Copper Stress Induces a Global Stress Response in *Staphylococcus aureus* and Represses *sae* and *agr* Expression and Biofilm Formation[▽][†]

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Copper is an important cofactor for many enzymes; however, high levels of copper are toxic. Therefore, bacteria must ensure there is sufficient copper for use as a cofactor but, more importantly, must limit free intracellular levels to prevent toxicity. In this study, we have used DNA microarray to identify *Staphylococcus aureus* copper-responsive genes. Transcriptional profiling of *S. aureus* SH1000 grown in excess copper identified a number of genes which fall into four groups, suggesting that *S. aureus* has four main mechanisms for adapting to high levels of environmental copper, as follows: (i) induction of direct copper homeostasis mechanisms; (ii) increased oxidative stress resistance; (iii) expression of the misfolded protein response; and (iv) repression of a number of transporters and global regulators such as Agr and Sae. Our experimental data confirm that resistance to oxidative stress and particularly to H_2O_2 scavenging is an important *S. aureus* copper resistance mechanism. Our previous studies have demonstrated that Eap and Emp proteins, which are positively regulated by Agr and Sae, are required for biofilm formation under low-iron growth conditions. Our transcriptional analysis has confirmed that *sae*, *agr*, and *eap* are repressed under high-copper conditions and that biofilm formation is indeed repressed under high-copper conditions. Therefore, our results may provide an explanation for how copper films can prevent biofilm formation on catheters.

Staphylococcus aureus is responsible for a diverse range of infections (12) and continues to be a significant health care problem worldwide. To be a successful pathogen, *S. aureus* must sense and respond to changing environmental stresses. A key environmental stress encountered during infection is the varying concentration of the transition metal, copper. Copper is an important cofactor for many enzymes, including those essential for catabolic pathways and energy metabolism; however, high levels of copper are toxic (30, 39). It has been proposed that toxicity can occur through copper catalyzing the generation of superoxide or other reactive oxygen species (ROS), leading to cell damage and death; binding to free thiol groups, affecting the function/folding of proteins; or competing with other metals for binding sites, leading to their displacement and enzyme inactivation (41). Therefore, bacteria must ensure that there is sufficient copper for use as a cofactor but, more importantly, must limit free intracellular levels to prevent toxicity.

Studies have shown that in a host environment, while free copper levels are very low, especially in the blood, copper concentrations are higher in some tissues like the lungs (3) and have been shown to increase during infection and inflamma-

tion (1). Therefore, it is possible that *S. aureus* and other bacteria will need to respond to increased copper levels in a host. This is supported by the fact that copper resistance mechanisms are important for virulence, as shown in *Listeria monocytogenes* and *Pseudomonas aeruginosa* (10, 33.), and transcription of the *Mycobacterium tuberculosis* copper-exporting P1-type ATPase gene is induced in mouse lung tissue and tubercle bacillus-infected macrophages (13, 40).

Bacteria have developed a number of mechanisms for dealing with excess copper. These mechanisms involve the efflux and sequestration of copper and a global adaptive response which can involve induction of other stress regulons (18, 41, 47). However, the mechanisms used and particularly the coordination of regulatory responses can vary significantly between species. Efflux mechanisms include the ubiquitous CopA/CopB P1-type ATPase transporters (44) and copper binding proteins, such as the CopZ family, which chaperone the copper ions intracellularly for incorporation/use by other copper binding proteins such as the efflux transporters (29). Most gram-negative bacteria also encode a multicopper oxidase (MCO), which is required for the periplasmic oxidation of Cu(I) to Cu(II) following P1-type ATPase transport of Cu(I) from the cytoplasm (31). Five types of bacterial copper-responsive regulators have been identified to date. CopY is a copper-responsive repressor in *Enterococcus* and *Streptococcus* spp. (29). In *Escherichia coli*, CueR is a MerR homologue and CusRS is a two-component regulator system, both of which activate transcription in response to excess copper (18), whereas CsoR is a copper-responsive repressor in *M. tuberculosis* (20) and *Bacillus subtilis* (38).

All sequenced *S. aureus* strains have an operon encoding a

TABLE 1. Bacterial strains and plasmids used in this study

S. aureus strain	Description	Source or reference
Newman	Wild type	9
SH1000	NCTC 8325-4 with *rsbU* mutation repair	15
SH1000 *copA*	SH1000 with partial *copA* deletion	37
SH1000 *ahpC*	SH1000 *ahpC*::tet	7
SH1000 *katA*	SH1000 *katA*::Tn*917*	7
SH1000 *ahpC katA*	SH1000 *ahpC*::tet *katA*::Tn*917*	This work
SH1000 *sodA*	SH1000 *sodA*::Tn*917*	6
SH1000 *sodM*	SH1000 *sodM*::tet	17
SH1000 *sodM sodA*	SH1000 *sodM*::tet/*sodA*::Tn*917*	17

TABLE 2. Primers used in this study

Gene	Primer	Sequence (5′–3′)
agrC	agrC-F	CATATTCGCAAATTGATGCG
	agrC-B	CCTAAACCACGACCTTCACC
sodM	sodM-F	CAATGGAGTTTCATCACGACA
	sodM-B	GTAGGCATGCTCCCAAACAT
copA	copA-F	ATATGACTTGTGCCGCATGTT
	copA-B	GCTTAATCAATTTATGTTGTAGCGC
copZ	copZ-F	CGGATCCATGTCACAAGAAATTTTAA
	copZ-B	GAAGCTTTTAAACGACATCGTAACCT
saeS	SaeS-F	TTGTTGCGCGAGTTCATTAG
	SaeS-F	ACGGAAATTACGCAAGCAAT
ahpF	SAS0357-F	CGGTGCTCATGTCTTCGTAA
	SAS0357-B	GTGGTGGTCCTGCTAGTGGT
oppA	SAV0990-F	AAACCGGTGTTAGGTGTTGC
	SAV0990-B	GCTCTATCAGCTGCCGTACC
katA	katF	GACCTCTTTTAATGCAAGATA
	katR	GAAACTACATATCAAATTTAT
16S	16S-F	GATCCTGGGTCAGGATG
	16S-R	CTAGAGTTGTCAAAGGATG

P1-type ATPase (*copA*) and a copper binding chaperone homologue (*copZ*) (37). The expression of the *copAZ* operon is induced by copper, and mutational analysis has shown that CopA transports copper from the cell and hence is required for *S. aureus* tolerance of excess copper (37). The identification of *copA* by signature-tagged mutagenesis in a murine renal abscess model (21), and the induction of *copA* expression following phagocytosis by human neutrophils (46) suggest that CopA is important for *S. aureus* virulence. A second putative copper-transporting P1-type ATPase (*copB*) and a multicopper oxidase (*mco*) have also been identified in a small number of *S. aureus* strains, and *mco* has been shown to have a role in copper and oxidative stress resistance (36). It is thought that the *copB* and *mco* genes are not essential for normal staphylococcal growth, as they are not found in all *S. aureus* strains. To date it is not known what other mechanisms are involved in *S. aureus* copper homeostasis, how gene expression is regulated in response to copper, and what roles these mechanisms have in *S. aureus* virulence. It is also has not been established how copper toxicity occurs in *S. aureus* or other gram-positive bacteria.

