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Regulation of DNA-binding activity of the *Staphylococcus aureus* catabolite control protein A by copper (II)-mediated oxidation

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Catabolite control protein A (CcpA) of the human pathogen *Staphylococcus aureus* is an essential DNA regulator for carbon catabolite repression and virulence, which facilitates bacterial survival and adaptation to a changing environment. Here, we report that copper (II) signaling mediates the DNA-binding capability of CcpA *in vitro* and *in vivo*. Copper (II) catalyzes the oxidation of two cysteine residues (Cys216 and Cys242) in CcpA to form intermolecular disulfide bonds between two CcpA dimers, which results in the formation and dissociation of a CcpA tetramer of CcpA from its cognate DNA promoter. We further demonstrate that the two cysteine residues on CcpA are important for *S. aureus* to resist host innate immunity, indicating that *S. aureus* CcpA senses the redox-active copper (II) ions as a natural signal to cope with environmental stress. Together, these findings reveal a novel regulatory mechanism for CcpA activity through copper (II)-mediated oxidation.

Staphylococcus aureus is a widespread human pathogen and is the main cause of hospital- and community-acquired infections. Infection by the bacterium causes a series of human diseases, ranging from minor skin infections to life-threating sepsis (1). Because of the overuse or abuse of antibiotics, the emergence of drug-resistance strains, such as methicillin-resistant and vancomycin-resistant *S. aureus*, poses a great threat to public health worldwide (2). The broad range of conditions caused by *S. aureus* is related to the expression of the virulence factors that allow the bacteria to invade the immune system and lead to harmful effects to the host (3). Therefore, understanding how *S. aureus* regulates its virulence in response to the host environment is crucial to devising effective treatment strategies.

Catabolite control protein A (CcpA) is a highly conserved DNA regulator in Gram-positive bacteria, and it plays an important role in bacterial carbon metabolism (4). In particular, the function of *S. aureus* CcpA (*Sa*CcpA) is also closely related to resistance to antibiotics, the formation of biofilms, and toxin expression, indicating its critical role as an important global regulator of bacterial virulence (5–7). The inhibition of *Sa*CcpA activity by silver ions or small molecule inhibitors markedly reduces *S. aureus* pathogenesis (8, 9). As with other Gram-positive bacteria, *Sa*CcpA regulatory activity is induced by carbon sources. It has been reported that 4 mM glucose is sufficient to fully activate *Sa*CcpA, which exhibits an increased affinity for catabolite-responsive element (*cre*) DNA sequences (5). Furthermore, a recent study demonstrated that *Sa*CcpA activity was also affected by Ser/Thr kinase Stk1, which inactivated *Sa*CcpA *via* the phosphorylation of two Thr residues (Thr-18 and Thr-33) located at the DNA-binding site (10). Whether alternative regulatory mechanisms that modulate the DNA binding property of *Sa*CcpA exist remains unknown.

Here, we report that copper (II) ions directly oxidize the two cysteine residues on *Sa*CcpA to form intermolecular disulfide bonds between two *Sa*CcpA dimer. The formation of the *Sa*CcpA tetramer resulted in the dissociation of *Sa*CcpA from the *cre* DNA sequence *in vitro* and *in vivo*. In addition, this mode of regulating the *Sa*CcpA function is essential for *S. aureus* to resist the host immune system. Our study demonstrates that the copper (II) ion could act as a natural signal in *S. aureus*, and copper (II)-mediated oxidation may be a novel regulatory mechanism for CcpA activity.

Results and discussion

Copper (II) triggers CcpA–DNA complex dissociation

Previous studies have demonstrated that *S. aureus* is equipped with several thiol-based oxidation-sensing regulatory proteins to cope with the toxic reactive oxygen species (ROS) produced by the host immune system, including MgrA, SarZ, and CymR (11–13). In particular, inspection of the *Sa*CcpA sequence indicated two unique Cys residues, Cys-216 and Cys-242, which are absent in other CcpA homologs (Fig. 1)

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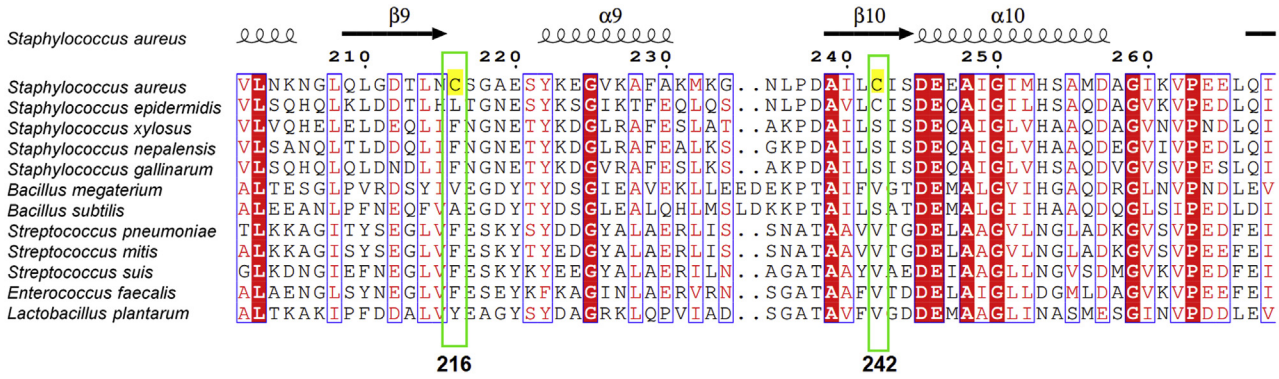

Figure 1. Sequence alignments of CcpA homologs from Gram-positive bacteria. The two unique cysteine residues (216 and 242) are highlighted in *green box*. CcpA, Catabolite control protein A.

This observation prompted us to hypothesize that *Sa*CcpA may use a similar thiol-based oxidation-sensing mechanism to regulate gene expression. To validate our hypothesis, we first investigated whether the DNA-binding property of *Sa*CcpA could be affected by oxidative stress.

Electrophoretic mobility shift assay was applied to a 35-nM *pckA* DNA probe (*cre* sequence of *pckA* gene encoding phosphoenolpyruvate carboxykinase) in the presence of 500 nM *Sa*CcpA (monomer concentration), which formed a protein–DNA complex with or without the incubation of different concentrations of oxidative reagents (these *Sa*CcpA and DNA concentrations were used for all of the following EMSA experiments unless otherwise noted). Intriguingly, the *Sa*CcpA–DNA complex remained stable even in the presence of cumene hydroperoxide or H_2O_2 up to 50 μM (Fig. 2, *A* and *B*). By contrast, copper (II) ions in as low an amount as 1 μM could remarkably perturb the *Sa*CcpA–DNA complex formation, and 5 μM copper (II) ions could completely abrogate the complex (Fig. 2*C*). In particular, adding reducing reagent such as DTT effectively restored the DNA-binding ability of *Sa*CcpA (Fig. 2*E*). By contrast, the double-mutant *Sa*CcpA^{2CS} (both Cys-216 and Cys-242 are mutated to Ser) was not sensitive to copper (II) ion treatment (Fig. 2*D*). Subsequently, we used EMSA to examine a panel of other metal ions, including nickel (II), iron (III), zinc (II), and cobalt (II). It is worth noting that copper (II) is the only transition metal ion to disrupt *Sa*CcpA–DNA complex (Fig. 2*F*). Together, the results indicated that low concentrations of copper (II) ions are sufficient to trigger the dissociation of *Sa*CcpA from its cognate promoter DNA, and the two unique Cys residues are necessary for this process.

Molecular mechanism of copper (II)-triggered CcpA inactivation

As a redox-active transition metal, copper has largely been studied as a static enzyme cofactor. However, recent studies have identified regulatory role played by labile copper ions as signaling molecules for diverse cellular processes, either through direct copper-enzyme interaction (14–16) or copper-catalyzed protein oxidation (17). We reasoned that the dissociation of the *Sa*CcpA–DNA complex caused by the copper (II) ion could be because of the copper (II) ion binding or to copper (II)-mediated oxidation of *Sa*CcpA.

