Miller, Z., Nakatani, E., Schulte, C. F., Tolmie, D. E., Kent Wenger, R., Yao, H., and Markley, J. L. (2008) BioMagResBank. *Nucleic Acids Res.* **36**, D402–D408 [Medline](#)
 101. Shen, Y., Delaglio, F., Cornilescu, G., and Bax, A. (2009) TALOS+: a hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. *J. Biomol. NMR* **44**, 213–223 [CrossRef Medline](#)
 102. Huang, Y. J., Tejero, R., Powers, R., and Montelione, G. T. (2006) A topology-constrained distance network algorithm for protein structure determination from NOESY data. *Proteins* **62**, 587–603 [Medline](#)
 103. Güntert, P., Mumenthaler, C., and Wüthrich, K. (1997) Torsion angle dynamics for NMR structure calculation with the new program DYANA. *J. Mol. Biol.* **273**, 283–298 [CrossRef Medline](#)
 104. Tjandra, N., Grzesiek, S., and Bax, A. (1996) Magnetic field dependence of nitrogen-proton J splittings in ¹⁵N-enriched human ubiquitin resulting from relaxation interference and residual dipolar coupling. *J. Am. Chem. Soc.* **118**, 6264–6272 [CrossRef](#)
 105. Linge, J. P., Williams, M. A., Spronk, C. A., Bonvin, A. M., and Nilges, M. (2003) Refinement of protein structures in explicit solvent. *Proteins* **50**, 496–506 [CrossRef Medline](#)
 106. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Crystallography and NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr. D Biol. Crystallogr.* **54**, 905–921 [CrossRef Medline](#)
 107. Bhattacharya, A., Tejero, R., and Montelione, G. T. (2007) Evaluating protein structures determined by structural genomics consortia. *Proteins* **66**, 778–795 [Medline](#)
 108. Huang, Y. J., Powers, R., and Montelione, G. T. (2005) Protein NMR recall, precision, and F-measure scores (RPF scores): structure quality assessment measures based on information retrieval statistics. *J. Am. Chem. Soc.* **127**, 1665–1674 [CrossRef Medline](#)
 109. Koradi, R., Billeter, M., and Wüthrich, K. (1996) MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph.* **14**, 51–55, 29–32 [CrossRef Medline](#)
 110. Schrödinger, L. (2015) *The PyMOL Molecular Graphics System*, Version 1.8, Schrödinger, LLC, New York
 111. Ashkenazy, H., Erez, E., Martz, E., Pupko, T., and Ben-Tal, N. (2010) ConSurf 2010: calculating evolutionary conservation in sequence and structure of proteins and nucleic acids. *Nucleic Acids Res.* **38**, W529–W533 [CrossRef Medline](#)
 112. Farmer, B. T., 2nd., Constantine, K. L., Goldfarb, V., Friedrichs, M. S., Wittekind, M., Yanchunas, J., Jr., Robertson, J. G., and Mueller, L. (1996) Localizing the NADP⁺ binding site on the MurB enzyme by NMR. *Nat. Struct. Biol.* **3**, 995–997 [CrossRef Medline](#)
 113. Fey, P. D., Endres, J. L., Yajjala, V. K., Widhelm, T. J., Boissy, R. J., Bose, J. L., and Bayles, K. W. (2013) A genetic resource for rapid and comprehensive phenotype screening of nonessential *Staphylococcus aureus* genes. *MBio* **4**, e00537–12 [Medline](#)