In this study, we have used DNA microarray to identify *S. aureus* copper-responsive genes. Transcriptional profiling of *S. aureus* SH1000 grown in excess copper identified a number of genes which fall into four groups, suggesting that *S. aureus* has four main mechanisms for adapting to high levels of environmental copper, including induction of the oxidative stress response. Our phenotypic analysis demonstrates that resistance to oxidative stress and particularly H_2O_2 scavenging are important for *S. aureus* growth in high levels of copper. Our microarray results also showed that copper represses two major global virulence gene regulators, Sae and Agr. This has important implications for the effect of copper on virulence and the antimicrobial uses of copper. Our previous studies have demonstrated that Eap and Emp proteins, which are positively regulated by Agr and Sae, are required for biofilm formation under low-iron growth conditions (16). Biofilm formation is an important *S. aureus* virulence factor, as *S. aureus* is a major pathogen associated with nosocomial infections due to its ability to colonize a wide range of medically implanted devices, which are colonized as biofilms. Copper films on catheters have been shown to prevent biofilm formation, but the underlying mechanisms involved were unknown (23). Thus, this study shows that excess copper represses the expression of positive regulators of biofilm formation *sae* and *agr* and a

biofilm factor, *eap*, and hence represses *S. aureus* biofilm formation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study are listed in Table 1 and were stored at −80°C in 10% (vol/vol) glycerol TSB (Trypticase soy broth; BBL). *S. aureus* strains were plated out fresh from frozen stocks onto 6% defibrinated horse blood agar for each biofilm assay. Strains for biofilm assays and copper growth assays were cultured under iron-restricted conditions in CRPMI, RPMI 1640 tissue culture medium (Sigma Ltd) which had been depleted of iron by batch incubation with 6% (wt/vol) Chelex 100 (Sigma Ltd) and then supplemented with 10% RPMI 1640 to provide trace elements required for growth (26). CRPMI cultures were incubated statically for 24 h at 37°C in 5% CO_2 in air. Various concentrations of $CuSO_4$, $MnCl_2$, $MgCl_2$, and $CaCl_2$ were added to achieve metal-replete conditions.

Transduction of mutations into *S. aureus* strain Newman. The *kat*::Tn*917* mutation was transferred to *S. aureus* strain SH1000 *ahpC*::tet by transduction with phage phi11 (5) from SH1000 *katA*. Transductants were selected on LK agar plates (LK medium consists of 1% tryptone, 0.5% yeast extract, 0.7% KCl, and 1.5% agar) containing 10 μg ml^{-1} of either kanamycin or tetracycline and 0.05% sodium citrate. Colonies containing the relevant mutation were confirmed by PCR using primers katF and katR (Table 2).

RNA isolation and quantification. RNA was isolated from three independently grown 20-ml cultures of *S. aureus*. *S. aureus* strains were grown by shaking them in defined medium (DM) at 37°C overnight, as described by Townsend and Wilkinson (42), and then diluted 1:200 into fresh DM. Bacteria were then pregrown in DM until mid-log phase (optical density at 600 nm [OD_{600}] = 0.4). A total of 100 μM copper sulfate was then added to create conditions of copper excess, whereas control cultures were grown in the absence of copper. Test and control cultures were then incubated at 37°C with shaking for a further 30 min. Ten milliliters of exponentially growing cultures was pretreated with an equal volume of RNAprotect (Qiagen Inc.) before cell harvesting at 8,000 × *g* for 15 min. The cell pellet was then resuspended in 1 ml Tri reagent (Sigma), transferred to a centrifuge tube containing 0.1-mm zirconia/silica beads (Biospec Products Inc.), and processed in an FP120 FastPrep cell disruptor (MP Biomedicals, Irvine, CA). BCP (1-bromo-3-chloropropane) was then added to the lysates, which were centrifuged at 16,000 × *g* for 15 min, and then the RNA was precipitated with 100% ethyl alcohol. RNA was further purified using DNase I and RNeasy minikits (Qiagen), according to the manufacturer's instructions. Following the final purification, RNA was dissolved in RNase-free water, and the concentration was determined by measuring the absorbance at 260 nm. Finally, the purity and integrity of RNA were checked through agarose gel electrophoresis.

Transcriptional profiling. DNA microarray experiments were performed using three independently cultured sample sets to isolate the RNA, and each set was run independently to generate triplicate data. *S. aureus* grown in the absence of copper served as the control, whereas *S. aureus* grown in excess copper as

described above served as the experimental set. Labeling of RNA was carried out using 5 µg of RNA, followed by synthesizing of cDNA using reverse transcriptase (Invitrogen), as described previously (27). Briefly, cDNA was generated using random hexamers as primers for reverse transcription. The primers were annealed at 70°C for 10 min, followed by snap-freezing in ice for 1 min to total RNA (5 µg), and were extended with SuperScript II reverse transcriptase (Invitrogen) with 0.1 M dithiothreitol-12.5 mM deoxynucleoside triphosphate-5-(3-aminoallyl)-dUTP mix (Ambion, Austin, TX) mix at 42°C overnight. Two independent sets were performed. In one experiment, the control sample was labeled with Cy3, and the test sample was labeled with Cy5. For the other experiment, Cy5 was used to label the control sample, and Cy3 was used to label the test sample. Fluorescently labeled cDNA was purified by the Qiagen PCR purification kit. After being dried, the cDNA was dissolved in a hybridization buffer composed of 30% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), and a 1:100 volume of sheared salmon sperm DNA and heated at 98°C for 2 min. The denatured cDNA was applied to a *S. aureus* genome microarray version 5.0 DNA chip (Pathogen Functional Genomic Resource Center—The Institute of Genomics Research [PFGRC-TIGR]) (http://pfgrc.jcvi.org/) under a glass slide, and hybridization was carried out overnight at 42°C. After hybridization, the slides were washed at room temperature in wash buffer I (1× SSC and 0.2% SDS) twice for 10 min, in wash buffer II (0.1× SSC and 0.1% SDS) once for 10 min, and in wash buffer III (0.1× SSC) twice for 10 min. The slides were dried by centrifugation at 500 rpm for 5 min.