To elucidate the molecular mechanism of inactivation of *Sa*CcpA by copper (II) ions, we first examined whether *Sa*CcpA could bind copper (II) ions. In brief, *Sa*CcpA was incubated with 3 M equivalents of copper (II). After removing the excess metal ions using a desalting column, the metal content of *Sa*CcpA was measured using inductively coupled plasma mass spectrometry (ICP-MS). No copper content could be detected in the treated *Sa*CcpA sample (Fig. S1). In addition, the isothermal titration calorimetry profile of *Sa*CcpA titrated with copper (I) confirmed that there is no interaction between *Sa*CcpA and copper (I) ions (Fig. S2). Collectively, the results excluded the possibility that copper (II) binding triggered *Sa*CcpA inactivation.

Subsequently, we investigated whether copper (II) caused the oxidation of *Sa*CcpA. As copper (II)-catalyzed protein oxidation could cause the reduction of copper (II) to copper (I), we monitored copper (I) production with a copper (I)-specific indicator, namely, bathocuproine disulfonate (BCS), which forms a Cu(I)-$(BCS)_2^{3-}$ complex with a characteristic UV-absorbance peak at 483 nm (18). Titration of the *Sa*CcpA-BCS solution (10 μM *Sa*CcpA and 200 μM BCS) with copper (II) ions revealed an increasing absorption peak at 483 nm. This peak leveled off with a stoichiometry of 1:2 between *Sa*CcpA monomer and copper (II), indicating the presence of copper (I) (Fig. 3*A*). By contrast, no copper (I) was detected after the titration of copper (II) ions into double mutant *Sa*CcpA^{2CS} (Fig. S3). To further confirm that the two unique Cys residues (Cys-216 and Cys-242) of *Sa*CcpA are involved in copper (II) reduction, 5,5′-dithio-bis-2-nitrobenzoic acid assay was applied to measure the free thiol content of *Sa*CcpA before and after copper (II) treatment. Indeed, no free thiols were detectable in *Sa*CcpA after copper (II) treatment. Similar results were obtained for the two single-residue mutants *Sa*CcpA^{C216S} and *Sa*CcpA^{C242S} (mutation of Cys-216 and Cys-242 to Ser individually). The results indicated that both of the Cys residues were oxidized by copper (II) (Fig. S4).

The molecular weight (MW) of the *Sa*CcpA monomer is 37 kDa. However, nonreducing SDS-PAGE analyses of *Sa*CcpA after copper (II) oxidation revealed a new band with

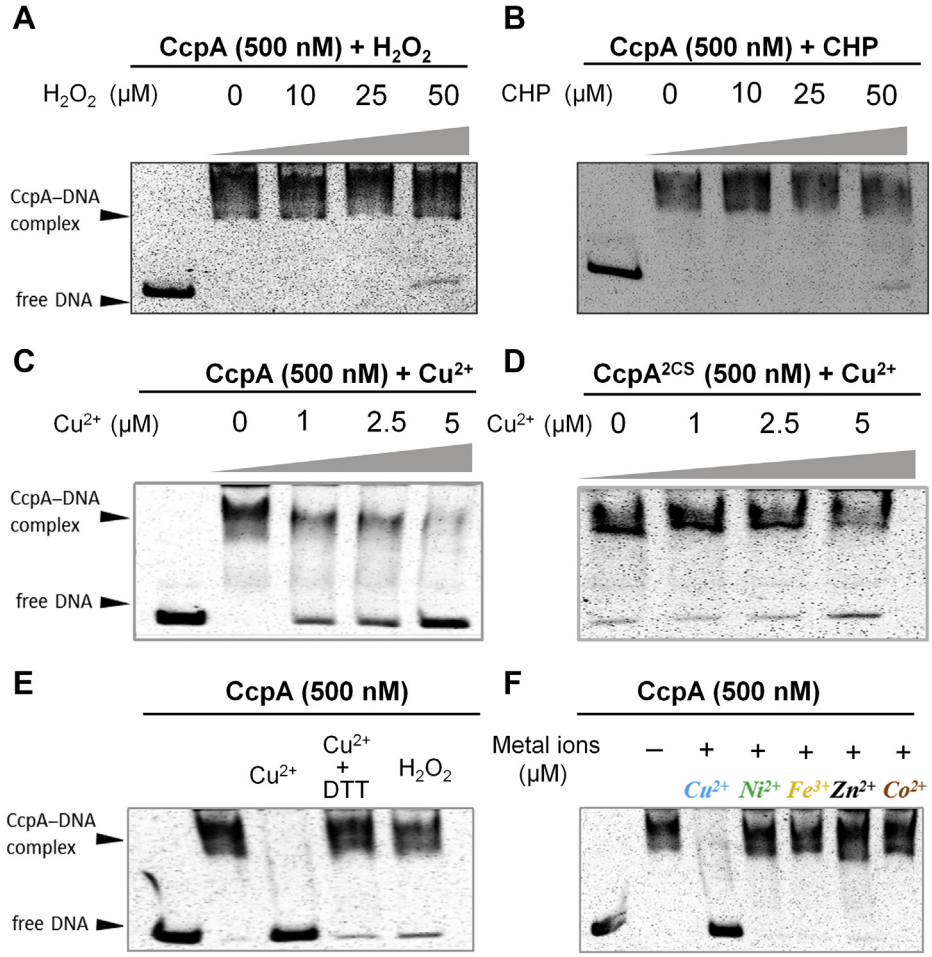

Figure 2. Copper (II) ion abrogates the DNA-binding capability of *Sa*CcpA *in vitro*. Electrophoretic mobility shift assay showing the association of *Sa*CcpA with *pckA* promoter DNA in the presence of increasing concentrations of (*A*) H_2O_2, (*B*) cumene hydroperoxide, and (*C*) Cu(II) ions. *D*, electrophoretic mobility shift assay of *Sa*CcpA in the presence of Cu(II), Cu(II) and DTT, H_2O_2. *E*, electrophoretic mobility shift assay of *Sa*CcpA^{2CS} mutant with *pckA* promoter in the presence of increasing concentrations of Cu(II) ion. *F*, electrophoretic mobility shift assay of WT *Sa*CcpA with *pckA* promoter in the presence of 5 μM $CuCl_2$, $NiCl_2$, $FeCl_3$, $ZnCl_2$, and $CoCl_2$, respectively. CcpA, Catabolite control protein A; SaCcpA, S. aureus CcpA.

an apparent MW of around 72 kDa, indicative of the formation of an intermolecular covalent bond between two *Sa*CcpA monomers. (Fig. 3*B*). Similar copper (II)-oxidized dimer bands were also observed for single-residue mutants *Sa*CcpA^{C216S} and *Sa*CcpA^{C242S} but not for the double mutant *Sa*CcpA^{2CS}. In particular, the dimer was abrogated by addition of DTT, which implies that both Cys residues are involved in intermolecular disulfide bond formation after copper (II) oxidation (Fig. S5). This observation is in line with the size-exclusion chromatography results. Wild-type *Sa*CcpA was eluted at 9.6 ml in a Tricorn Superdex 75,100/300 Gl column with a calculated MW of 62.2 kDa, corresponding to a dimeric form. After copper (II) oxidation, a new elution peak appeared at 8.6 ml with a calculated MW of 159 kDa, indicative of *Sa*CcpA tetramer formation (Fig. 3*C*). Similar size-exclusion chromatography profiles were also observed for *Sa*CcpA^{C216S} and *Sa*CcpA^{C242S} mutants (Fig. S6).

To further validate the nature of the formation of the *Sa*CcpA tetramer after copper (II) oxidation, protein mass spectroscopy was used to analyze the residues involved in covalent bond formation. In brief, after copper (II) oxidation, WT *Sa*CcpA was subjected to nonreducing SDS-PAGE, and then the dimeric band was collected and digested in gel with Glu-C and Asp-N endoproteinases. The digested peptides were extracted and subjected to mass spectrometry (MS) analysis using a Thermo Orbitrap high resolution liquid chromatography–mass spectrometry. We identified two 2^+ charged peaks with m/z values of 733.37 and 908.35, corresponding to disulfide-containing peptides cross-linked between the two unique Cys residues (Cys216 and Cys216′ on two Asp-Ala-Ile-Leu-Cys-Ile-Ser peptides with a calculated MW of 1464.75 Da; Cys242 and Cys242′ on two Asp-Thr-Leu-Asn-Cys-Ser-Gly Ala-Glu peptides with a calculated MW of 1814.72 Da) (Figs. 3, *D* and *E* and S7). Collectively, the results indicate that copper (II) may trigger the formation of a covalent dimer-of-dimer of *Sa*CcpA *via* Cys-216 and Cys-242 residues in catalytic fashion.

Crystal structure of dimeric Sa*CcpA*

To obtain additional insight into the molecular mechanisms of the copper (II)-mediated oxidation of *Sa*CcpA, we tried to resolve the crystal structure of *Sa*CcpA. Despite our efforts, we

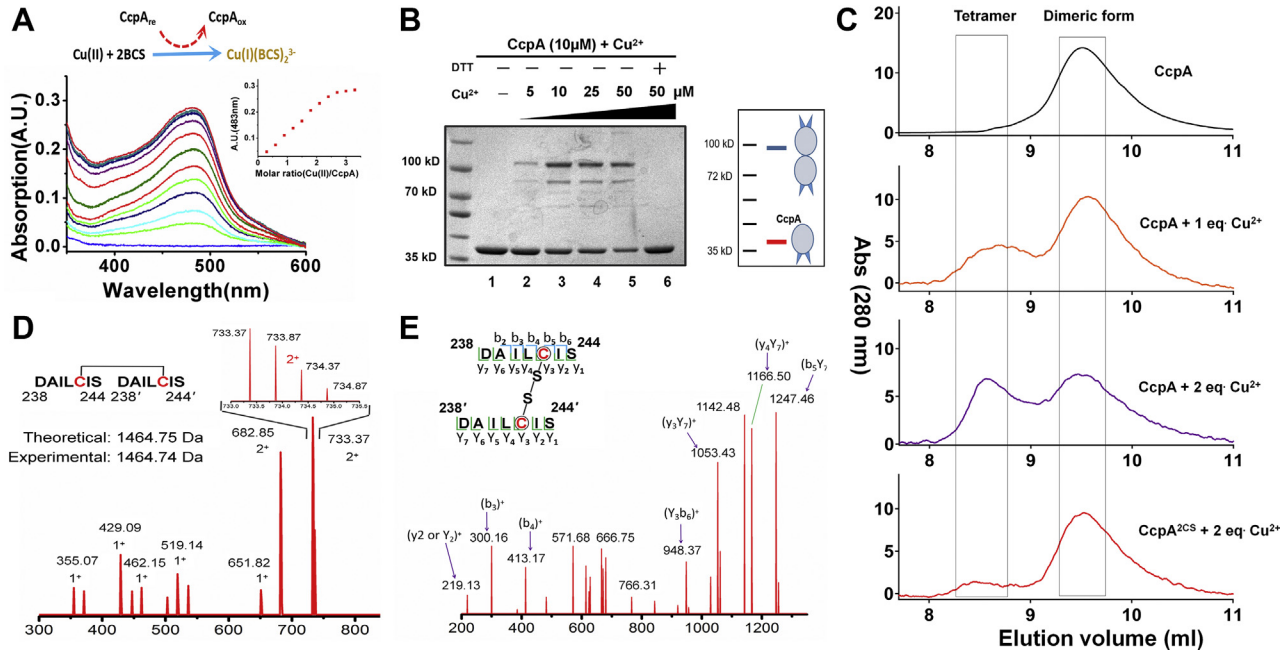

Figure 3. The molecular response of CcpA to copper (II) *in vitro*. *A*, titration of aliquots of $CuCl_2$ into the *Sa*CcpA-BCS solution (10 μM *Sa*CcpA monomer and 200 μM BCS). Absorption at 483 nm (the characteristic absorption peak of the Cu(I)–$(BCS)_2^{3-}$ complex) was plotted against the copper (II)/protein ratio. *B*, nonreducing SDS-PAGE of *Sa*CcpA after incubation with increasing concentrations of copper (II) ions with or without DTT treatment. *C*, size-exclusion chromatography analysis of WT *Sa*CcpA and *Sa*CcpA^{2CS} mutant after incubation with 1 or 2 M equivalents of copper (II) ions. *D*, mass spectrum of an unfractionated tryptic peptide mixture of the copper (II)-oxidized *Sa*CcpA protein. *Inset*, the 2^+ charged peak (m/z: 733.37–734.87) corresponding to the disulfide-containing peptide of interest (theoretical molecular mass: 1464.75 Da). *E*, MS/MS fragmentation of the 2^+ charged peptide (*m/z*: 733.37). AU, absorbance unit; BCS, bathocuproine disulfonate; CcpA, Catabolite control protein A; SaCcpA, S. aureus CcpA.

were unable to obtain crystals to determine the structure of copper-oxidized *Sa*CcpA tetramer. However, the untreated dimeric *Sa*CcpA yielded three-dimensional crystals with sufficient quality for an X-ray diffraction experiment. The crystals of dimeric *Sa*CcpA belong to the I_{422} space group and diffract to a resolution of 2.5 Å. The initial phases were obtained by molecular replacement using *Bacillus subtilis* CcpA (*Bs*CcpA, PDBID:1ZVV) as a search model. The final structural model contains three polypeptide chains of *Sa*CcpA in one asymmetric unit with a R_{work} and R_{free} of 23% and 25%, respectively (Table S2). Among these, chain B and C form a non-crystallographic homo-dimer, and chain A forms a crystallographic homo-dimer with a symmetry-related chain A. These dimers resemble that of LacI-GalR members LacI and PurR. Typically, each *Sa*CcpA monomer consists of a DNA-binding domain (residues 1–50) and a dimerization domain (residues 60–329). The later dimerization domain has a two-domain architecture, consisting of an N subdomain and a C subdomain (Fig. 4*A*) (19, 20). The structural superimposition of *Sa*CcpA with *Bs*CcpA reveals that their dimerization domains are highly similar with an average r.m.s. deviation of 2.94 Å for all C_α atoms. By contrast, the DNA-binding domains of the two structures have distinct orientations. In *Bs*CcpA, the four helices of the DNA-binding domain are clearly visible. However, the hinge helix of the *Sa*CcpA DNA-binding domain (residues 50–57) is disordered, and the DNA-binding domain swings up to contact the dimerization domain (Fig. 4*B*).

The unique Cys residues of *Sa*CcpA, namely, C216 and C242, are both located at the C-subdomain of *Sa*CcpA dimerization domain (Fig. 4*C*). Solvent accessibility analyses using the AREAIMOL software from CCP4 package revealed that C216 is a solvent-exposed residue, whereas the C242 residue is relatively buried. However, copper (II)-mediated oxidation experiments indicated that both of the two Cys residues are accessible to copper (II) ion, which implies that the *Sa*CcpA structure could probably undergo conformational change or partial unfolding in the presence of copper (II), exposing the relatively buried C242 residue. Furthermore, as the two Cys residues are both located at the C-subdomain of the dimerization domain, it is reasonable to conclude that the homo-tetramer formation of *Sa*CcpA after copper (II)-oxidation could prevent the conformational rotation of the C-subdomain of the *Sa*CcpA dimerization domain, which is required for the allosteric DNA-binding activation of CcpA (21, 22), such that the *Sa*CcpA loses its DNA-binding capability after the formation of homo-tetramers.