Data analysis. Hybridization signals were scanned using a Axon 4000B scanner with Acuity 4.0 software. Scans were analyzed using TIGR Spotfinder software, and the data set was normalized by applying the LOWESS algorithm using TIGR-MIDAS software (www.tigr.org/software/). Significant changes of gene expression were identified with significance analysis of microarrays (SAM) software using one class mode. SAM assigns a score to each gene on the basis of the change in gene expression relative to the standard deviation of repeated measurements. The "q value" assigned to each gene corresponds to the lowest false discovery rate at which the gene is called significant (43). Three independent cultures were used to prepare RNA.

RNA extraction and Northern blotting. Exponentially growing cultures in CRPMI were pelleted and resuspended in RNAlater, as directed by the manufacturer (Ambion). Total RNA was then extracted using the hot phenol method, as described by Schmitt et al. (32), but with 300 µg ml^{-1} lysostaphin added to the initial cell lysis step. A total of 10 µg RNA was electrophoresed on a denaturing 1.5% formaldehyde gel, and the RNA was transferred to a nylon filter by Northern blotting overnight. The filters were hybridized overnight at 65°C in Church Gilbert's buffer (0.5 M Na$_2$HPO$_4$, 0.5 M NaH$_2$PO$_4$, 7% [wt/vol] SDS, 1 mM EDTA) with probes labeled with [α-^{32}P]dCTP (PerkinElmer), using the Klenow fragment of DNA polymerase I and random hexamers as primers. Probes were constructed by PCR using the appropriate primer pairs shown in Table 2. Filters were washed with 3× SSC/0.1% (wt/vol) SDS and exposed to X-ray film at −80°C. The blots were then stripped of probes by washing the filters with 2 to 3 changes of boiling 0.1% (wt/vol) SDS and rinsing with 2× SSC. Transcripts were evaluated using ImageJ 1.41 software (downloaded from http://rsbweb.nih.gov/ij/). Values were normalized against 16S rRNA transcripts and expressed as a percentage of the level of transcription in the wild type.

MIC determination. The MIC of Cu was calculated as the lowest concentration at which there was no growth after 24 h of exposure. *S. aureus* was grown overnight in CRPMI at 37°C in 5% CO$_2$ in air, diluted to an optical density at 595 nm of 0.05 in fresh warmed CRPMI, and inoculated into triplicate wells of 96-well flat-bottomed tissue culture plates (Nunc) with differing concentrations of copper chloride. A total of 500 U/ml of catalase and 10 U/ml of superoxide dismutase (SOD) were added when required. The plates were then incubated statically at 37°C in 5% CO$_2$ in air for 24 h. After incubation, the plates were read at 595 nm in a plate reader (Bio-Rad), and the MIC was determined. Grown cultures were blanked with medium containing the equivalent copper concentration. The mean results and standard deviations shown have been calculated from at least three independent experiments. A two-tailed Student's *t* test was used to determine the differences in growth between mutant and wild-type strains. Differences were considered statistically significant when *P* was <0.05.

Biofilm formation assay. Quantitative measurement of staphylococcal adherence to polystyrene, indicative of biofilm formation, was done in a microtiter assay as described previously (8), with the following modifications. Bacteria were grown overnight in CRPMI at 37°C in 5% CO$_2$ in air, diluted to an optical density at 595 nm of 0.1 in fresh CRPMI, and inoculated into quadruple wells of 96-well flat-bottomed tissue culture plates (Nunc). Appropriate concentrations of CuSO$_4$, MnCl$_2$, MgCl$_2$, or CaCl$_2$ were added, and the plates were then incubated statically at 37°C in 5% CO$_2$ in air for 24 h. After incubation, plates
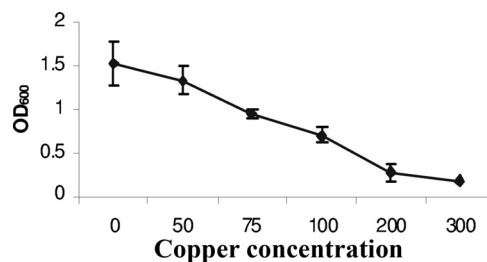

FIG. 1. Graph showing growth of *S. aureus* SH1000 in DM containing various concentrations of copper sulfate (in µM).

were read at 595 nm in a plate reader (Bio-Rad) to monitor growth. Wells were then washed three times with sterile PBS (phosphate-buffered saline) to remove nonadherent cells, dried at 65°C, and stained with 1% (wt/vol) safranine. After being stained, plates were read in a plate reader at 490 nm to determine the extent of biofilm formation. The mean results and standard deviations shown have been calculated from at least four independent experiments. A two-tailed Student's *t* test was used to determine the differences in *S. aureus* biofilm formation among the different metal ions. Differences were considered statistically significant when *P* was <0.05.

RESULTS

Excess environmental copper causes a global adaptive response involving a number of stress responses and virulence gene regulators. To investigate the *S. aureus* response to sudden copper stress, microarray analysis was used to determine the transcriptome of exponentially growing *S. aureus* exposed to excess copper. To determine the appropriate concentration of copper for the transcriptional profiling, *S. aureus* SH1000 was first grown in chemically defined medium (DM) with the addition of various concentrations of CuSO$_4$ (Fig. 1). *S. aureus* grew to maximal levels in DM in the absence of added copper, whereas the MIC of CuSO$_4$ in the defined media was 200 µM. Therefore, the subinhibitory concentration of 100 µM CuSO$_4$ was added to DM under the transcriptional profiling excess copper condition. A doubling time of 90 min was observed in the presence of 100 µM CuSO$_4$ compared to that of 60 min in the absence of copper.