Copper (II) ion negatively regulates CcpA DNA-binding property in vivo

All of the *in vitro* results demonstrate that copper (II) abolishes the DNA-binding property of *Sa*CcpA by directly oxidizing Cys-216 and Cys-242 residues. We examined whether the copper (II) ion could regulate the activity of *Sa*CcpA *in vivo*. To validate the regulatory mechanism *in vivo*, we needed to find a way to increase the level of bacterial intracellular copper (II) without significantly perturbing the bacterial growth. *S. aureus* features a complete copper homeostasis system, including the metallochaperone CopZ and

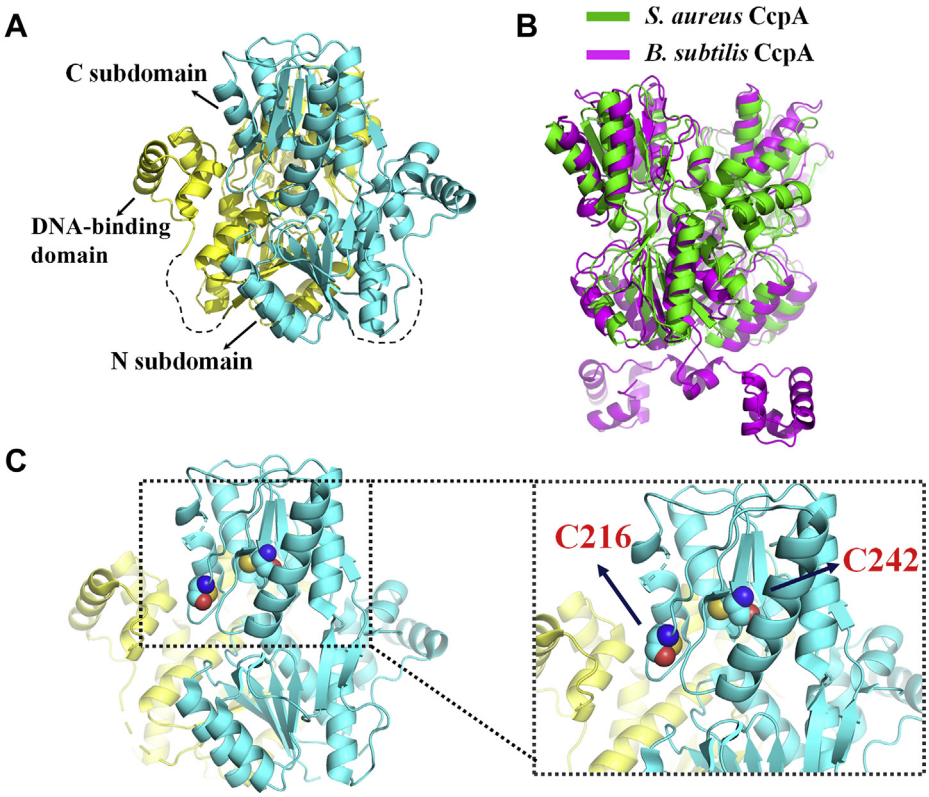

Figure 4. Crystal structure of WT SaCcpA. *A*, overall structure of dimeric *Sa*CcpA structure is shown in *cartoon*. The two monomers are colored *yellow* and *cyan*, respectively. DNA-binding domain, N subdomain, and C subdomain of the dimerization domain are labeled. The missing regions (residue 50–57) are show as *dashed line*. *B*, structure superimposition of *Staphylococcus aureus* CcpA (*Sa*CcpA, PDBID: 7E5W) and *Bacillus subtilis* CcpA (*Bs*CcpA, PDBID:1ZVV). The *Sa*CcpA and *Bs*CcpA structures are colored in *magenta* and *green*, respectively. *C*, the two unique cysteine residues (Cys216 and Cys242) of one *Sa*CcpA monomer are represented in *spheres*. CcpA, Catabolite control protein A; SaCcpA, S. aureus CcpA.

the copper-response transcriptional regulator CsoR (23, 24). The free copper content is estimated to exist only at the atomolar range in the bacterial cytosol under normal conditions (25). However, recent studies have demonstrated that a certain antibiotic treatment of *Escherichia coli* can produce organic hydroperoxide, which impairs bacterial cytoplasmic membrane-bound copper proteins, resulting in the release of free copper (I). The liberated copper (I) together with intracellular ROS leads to elevated intracellular copper (II) (17). Therefore, we incubated *S. aureus* with a series of antibiotics or copper (II) ions and monitored the intracellular level of copper (I) using a cell-permeable fluorogenic copper (I) probe CF4 (26). As with *E. coli*, treatment of *S. aureus* with low concentrations of ampicillin, cefoperazone, or copper (II) could lead to substantially elevated levels of intracellular copper, as indicated by the CF4 fluorescent signals. By contrast, no obvious increase was observed for *S. aureus* when treated with levofloxacin, gentamycin, or chloramphenicol (Fig. S8). However, even low concentrations of ampicillin and cefoperazone treatment significantly perturbed the growth of *S. aureus* RN4220 or Newman strains. However, incubation with low concentrations of copper (II) ions (30 μM) had no observable impact on the growth of *S. aureus*. Therefore, we choose this condition for the following *in vivo* experiments.

As a global transcription factor, *Sa*CcpA can bind to cognate *cre* promoter sequences, such as *pckA* (encoding the phosphoenol-pyruvate carboxykinase), *hla* (encoding α-hemolysin), and *citZ* (encoding the citrate synthase) promoters. Previous studies have demonstrated that CcpA binding to the *cre* sequences could result in either positive or negative regulation of controlled genes. For example, *Sa*CcpA binding to the corresponding DNA promoter reduced the transcription of *pckA* but increased the transcription of *hla* (27). Therefore, we first investigated the change in transcription level for the *pckA* and *hla* genes in *S. aureus* treated with copper (II) ions. In brief, the WT *S. aureus* Newman strain in the exponential phase was incubated with 20 and 30 μM copper (II) ions for 3 h. Total cellular RNA was extracted, and the change in transcription levels of *pckA* and *hla* genes was determined using quantitative real-time PCR (qPCR) with rrsA (16S rRNA) as an internal control. As shown in Figure 5, *A* and *B*, a substantial increase in the transcription of the *pckA* gene was observed after copper (II) treatment. Typically, the transcription level of *pckA* increased 1.6-fold after treatment with 30 μM copper (II). Similarly, a relatively small but obvious decline in the *hla* transcription level was also observed after treatment with 30 μM copper (II). Quantitative analyses using fluorescence Western blotting demonstrated that *Sa*CcpA protein level remained unchanged after copper (II) treatment (Fig. S9). It is implied that the change in transcription level for the *pckA* and *hla* genes could probably be attributed to the dissociation of *Sa*CcpA from the gene promoters mediated by the copper (II) ion.