To determine the effect of excess copper on *S. aureus* gene expression, exponentially growing *S. aureus* was exposed to excess copper for 30 min as described in previous studies (41). Very little growth occurred in the copper-containing culture after 30 min (the OD$_{600}$ was from 0.4 to 0.44), whereas some growth occurred in the control culture (the OD$_{600}$ increased from 0.4 to 0.53). This reflects a 17% inhibition in the growth of the test culture during the 30-min copper exposure time. Total RNA was extracted from exponentially growing SH1000 grown in DM for 30 min with or without the addition of 100 µM CuSO$_4$. cDNA samples were hybridized with the PFGRC *S. aureus* genomic microarray. After statistical evidence with a ≥2.0-fold difference as the threshold change and a *P* value of less than 0.05 was applied, microarray analysis showed that exposure to excess copper induced 39 genes and repressed 58 genes which fall into four main groups (see Table S1 in the supplemental material). As shown in Fig. 2, excess copper induces a copper stress response, which includes copper export and chaperone mechanisms and the misfolded protein and oxidative stress responses. The microarray analysis also shows that there is repression of the protein biosynthesis genes, a
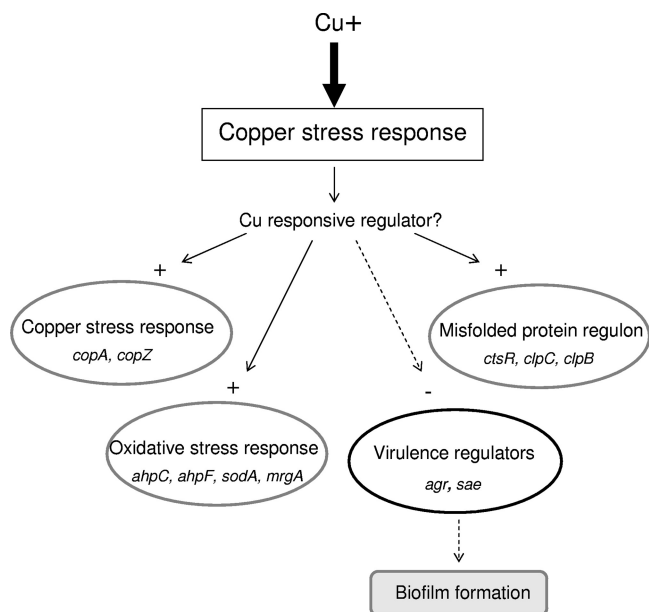
FIG. 2. Model of the *S. aureus* adaptive copper response. Excess copper induces the copper, protein misfolding, and oxidative stress regulons, represses virulence gene regulators Agr and Sae, and hence, represses biofilm formation. Arrows indicate positive regulation, and dotted arrows indicate negative regulation.

number of transporters, and the virulence gene global regulators *agr* and *sae*. There are also a large number of hypothetical genes which are differentially regulated by excess copper (Tables 3 and 4).

Excess copper induces copper homeostasis mechanisms. Our previous studies identified a direct mechanism for copper homeostasis that involved induction of the *copAZ* operon, which encodes the CopA and CopZ proteins, both of which have metal binding domain motifs MXCXXC (37). The tran-

scriptional profiling experiments showed that both *copA* and *copZ* are significantly induced by copper (fold changes of 6.6 and 6.8, respectively), thus demonstrating that the microarray results are representative of the global response to excess copper (36) (Table 3). However, the transcriptional profiling did not identify any other known copper homeostasis homologues or any other proteins with metal binding motifs, although it did identify a number of hypothetical genes with increased expression in excess copper of the same order as that of the *copA* and *copZ* genes (see Table S1, MW1746, 5.8-fold; SACOL1858, 4.61-fold; and SAV0454, 4.05-fold, in the supplemental material). These may represent components of novel *S. aureus* copper homeostasis mechanisms and require further investigation.

The protein translation is downregulated and the misfolded protein and oxidative stress responses are induced by excess copper. Our transcriptional profiling suggests that an *S. aureus* adaptive response to copper shock is the temporary downregulation of protein synthesis. The addition of copper to exponentially growing *S. aureus* resulted in a decrease in the expression of at least 20 genes encoding ribosomal proteins, translation initiation factors, and the RNA polymerase *rpoA* subunit (Table 4), suggesting that there is a corresponding decrease in protein synthesis upon copper shock.

Copper shock also resulted in the induction of the CtsR-regulated misfolded protein response, as addition of copper induced the expression of the *ctsR* repressor gene as well as that of the genes encoding the energy-dependent proteolytic complex *clpC* and *clpP* and encoding the *clpB* chaperone (Table 3). In gram-positive bacteria, the Clp proteolytic complex plays an indispensable role in cellular protein quality control by refolding or degrading damaged proteins and is essential for virulence and survival under a number of stress conditions in several pathogenic bacteria, including *S. aureus* (11). Therefore, these results suggest that sudden exposure to copper damages staphylococcal proteins. Hence, the downregulation

TABLE 3. Genes upregulated in excess copper

Locus tag	Gene[a]	Description	Fold change
Direct copper homeostasis mechanism genes			
SACOL2573	*copZ*	Copper ion binding protein	6.8
SAV2557	*copA*	Copper-transporting ATPase	6.64
SAS2591	NA	DNA binding protein	2.24
SAS1326	*phoU*	Putative phosphate transport system protein	2.22
SAS1804	NA	Putative exported protein	2.1
Oxidative stress resistance genes			
SAV1713	NA	Probable thiol peroxidase	2.77
SAS2042	*mrgA*	Putative non-heme-containing ferritin	2.71
SAS0357	*ahpF*	Alkyl hydroperoxide reductase subunit F	2.68
SAR0135	*sodM*	Superoxide dismutase	2.56
SACOL1155	*trxA*	Thioredoxin	2.42
SAV0764	*trxB*	Thioredoxin reductase	2.20
SAV2187	NA	Quinine oxidoreductase	2.14
Misfolded protein response genes			
SACOL0570	*clpC*	ClpC ATPase	3.25
SACOL0833	*clpP*	ATP-dependent ClpP protease	2.58
SAV0975	*clpB*	ClpB chaperone	2.13
SAS0479	*ctsR*	CtsR transcriptional repressor	2.44

[a] NA, not applicable.