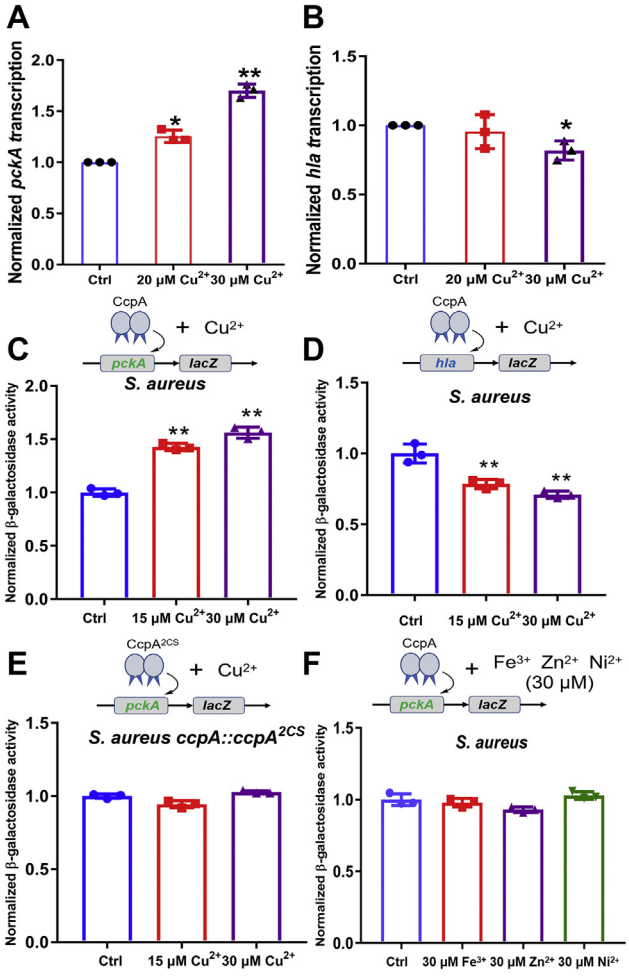

Figure 5. Copper (II) ions regulate DNA-binding capability of *Sa*CcpA *in vivo*. Quantitative real-time PCR (qPCR) analysis of (*A*) *pckA* and (*B*) *hla* gene transcription levels in *Staphylococcus aureus* Newman strain treated with 20 μM and 30 μM copper (II), respectively. β-galactosidase activities of the (*C*) *pckA*::*lacZ* and (*D*) *hla*::*lacZ* reporters in WT *S. aureus* RN4220 strain incubated with copper (II) ion. *E*, β-galactosidase activities of the *pckA*::*lacZ* reporter in *S. aureus ccpA*::*ccpA*^{2CS}RN4220 mutant strain incubated with copper (II) ion. *F*, β-galactosidase activities of the *pckA*::*lacZ* reporter in WT *S. aureus* RN4220 strain incubated with 30 μM $FeCl_3$, $ZnCl_2$, and $NiCl_2$, respectively. The mean value of the control group was set as 1. The values in other groups were normalized to that of control group. All experiments were performed in triplicate. The results are shown as mean ± sd. The statistical difference is determined by two-tailed Student's *t* test. *$p < 0.05$, **$p < 0.01$. CcpA, Catabolite control protein A; SaCcpA, S. aureus CcpA.

To further examine the effects of copper (II) ion on *Sa*CcpA function *in vivo*, a β-galactosidase (β-Gal) assay was used. In brief, we individually mutated the two Cys residues C216 and C242 of *Sa*CcpA to alanine in the *S. aureus* genome using the reported CRISPR/Cas9-based genome editing tool (28). All of the mutants *S. aureus ccpA::ccpA*^{C216S}, *S. aureus ccpA::ccpA*^{C242S}, and *S. aureus ccpA::ccpA*^{2CS} (both Cys residues were mutated to Ala) were created and obtained in the RN4220 strain. quantitative real-time PCR analyses indicated that the transcription levels of *ccpA* and *pckA* genes in *S. aureus* mutants were almost the same as those in WT *S. aureus*, indicating that site-directed mutagenesis did not perturb the physiological function of *Sa*CcpA (Fig. S10). Then, we created a reporter plasmid by fusing the *S. aureus pckA* (or *hla*) promoter with the *lacZ* gene. The constructed plasmid containing the *pckA::lacZ* (or *hla::lacZ*) reporter gene was transformed into WT *S. aureus* RN4220 and three mutant strains, respectively. The transformed bacterial strains were subsequently incubated with 15 or 30 μM copper (II) ions for 3 h followed by freeze-thaw cell lysis. The β-Gal activities in cell lysates were determined, as described previously (29).

For WT *S. aureus* bearing the *pckA::lacZ* reporter gene, copper (II) ion treatment led to a dose-dependent increase in β-Gal activity (Fig. 5*C*), whereas for WT *S. aureus* with the *hla::lacZ* reporter gene, copper (II) ion caused reduced β-Gal activity (Fig. 5*D*). Similarly, copper (II) treatment could also cause the β-Gal activity change in *S. aureus ccpA::ccpA*^{C216S} and *S. aureus ccpA::ccpA*^{C242S} mutants that were transformed with the two reporter genes (Fig. S11). By contrast, transformed *S. aureus ccpA::ccpA*^{2CS} mutants exhibited no observable change on the β-Gal after incubation with copper (II) (Figs. 5*E* and S12). It is worth noting that the change in β-Gal activity was specific to the copper (II) ion because iron (III), zinc (II), and nickel (II) treatment could not perturb the β-Gal activity in the WT *S. aureus* cell lysate (Fig. 5*F*). Together, these observations indicated that the copper (II) ion could also regulate the DNA-binding capability of *Sa*CcpA *in vivo*, probably by the oxidation of the two Cys residues of *Sa*CcpA.

Cys residues of SaCcpA *are important for* S. aureus *to resist host innate immunity*

As a successful human pathogen, *S. aureus* has the intrinsic metabolic flexibility to quickly adapt to the diverse host niches to promote its survival (30). In particular, the metabolic reprogramming of *S. aureus* allows for the production of various virulence factors that combat the host immune system (31). Therefore, the functional regulation of *Sa*CcpA may also affect the bacterial susceptibility to host immunity. Our results demonstrated that copper (II)-mediated oxidation could be a new mode of regulation of *Sa*CcpA function *in vitro* and *in vivo*. We are promoted to further investigate whether this regulatory mechanism could contribute to the bacterial adaption to host inches.

Because the ROS such as O_2^- and H_2O_2 generated by neutrophils are a crucial part of the host defense mechanism against *S. aureus*, we first compared the antioxidative capabilities of WT *S. aureus* and three *ccpA* mutants strains (*S. aureus ccpA::ccpA*^{C216S}, *S. aureus ccpA::ccpA*^{C242S}, and *S. aureus ccpA::ccpA*^{2CS}) using H_2O_2, as described previously (32). In brief, WT *S. aureus* and mutants were incubated with 10 mM H_2O_2 for 30 min. Bacterial viability was assessed with dilutions on Tryptic Soy Agar (TSA) plates for the enumeration of the surviving colony-forming units (CFUs). As shown in Figure 6*A*, the survival rate of WT *S. aureus* after H_2O_2 treatment was around 70%. The two bacterial mutants *S. aureus ccpA::ccpA*^{C216S} and *S. aureus ccpA::ccpA*^{C242S} exhibited lower tolerance to H_2O_2 than the WT strain, with survival rates of 48.4% and 56.2%, respectively. By contrast, the double mutant *S. aureus ccpA::ccpA*^{2CS} was the most susceptible strain to oxidative stress, with a lower survival rate of 36.9%. Besides

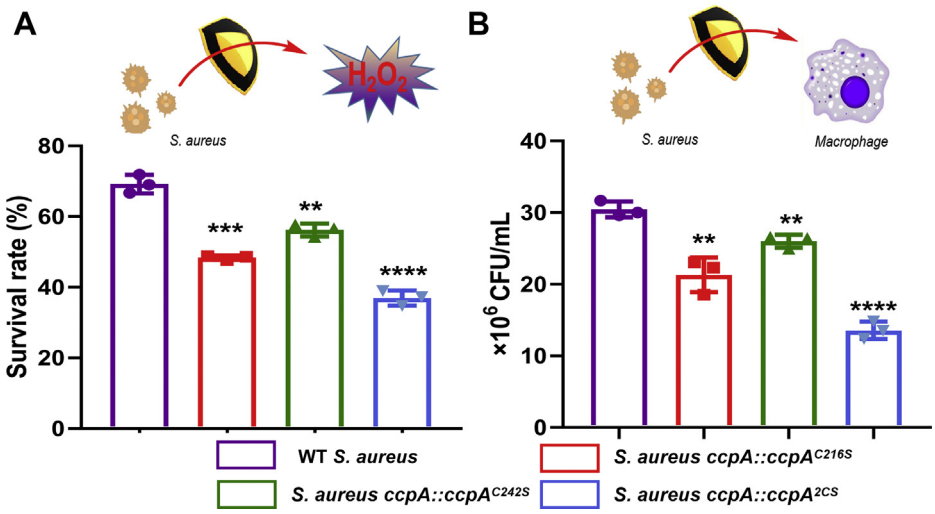

Figure 6. The cysteine residues of *Sa*CcpA are essential for *Staphylococcus aureus* to survive under environmental stress. Comparison of resistance capability of *S. aureus* RN4220 and different mutants against H_2O_2 (*A*) and macrophage phagocytosis (*B*). Mutagenesis of the Cys residue of *Sa*CcpA significantly attenuates the resistance capability of *S. aureus* against H_2O_2 and macrophage phagocytosis. Each experiment was performed in triplicates. The data are shown in mean ± sd. The statistical difference is determined by two-tailed Student's *t* test. $^{**}p < 0.01$, $^{***}p < 0.0005$, $^{****}p < 0.0001$. CcpA, Catabolite control protein A; SaCcpA, S. aureus CcpA.

oxidative stress, phagocytes such as macrophages also play a vital role in restricting and destroying *S. aureus* (33). Therefore, macrophage-killing experiments were also carried out with WT *S. aureus* and *ccpA* mutants. In line with the H_2O_2-resistance assays, the viabilities of *S. aureus* mutants were significantly lower than those of WT *S. aureus* after macrophage phagocytosis. In particular, the viability of *S. aureus ccpA::ccpA*^{2CS} was only one-third of that of WT *S. aureus* (Fig. 6*B*), which implies that free cysteine plays an important role in *Sa*CcpA to protect *S. aureus* against macrophage phagocytosis.

Conclusion

In this study, we showed that the CcpA is a thiol-based, copper (II)-sensing regulator in *S. aureus*. *Sa*CcpA directly senses copper (II) ion by the oxidation of the Cys residues C216 and C242 to form inter-molecular disulfide bonds. Copper (II)-mediated oxidation leads to the dissociation of *Sa*CcpA from its cognate *cre* promoters and impacts the transcription of downstream regulons, including the genes involved in carbon metabolism and virulence genes. Furthermore, the copper (II)-mediated regulatory mechanism of *Sa*CcpA function is essential for the survival of *S. aureus* in host innate immunity.

As an important global DNA regulator for the human pathogen *S. aureus*, *Sa*CcpA has an essential regulatory function for controlling bacterial carbon metabolism and virulence expression (5, 27, 34). The classic regulatory mechanism of CcpA function involves the coregulator Hpr, which is phosphorylated on residue Ser46 by its cognate kinase HprKP in the presence of glucose. Catabolite control protein A and Hpr-Ser(P)-46 form a complex and exhibit enhanced DNA-binding affinity (35). In addition, *Sa*CcpA could be directly phosphorylated by a Ser/Thr protein kinase Stk1 on two Thr residues on the DNA-binding domain, which inhibits DNA-binding activity toward its regulon. The data we present here strongly suggest that copper (II)-mediated oxidation of Cys residues (Cys216 and Cys242) is a novel regulatory mechanism for *Sa*CcpA function (Fig. 7). These multiple regulatory modes of *Sa*CcpA may contribute to the remarkable capacity of *S. aureus* to adapt rapidly to changes in environmental conditions and regulate the expression of a large array of virulence factors (31, 36–38).

It should be noted that Cys residues are not conserved because the Cys at positions 216 and 242 of CcpA are only found in two species closely related to *S. aureus* (*Staphylococcus argenteus* and *Staphylococcus schweitzeri*) but not in

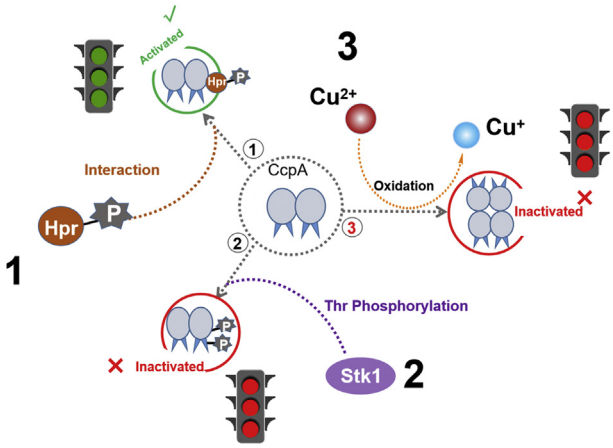

Figure 7. Proposed regulatory mechanism of *Sa*CcpA as a molecular switch in carbon catabolite repression and virulence gene expression in *Staphylococcus aureus*. (1) *Sa*CcpA binding to DNA is modulated *via* the HprKP/Hpr system. The binding of *Sa*CcpA to its DNA targets is enhanced once forms a complex with Hpr-Ser(P)-46 protein. (2) the Stk1-mediated phosphorylation of *Sa*CcpA abrogates the DNA-binding capability of *Sa*CcpA and prevents the transcriptional control of its target genes. (3) intracellular copper (II)-mediated oxidation of *Sa*CcpA lead to the formation of inter-molecular disulfide bonds. The *Sa*CcpA tetramer formation inactivates its DNA binding capability. CcpA, Catabolite control protein A; SaCcpA, S. aureus CcpA.

other *Staphylococcus* species or *B. subtilis* (Fig. S13). An implied oxidation-mediated regulatory mechanism of CcpA is quite unique for *S. aureus*. We speculate that there may be an evolutionary advantage to maintaining the unique Cys residues in *Sa*CcpA sequence because *S. aureus* but not *B. subtilis* is pathogenic in humans. As a human pathogen, *S. aureus* must cope with the oxidative stress generated by the human immune system. Therefore, several thiol-based oxidation-sensing regulatory proteins have been identified in *S. aureus* species, such as DNA regulators MgrA, SarZ, and CymR and the two-component system SrrAB (11, 13, 39, 40). Our data confirmed that the mutation of Cys to Ser residue did not perturb the regulatory function of *Sa*CcpA in a glucose-replete medium. However, the mutagenesis substantially impaired bacterial resistance to the host innate immune system, which implies that the oxidation-sensing function of *Sa*CcpA is important for the bacterial adaption to hostile environment.

Redox-active transition metal ions such as copper and iron were primarily studied as static enzyme cofactors. However, several studies have demonstrated that transition metal ion copper could also serve as a mediator for cellular signaling in mammalian cells (14–16, 41). We also identified that copper (II) acted as an intracellular signal molecule to regulate the DNA-binding capability of *Sa*CcpA. Recent studies have found that macrophage phagosome accumulated copper ions during *S. aureus* infection, which constitutes an important phagocyte-killing mechanism (42). The engulfed *S. aureus* is therefore exposed to relatively high concentrations of copper, which might lead to elevated intracellular copper (I) level. Indeed, we observed enhanced fluorescent signals from a CF4 probe after incubation of *S. aureus* with free copper (II) in culture medium. The CF4 probe selectively recognized Cu(I) with a dissociation constant (K_D) of 2.9×10^{-13} M, which is relatively weaker than that of Cu(I)-binding proteins (43). Therefore, the increased fluorescent signals indicate an increased labile copper pool in the bacterial cytosol.