TABLE 4. Genes downregulated in excess copper

Locus tag	Gene	Description	Fold change
Transport genes			
SAV0990	*oppA*	Oligopeptide transporter binding protein	−2.16
SAV2316	*potE*	Transport protein	−2.13
SAV0950	*mnhC*	Putative monovalent cation/H$^+$ antiporter subunit C	−2.1
SAS2358	*opp-1A*	Oligopeptide transporter binding protein	−2.09
Regulator genes			
SACOL2024	*agrD*	Accessory gene regulator protein D	−2.17
SACOL0765	*saeS*	Sensor histidine kinase SaeS	−2.00
SACOL0766	*saeR*	Response regulator SaeR	−2.01
Cofactor and carrier biosynthetic genes			
SAV1769	*ribA*	Riboflavin biosynthesis	−2.48
SACOL0538	*ispE*	4-Diphosphocytidyl-2C-methyl-D-erythritol kinase	−2.21
SAV1771	*ribD*	Riboflavin-specific deaminase	−2.11
SACOL1819	*ribE*	Riboflavin synthase, alpha subunit	−2.00
Protein biosynthesis genes			
SACOL0590		30S ribosomal protein L7	−2.09
SACOL0591	*rpsL*	Ribosomal protein S12	−2.25
SACOL0592	*rpsG*	Ribosomal protein S7	−2.36
SACOL0593	*fusA*	Translation elongation factor	−2.29
SACOL2212	*rplQ*	Ribosomal protein L17	−2.17
SACOL2213	*rpoA*	DNA-directed RNA polymerase	−2.27
SACOL2216	*rpmJ*	Ribosomal protein L36	−2.75
SACOL2217	*infA*	Translation initiation factor IF-1	−2.18
SACOL2220	*rplO*	Ribosomal protein L15	−2.02
SACOL2224	*rplF*	Ribosomal protein L6	−2.18
SACOL2225	*rpsH*	Ribosomal protein S8	−2.19
SACOL2226	*rpsN2*	Ribosomal protein S14	−2.33
SACOL2227	*rplE*	Ribosomal protein L5	−2.65
SACOL2228	*rplX*	Ribosomal protein L24	−3.09
SACOL2229	*rplN*	Ribosomal protein L2	−2.14
SAV2247	*rplB*	Ribosomal protein L36	−2.75
SACOL2234	*rplV*	Ribosomal protein L22	−2.09
SACOL2235	*rpsS*	Ribosomal protein S19	−2.17
SACOL2238	*rplD*	Ribosomal protein L4	−2.24
SACOL2239	*rplC*	Ribosomal protein L3	−2.75

of *S. aureus* protein synthesis could be in direct response to the protein damage caused by the presence of copper.

Our microarray analysis also shows that the expression of the oxidative-stress resistance genes alkyl hydroperoxide reductase subunit F (*ahpF*), superoxide dismutase (*sodM*), and *mrgA* is induced in *S. aureus* more than 2-fold by excess copper (Table 3). *S. aureus* has a number of mechanisms for protection against oxidative stress. *S. aureus* MrgA is an important nucleoid protein which demonstrates induced expression during oxidative stress and is important for the compaction of the nucleoid during oxidative stress, which may protect the DNA from damage due to reactive oxygen species (25). SodM is one of two *S. aureus* superoxide dismutases, SodA and SodM, which catalyze the conversion of the highly reactive O_2^- to O_2 and H_2O_2 (17). The H_2O_2 is then reduced to H_2O and O_2 by the compensatory functions of catalase (*katA*) and alkyl hydroperoxide reductase (*ahpC*) (7). AhpF is a homodimeric flavoenzyme carried by the same operon as *ahpC*, which catalyzes the NADH-dependent reduction of AhpC, therefore regenerating AhpC with every catalytic cycle. Northern blot analysis confirmed the transcriptional profiling results, as *ahpF* and *sodM* transcription increased in DM with excess copper (Fig. 3A). This induction of oxidative stress genes agrees with the
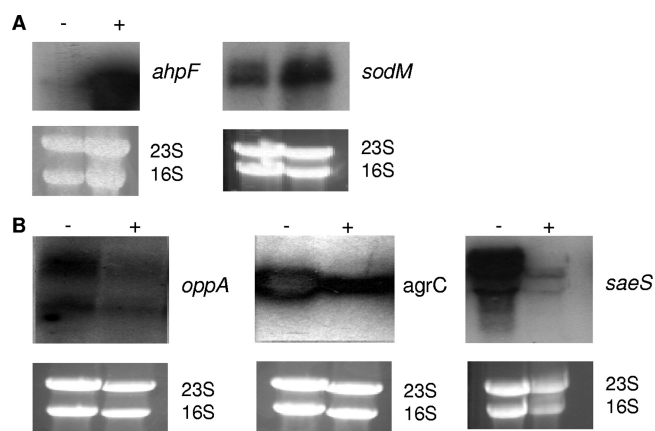

FIG. 3. Northern blot analysis was used to confirm differential regulation of genes in response to excess copper identified by the microarray analysis. *S. aureus* SH1000 was grown in DM until an OD$_{600}$ of 0.4 was reached. A total of 100 μM copper was then added, and following 30 min of incubation, total RNA was extracted. Ten micrograms of RNA was resolved by agarose gel electrophoresis and hybridized with radiolabeled *ahpF* and *sodM* DNA probes (A) and radiolabeled *oppA*, *agrC*, and s*aeS* DNA probes (B). Ethidium bromide-stained 23S and 16S rRNA provided loading controls.

proposal that copper toxicity is caused partly by Cu^{1+} catalyzing the formation of highly reactive hydroxyl radicals from H_2O_2 via the Haber-Weiss reaction (41). However, our microarray study suggests that H_2O_2 stress is not the only toxic effect of excess copper, as the thioredoxin *trxA* and thioredoxin reductase *trxB* genes which are required for combating thiol oxidation are also induced by excess copper. These genes are induced in response to oxidative stress agents that result in increased disulfide bond formation but are not induced by H_2O_2 (45), suggesting that excess copper also causes thiol oxidation in *S. aureus*.

Resistance to oxidative stress is important for *S. aureus* growth in excess copper. Oxidative stress genes have also been shown to be induced in response to copper stress in *P. aeruginosa* and *M. tuberculosis* (41, 47), but the role of the oxidative stress genes in copper resistance has not actually been shown for any bacterium to date. Therefore, to determine the role of the oxidative stress response in *S. aureus* copper resistance, we investigated the effect of deleting the oxidative stress genes *ahpC*, *katA*, *sodA*, and *sodM* on *S. aureus* growth under low- and high-copper conditions. *S. aureus* SH1000 and the isogenic mutants were grown in CRPMI for 24 h with or without the addition of 50 or 100 μM $CuSO_4$. The copper MIC for SH1000 in CRPMI is 100 μM. This is in contrast to the MIC of 200 μM in the DM used under the microarray conditions, where 100 μM copper only partially inhibited growth (Fig. 1). This is reflective of the increased sensitivity of bacteria to copper grown under nutrient-deprived growth conditions (37).