Copper stress could also lead to intracellular ROS generation, as reported previously (44). It is reasonable to speculate that intracellular copper concentration would increase under this condition. Therefore, it is not surprising that *S. aureus* could adapt a unique mechanism to use *Sa*CcpA to directly sense the copper (II) signal and cope with environmentally derived stress. It is noteworthy that similar copper-mediated regulatory mechanisms have also been reported for the multiple antibiotics resistance regulator MarR in Gram-negative bacterial *E. coli* (17) implying that redox-active copper ion might be a general regulatory signal in bacterial species. Given the essential role that *Sa*CcpA plays in the central metabolism and virulence of *S. aureus*, the involvement of copper (II) ions in its function regulation suggests crosstalk among bacterial carbon metabolism, virulence factor expression, and metal homeostasis, which merits further investigation. Our data here also presage opportunities to target copper homeostasis to alter bacterial metabolic state and virulence, which may lead to new avenues for therapeutic intervention into bacterial infectious diseases.

Experimental procedures

Electrophoretic mobility shift assays

The DNA probe containing the promoter region of *pckA* for EMSA was PCR-amplified using *S. aureus* Newman chromosomal DNA as a template with primers pairs listed in Table S1. For oxidative reagents perturbation assays, *Sa*CcpA or *Sa*CcpA^{2CS} mutant (final concentration: 500 nM) was incubated with *pckA* probe (final concentration: 35 nM) in the presence of different concentrations of oxidative reagents for 30 min at room temperature in binding buffer (25 mM Tris–HCl, 80 mM NaCl, 35 mM KCl, 10 mM $MgCl_2$, 0.1% Triton X-100, and 10% glycerol, pH 7.5). Subsequently, 10 μl of the reaction mixture was loaded onto a native 6% (w/v) polyacrylamide TBE Gel and electrophoresed in 0.5 × TBE (v/v) buffer for 45 min at 200 V.cm-1. Gels were stained in a 10,000-fold diluted Gene-Finder nucleic acid staining solution (Xiamen Zhishan Ltd) for 5 min. The DNA bands were visualized with a blue light transilluminator (Syngene).

Copper content determination of SaCcpA

To determine the copper-binding capability of *Sa*CcpA protein, approximately 3 M equivalents of copper (II) ions were added into 100 μM *Sa*CcpA in Tris–HCl buffer (20 mM Tris–HCl, 150 mM, pH 7.4). After incubation for 30 min at room temperature, the samples were subsequently loaded on a Hitrap desalting column to remove the excess amount of copper (II) ions. The eluted protein concentration was measured by bicinchoninic acid assay. The bound metal contents of protein were determined by ICP-MS on a Thermo Scientific iCAP Q ICP-MS spectrometer. The copper content measurement for each sample was carried out in triplicate.

Isothermal titration calorimetry

Isothermal titration calorimetry experiments were performed on a Malvern MicroCal iTC200 at 25 °C. *Sa*CcpA was prepared in Tris–HCl buffer (20 mM Tris–HCl, 150 mM, pH 7.4) with a final concentration of 50 μM. The cuprous titrant was prepared by dissolving tetrakis(acetonitrile)-copper(I) hexafluorophosphate (sigma) in Tris–HCl buffer with a concentration of 1 mM. In general, 40 μl of cuprous titrant was titrated into 200 μl protein sample with 150 s interval between each injection. All the isothermal titration calorimetry data were analyzed using the Origin software provided.

Copper (II) reduction monitored by UV-vis spectroscopy

To monitor the reduction of copper (II) by SaCcpA protein, aliquots of $CuSO_4$ (from 2 μM to 20 μM) were titrated into the protein-BCS solution containing 10 μM *Sa*CcpA (monomer concentration) protein and 200 μM BCS in a final volume of 600 μl. The absorption at 483 nm (characteristic absorption of Cu(I)–$(BCS)_2^{3-}$complex) were plotted *versus* the molar ratio of copper (II) to protein (monomer concentration). All the experiments were performed in 20 mM Tris–HCl buffer (pH 7.4) on a Shimadzu UV-3600 spectrometer.

5,5′-dithiobis-2-nitrobenzoic acid assay

The free thiol contents of native and copper (II)-oxidized *Sa*CcpA or mutants were determined by the 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) assay. In brief, 50 μM *Sa*CcpA or mutants were incubated with 250 μM CuSO4 for 20 min at room temperature in Tris–HCl buffer (20 mM Tris–HCl, 150 mM, pH 7.4). Excess copper (II) was removed by HiTrap desalting column (GE healthcare), and then excess amount of DTNB was added to a final concentration of 160 μM. After further incubation for 20 min, the absorption at 412 nm of each sample was recorded and free thiol concentrations were calculated.

Protein oligomerization state analysis

The oligomerization states of *Sa*CcpA and mutants were analyzed by size-exclusion chromatography. In brief, *Sa*CcpA or mutants were incubated with different equivalent of copper (II) ions at room temperature for 30 min. The elution volumes of samples were subsequently measured on a Tricorn Superdex 75 10/300 Gl column (GE Healthcare) pre-equilibrated with Gel-filtration buffer (20 mM Tris–HCl, 300 mM NaCl, pH 7.4). The column was calibrated using the GE LMW calibration kit dissolved in the same buffer.

Disulfide bond detection by MS

Purified WT *Sa*CcpA (10 μM) were incubated with 50 μM $CuSO_4$ at room temperature for 20 min. Protein samples were run on a nonreducing, denaturing SDS-PAGE gel. The covalently linked dimer band was separately excised from the SDS-PAGE gel, destained, and digested with Glu-C and Asp-N enzyme at 37 °C for 12 h. The resulting tryptic peptide was extracted and subjected to mass spectra analysis using a Thermo Orbitrap Fusion Lumos mass spectrometer equipped with a nano-HPLC system. Mobile phase A consisted of 0.1% formic acid, and mobile phase B consisted of 100% acetonitrile and 0.1% formic acid. The single full-scan mass spectrum was acquired in the Orbitrap followed by three MS/MS scans in the quadruple collision cell using the collision-induced dissociation.

Protein crystallization and structure determination

Approximately, 2 μl *Sa*CcpA protein (12 mg/ml) in Tris–HCl buffer (20 mM Tris–HCl, 300 mM NaCl, 1 mM DTT, pH 7.5) was mixed with equal volume of reservoir solution containing 2.0 to 3.0 M $(NH_4)_2SO_4$, 0.1 M NaOAc, pH 5.5. Crystals appeared after 2 days by sitting drop diffusion. Protein crystals were transferred into a cryoprotective solution containing the reservoir solution supplemented with 15 to 20% glycerol (v/v) and flash frozen in liquid nitrogen. Diffraction data of crystals were collected at Shanghai Synchrotron Radiation Facility on beamline 17U1 (BL17U1). Raw data images were processed with HKL2000. The structures were solved by molecular replacement with PHENIX program, and the *B. subtilis* CcpA structure (PDBID 1ZVV) was used as a searching model. Subsequent model building and refinement were carried out in COOT and PHENIX. Crystallographic data statistics are summarized in Table S2. All the figures are prepared using the PyMOL software. The atomic coordinates and structural factors of *Sa*CcpA have been deposited into the Protein Data Bank with accession code 7E5W.

Copper signal detection in S. aureus

A specific copper (I) fluorescence sensor CF-4 (from Christopher J. Chang lab) was used to detect the presence of copper (I) ions inside *S. aureus* upon exposure to various stimulants. Generally, overnight *S. aureus* culture was inoculated (1:100) in 5 ml fresh Tryptic Soy Broth (TSB) medium and was grown to an A_{600} value of 0.6 before being treated by copper sulfate (25 μM), various antibiotics (1 μg/ml ampicillin, 2.5 μg/ml cefoperazone, 5 μg/ml chloramphenicol, 2.5 μg/ml levofloxacin, and 5 μg/ml gentamicin) at 37 °C for 2 h. After washing with 50 mM PBS buffer twice, bacterial samples were incubated with 5 μM CF-4 for 25 min at 37 °C followed by additional washing with PBS buffer twice. Change of fluorescence of the bacterial cultures was monitored using a Biotek Cytation3 plate reader.