There was no significant difference between the growth of the wild-type SH1000 and that of the single mutant strains in CRPMI with 100 μM copper, as the growth of all the strains was inhibited (Fig. 4A). There were also no significant differences between the wild-type and single mutant strain growth with 50 μM copper added, as all strains showed a 40 to 50% decrease in growth (Fig. 4A). However, previous publications have shown that AhpC and KatA have compensatory roles, as significant phenotypes were seen only with the double mutant strains (7), and superoxide dismutase (SOD) activity in *S. aureus* can be provided by both SodA and SodM (17). Therefore, we compared the growth of the double mutant strains SH1000 *ahpC katA* and SH1000 *sodA sodM* with wild-type SH1000 in low and high levels of copper. In CRPMI plus 50 μM copper, there was a significant decrease in the growth of SH1000 *ahpC katA* compared to wild-type SH1000 ($P = 0.00002$) (Fig. 4A), which suggests that H_2O_2 scavenging is an important *S. aureus* copper resistance mechanism.

The importance of H_2O_2 in *S. aureus* copper toxicity was further confirmed by the observation that in CRPMI with 100 μM copper, SH1000 *sodA sodM* mutant growth was significantly increased compared to wild-type SH1000 ($P = 0.0002$). Therefore, these results confirm the microarray results and show that a proportion of *S. aureus* copper toxicity is due to oxidative stress and the production of free radicals and that superoxide dismutase activity is actually contributing to copper toxicity. Furthermore, these results show that the oxidative stress mechanisms are required for continued growth in high levels of copper and do not just counteract the initial copper shock.

To further investigate the role of oxidative stress in *S. aureus*

copper toxicity, we compared wild-type SH1000, SH1000 *ahpC katA* and SH1000 *sodA sodM* growth under high- and low-copper conditions with and without the addition of exogenous catalase and SOD (Fig. 4B). The addition of external oxidative stress quenchers will determine if the redox reactions are occurring intracellularly only or also at the cell surface. The presence of these ROS quenchers had no effect on growth of *S. aureus* SH1000 under low-copper conditions. However, *S. aureus* SH1000 growth was significantly increased when either catalase or SOD was present under high-copper growth conditions (Fig. 4B) ($P = 0.03$ and 0.01, respectively). Exogenously added catalase or SOD is likely to remain extracellular; therefore, these data suggest that extracellular copper has a toxic effect on *S. aureus* growth, as exogenous catalase and SOD cannot protect the cell from excess intracellular copper. Addition of catalase to SH1000 *ahpC katA* also had a protective effect, as there was a significant increase in growth of the mutant in 100 μM copper ($P = 0.005$). However, exogenous SOD did not increase the survival of the *ahpC katA* mutant, suggesting that catalase and AhpC are required to convert the H_2O_2 produced by SOD to H_2O and O_2. Interestingly, exogenous catalase did not increase SH1000 *sodA sodM* survival, and in fact, the double mutant was more sensitive to copper in the presence of exogenous SOD ($P = 0.04$) than in its absence. This suggests that the H_2O_2 produced by the exogenous SOD is not dealt with correctly in the SH1000 *sodA sodM* mutant. Therefore, our data suggest that extracellular copper is toxic to *S. aureus*.

Repression of biofilm formation by excess copper is mediated by the repression of positive biofilm regulators *agr* and *sae*. In addition to induction of the misfolded protein and oxidative stress responses, the microarray analysis also showed that excess copper results in the downregulation of a number of genes, including *mnhC*, a monovalent cation/H^+ antiporter subunit, *potE*, a putative amino acid transporter, and oligopeptide transporters *oppA* and *opp-1A* (Table 4). The repression of *oppA* in response to excess copper was confirmed by Northern blot analysis (Fig. 3B). The OppBCDFA transporter has been shown to be required for the transport of oligopeptides (14), but the function of Opp-1A is unknown. Opp-1A does have 35% homology with the *Escherichia coli* NikA nickel transporter (14); therefore, it may have a role in transporting metal ions into the cytoplasm, which may be deleterious when there is excess copper. Hence, its expression is repressed. It is not clear why the expression of these transporters is regulated by copper. The effect of copper on these transport systems may be direct or may be caused by excess copper levels affecting intracellular ion levels per se.

Excess copper also repressed the two-component regulator genes *agr* and *saeRS* (Table 4). Agr and Sae are particularly important virulence gene regulators and are essential for *S. aureus* virulence. The *agr* system represses the expression of cell wall-associated proteins and induces the expression of several exoproteins during postexponential growth, while the *saeRS* locus also induces the expression of exoproteins, including α-hemolysin and protein A (2), and is essential for innate immune evasion (46).

Our previous studies have shown that Agr and SaeRS are required for the induced expression of the Emp and Eap extracellular proteins, which are important for biofilm for-
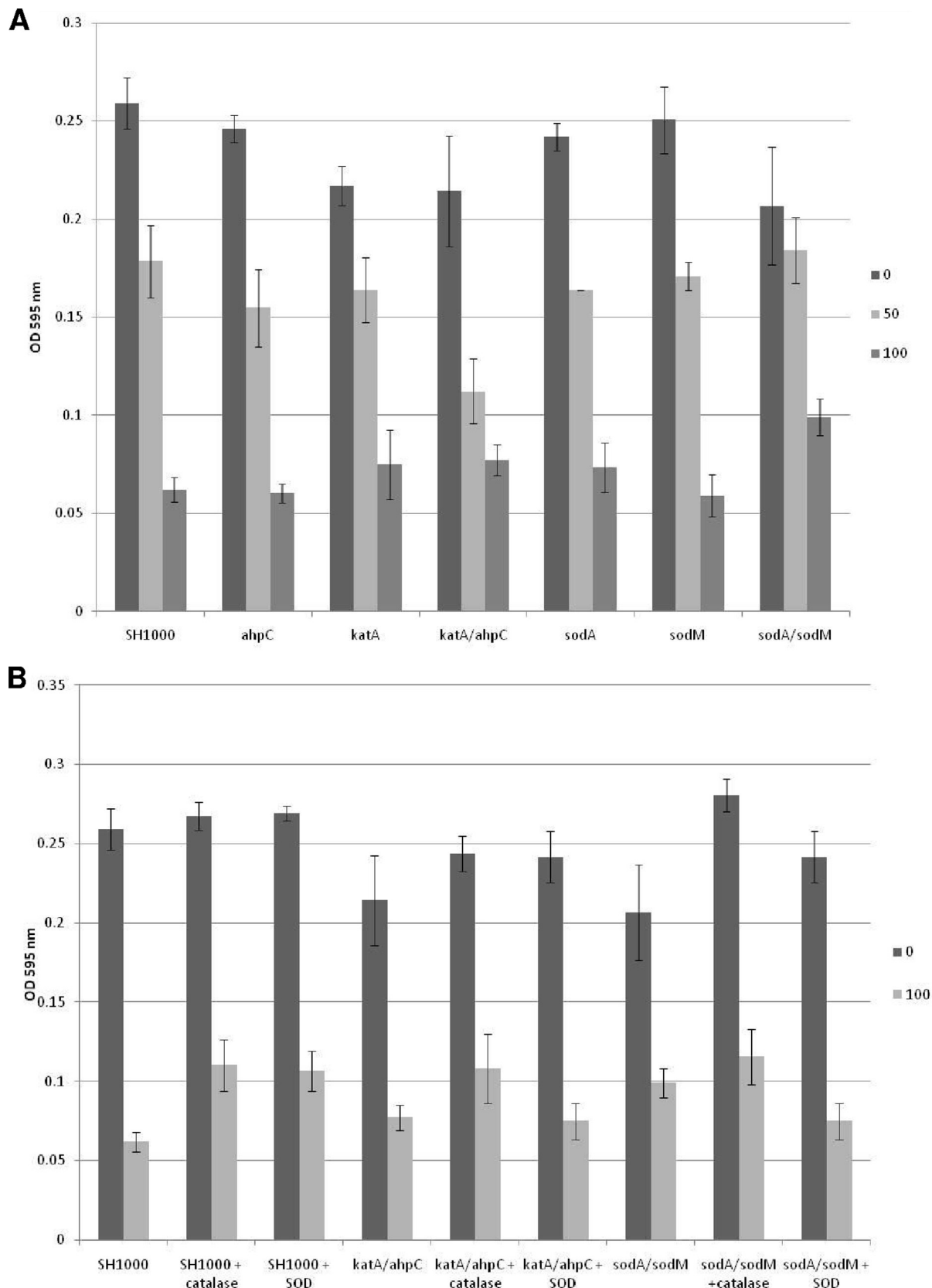
FIG. 4. Effect of oxidative stress resistance gene mutations (A) and the addition of exogenous ROS quenchers (B) on growth in excess copper. The *S. aureus* SH1000 wild type and SH1000 *ahpC*, *katA*, *ahpC katA*, *sodM*, *sodA*, and *sodM sodA* mutant cultures were grown overnight in CRPMI, then diluted to an OD_{600} of 0.05 in fresh CRPMI with and without 50 or 100 μM copper, inoculated into quadruple wells of 96-well flat-bottomed tissue culture plates, and incubated statically at 37°C for 24 h. After incubation, plates were read at 595 nm to monitor growth. Each point represents the mean result and standard deviation of at least 4 independent experiments. (B) A total of 500 U/ml of catalase or 10 U/ml of superoxide dismutase (SOD) enzymes were added before incubation at 37°C, where indicated.
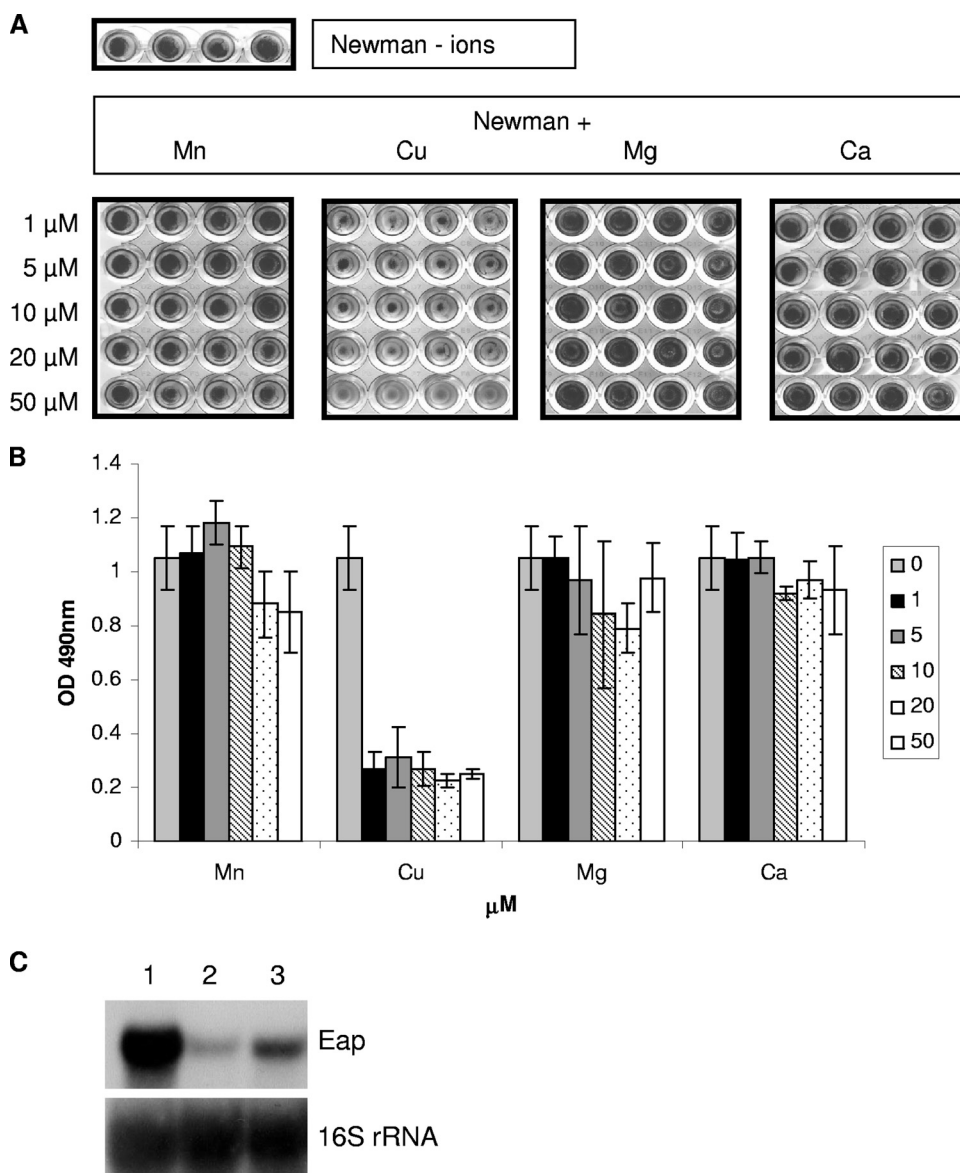
FIG. 5. Biofilm (A and B) and Northern blot analysis (C) of *S. aureus* Newman grown for 24 h in CRPMI, showing the effect of copper and other metal ions on biofilm formation and Eap expression. (B) Results represent the mean and standard deviation of at least four independent experiments. (C) A total of 10 μg of total RNA prepared from *S. aureus* Newman cells growing exponentially in CRPMI (lane 1), CRPMI with 50 μM Fe$_2$(SO$_4$)$_3$ (lane 2), or 10 μM copper chloride (lane 3) was resolved by agarose gel electrophoresis and hybridized with *eap* DNA probe. The blot was then stripped and rehybridized with the 16S rRNA control probe.