Measurement of gene transcription by quantitative real time PCR

S. aureus Newman, *S. aureus* RN4220, or *S. aureus* RN4220 mutants were grown overnight in TSB medium. The overnight cultures were diluted 100-fold in freshly prepared glucose-replete LB medium with or without Cu^{2+} treatment. After additional incubation for 3 h, the bacteria were harvested and total RNA was extracted using the SV total RNA isolation kit (Promega) according to the manufacturer's instructions. Complementary DNA was generated by reverse transcription using GoScript Reverse Transcriptase (Promega). The transcription level of detected gene was subsequently determined by real-time PCR using GoTaq qPCR Master Mix kit (Promega) on a StepOnePlus Real-time PCR system (Life Technologies). The *rrsA* (16S rRNA) was used as an internal control. All the experiments were conducted in triplicate and relative expression levels were measured using the $2^{-\Delta\Delta Ct}$ method. The mean value of control group was set as 1 and the values of experiment groups were normalized to that of control group. The following primers were used for real-time PCR: 16S rRNA-For and 16S rRNA-Rev, *ccpA*-For and *ccpA*-Rev, *hla*-For and *hla*-Rev, *pckA*-For and *pckA*-Rev (Table S1).

Western blot analysis

S. aureus RN4220 or mutant strains were grown at 37 °C overnight in TSB. The overnight cultures were diluted 100-fold in fresh prepared glucose-replete LB medium. The cultures were further grown at 37 °C with agitation until A_{600} reached 1.0. Subsequently, the bacterial cultures were treated with 20 μM or 30 μM Cu^{2+} at 37 °C for additional 3 h. The bacterial cells were harvested and lysed by three freeze-thaw cycles using liquid nitrogen. The cell lysates were centrifuged at 15,000*g* for 10 min at 4 °C to remove the pellets. And the same amounts of supernatants were

loaded to gel electrophoresis followed by immuno-blotting using CcpA antibodies followed by IRDye 800 CW goat anti-rabbit secondary antibodies (1:20,000 dilution). The fluorescence images were recorded on a LI-COR Odyssey infrared imaging system and quantified using the image studio software.

S. aureus *RN4220 mutant construction*

The CRISPR/Cas9 plasmid pCasSA was used to obtain the *S. aureus* RN4220 *ccpA::ccpA*^{2CS}, *ccpA::ccpA*^{C216S} and *ccpA::ccpA*^{C242S} mutants. The primers used to PCR-amplification of spacers and repair arms for homologous-recombination-mediated repair are listed in Table S1. The spacer and the repair arm DNA fragments were subsequently subcloned into the pCasSA to generate the editing plasmids pCasSA-*ccpA::ccpA*^{2CS}, pCasSA-*ccpA::ccpA*^{C216S}, and pCasSA-*ccpA::ccpA*^{C242S}, respectively. The editing plasmids were then transferred into *S. aureus* RN4220 competent cells using the electroporation method. The transferred cells were plated on a TSB agar plate supplemented with 10 μg/ml chloramphenicol at 30 °C until bacterial colonies appeared. The colonies were then picked from the plate and cultured in TSB medium 30 °C overnight. The genomic DNA of the picked colonies was extracted, and the ccpA DNA fragment was PCR-amplified using the genomic DNA as a template. The site-directed mutagenesis was further confirmed by sequencing.

*Construction of pALC-*pckA::lacZ *and pALC-*hla::lacZ *reporter plasmids*

Previous study reported a shuttle plasmid pALC1434, which contains a promoterless *gfp*UV gene. We modified the plasmid by replacing the *gfp*UV gene with a β-Gal gene (*lacZ*) to construct the pALC-*lacZ* reporter plasmid. *S. aureus pckA* (-370 to +37 of the start codon) and *hla* (-802 to +34 of the start codon) promoter regions were amplified by PCR with the primers listed in Table S1. The amplified promoter gene was cloned upstream the lacZ to construct the pALC-*pckA::lacZ* and pALC-*hla::lacZ* plasmids. The fusion plasmids were transferred into *S. aureus* RN4220 or mutants by electroporation, respectively.

β-Gal assays

The *S. aureus* RN4220 or mutant strains containing the lacZ reporter plasmid (pALC-*pckA::lacZ* or pALC-*hla::lacZ*) were used for this assay. In brief, overnight culture of the bacteria was diluted into fresh glucose-replete LB medium (containing 50 mM Hepes, 10 mM glucose). The culture was grown at 37 °C until at A_{600} reached approximately 1.0. To examine the effects of metal ions on the regulatory function of CcpA, the bacteria were then treated with copper (II) or other metal ions as indicated and cultured for additional 3 h. Subsequently, the bacteria were collected and cell density was recorded. After cell lysis, the β-Gal activity of the cell lysate was determined using the 4-methylumbelliferyl-β-d-galactoside as substrates. Fluorescence at 460 nm was monitored for 30 min to determine the produced 7-hydroxy-4-methylcoumarin with an excitation wavelength of 365 nm. All experiments were performed in triplicate. The fluorescence was normalized to bacterial density to obtain the relative β-Gal activity.

H_2O_2-resistance capability assays

S. aureus RN4220 or different mutant strains were incubated in glucose-replete LB medium at 37 °C and collected in the exponential phase. Bacterial cells were then washed twice with PBS buffer and diluted to a concentration of 5×10^6 CFU. Hydrogen peroxide was added to a final concentration of 10 mM. The bacterial mixture was incubated at 0 °C in dark. After 30 min, the excess amount of H_2O_2 was depleted by addition of exogenous catalase (Sigma-Aldrich) to reach a final concentration of 4 U/ml. After further incubation of 20 min at 37 °C, bacterial viability was assessed by dilutions on TSA plates for enumeration of surviving CFUs.

Macrophage phagocytosis assays

For macrophage phagocytosis assay, murine macrophage J774 cells were cultured in DMEM supplemented with 10% fetal bovine serum, and approximately, 5×10^5 cells were seeded into each well of 24-well plates and further incubated for 24 h. *S. aureus* RN4220 and different mutant strains were grown in glucose-replete LB medium at 37 °C until A_{600} reached 1.0. Bacterial cells were collected, washed twice with PBS, and diluted to a bacterial suspension with a concentration of 10^9 CFU/ml. Twenty five microliter of the *S. aureus* bacterial suspension was added into each well of J774 cell culture with a final volume of 500 μl DMEM medium supplemented with 10 % fetal bovine serum. The cell culture plates were centrifuged at 200*g* for 5 min and incubated at 37 °C for 1 h. After that, each well was washed five times with PBS gently to remove excess bacteria. Subsequently, 1 ml of DMEM supplemented with 300 μg/ml gentamicin was added to each well and incubated at 37 °C for additional 1 h to remove *S. aureus* cells in medium. The J774 cells were then lysed in 0.025% saponin and the bacterial viability was assessed by cell lysate dilutions on TSA plates for enumeration of bacterial CFUs.

Data availability

The structure was deposited into the Protein Data Bank (PDB) with accession codes 7E5W for *S. aureus* CcpA.

Supporting information—This article contains supporting information (Supplemental Tables, Figures, and Data).

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writing–review and editing; W. X. conceptualization; W. X. and H. S. supervision; W. X. project administration; W. X., Z.-W. M., and H. S. funding acquisition.

Conflicts of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: β-Gal, β-galactosidase; BCS, bathocuproine disulfonate; *Bs*CcpA, *Bacillus subtilis* CcpA; CcpA, Catabolite control protein A; CFU, colony-forming unit; *cre*, catabolite-responsive element; ICP-MS, inductively coupled plasma mass spectrometry; MS, mass spectrometry; MW, molecular weight; qPCR, quantitative real-time PCR; ROS, reactive oxygen species; *Sa*CcpA, *Staphylococcus aureus* CcpA; TSA, Tryptic Soy Agar; TSB, Tryptic Soy Broth.

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