mation under iron-restricted, nutrient-poor growth conditions (16). Hence, excess copper represses the expression of positive regulators of biofilm formation. Northern blot analysis confirmed the repression of *agr* and *sae* expression under excess copper conditions, thus confirming the microarray data (Fig. 3B). Therefore, we investigated the effect of copper as well as other divalent cations on *S. aureus* strain Newman biofilm formation. Biofilm assays were performed using *S. aureus* Newman grown in CRPMI with differing concentrations of copper, manganese, magnesium, and calcium (Fig. 5). There was no significant effect on biofilm formation due to the addition of manganese, magnesium, and calcium. However, there was a significant decrease in

biofilm formation with even the addition of only 1 μM copper. This was not due to any copper toxicity, as there was no significant difference in *S. aureus* Newman growth when 1 to 20 μM copper was added, although the addition of 50 μM copper to CRPMI resulted in a 35% decrease in growth (data not shown). Therefore, these results suggest that copper prevents the formation of *S. aureus* biofilms through the repression of the positive biofilm regulators Agr and Sae. This was further confirmed by Northern analysis, which demonstrated that transcription of the *eap* gene, which is positively regulated by Agr and Sae and is important for low-iron-induced biofilm formation, is also repressed by excess copper (Fig. 5C).

DISCUSSION

In this study, we show that the *S. aureus* response to high levels of copper includes the induction of the copper stress, misfolded protein, and oxidative stress responses, as well as the repression of the global virulence gene regulators *agr* and *sae*. These expression studies suggest that copper toxicity in *S. aureus* is caused by protein damage and oxidative stress through thiol oxidation and the production of ROS. Our phenotypic studies show that the oxidative stress genes are required for *S. aureus* adaptive growth under high-copper conditions and initial copper shock, therefore demonstrating that oxidative stress has an important role in *S. aureus* copper toxicity. Furthermore, we show that H_2O_2 scavenging is a particularly important stress response and that extracellular copper toxicity occurs in *S. aureus*. These studies also show that the repression of *agr* and *sae* leads to the repression of proteins required for the important *S. aureus* virulence factor, biofilm formation.

Our microarray studies show that protein damage is an important feature of copper stress in *S. aureus*, as sudden exposure to copper results in the downregulation of *S. aureus* protein synthesis and the induction of the misfolded protein response. Misfolded protein responses are also upregulated by other bacteria such as *E. coli* (18), *P. aeruginosa* (41), and *M. tuberculosis* (47) in response to exposure to copper. In gram-positive bacteria, the Clp proteolytic complex plays an indispensable role in cellular protein quality control by refolding or degrading damaged proteins (11). However, as well as being involved in protein quality control, the CtsR regulon could also control the proteome in response to environmental change through the degradation of key transcriptional regulators. Previous studies suggest that the *S. aureus* ClpP protease (24) and ClpC ATPase (4) may be involved in regulating copper homeostasis, as a number of genes differentially regulated by copper are also regulated by ClpP and ClpC, such as the *copA*, *copZ*, *ctsR*, *clpB*, *clpC ahpC*, *sodM*, *sodA*, and *trxA* genes. The CtsR regulon may be induced by copper due to the presence of misfolded proteins, or the modulators of CtsR repressor activity may sense copper directly. In *B. subtilis*, the CtsR transcriptional regulator is targeted by McsA and McsB proteins for degradation by the ClpCP protease in the presence of misfolded proteins, hence modulating CtsR action (19). The *S. aureus mcsA* homologue contains a MXCXXC metal binding motif which may bind, and hence sense, excess copper, thus modulating CtsR activity and therefore inducing a copper response. This possibility needs to be investigated further, particularly as the role of McsA and McsB has not been determined in *S. aureus* to date.

Our microarray data also show that as well as there being some similarities in the copper response in *S. aureus* and other bacterial species, there are also significant differences, suggesting that *S. aureus* has a unique adaptive response to copper. In *E. coli* and *P. aeruginosa*, iron homeostasis is significantly affected by copper exposure, as a number of iron transporters and siderophore biosynthetic genes are induced by copper (18, 41). Furthermore, the iron-dependent global regulator *fur* is also induced by copper in *E. coli*, *P. aeruginosa*, and *M. tuberculosis*, demonstrating a link between copper and iron homeostasis in these organisms. However, our transcriptional profiling shows that while the *S. aureus* copper regulon con-

tains a number of genes that are also regulated by iron, these are genes involved mainly in the oxidative stress response. In *S. aureus*, there is no induction of iron uptake transporters, siderophore biosynthetic genes, or *fur* in response to excess copper, suggesting that copper and iron homeostasis may not be linked in *S. aureus*. Furthermore, in *S. aureus*, no homologues of copper-dependent transcriptional regulators are induced in response to high levels of copper. This is in comparison to transcriptional profiling of *M. tuberculosis* and *P. aeruginosa*, where high levels of copper induce the expression of the copper-responsive regulators CsoR and CueR and the CopRS two-component regulator system, respectively (41, 47), suggesting that there may be novel mechanisms for induction of the copper response in *S. aureus*.

Oxidative stress has been implicated in copper toxicity in bacteria such as *P. aeruginosa* and *M. tuberculosis*, as transcriptional profiling studies have shown that oxidative stress resistance genes are induced in response to copper stress (41, 47). However, studies in *E. coli* have shown that copper toxicity is not caused by DNA damage through oxidative stress but occurs mainly through inactivation of the iron-sulfur clusters of the dehydratase enzymes, leading to defective amino acid biosynthetic pathways (22). It is likely that copper-mediated inactivation of biosynthetic enzymes does occur in *S. aureus*, as SH1000 copper sensitivity is increased in CRPMI, which is an extremely nutrient-poor medium compared to other richer media such as DM and TSB (37). However, both our microarray and phenotypic studies demonstrate that oxidative stress resistance is important for *S. aureus* exposed to high-copper conditions.

In the absence of KatA and AhpC, *S. aureus* demonstrated an increased sensitivity to copper, whereas the absence of SodA and SodM decreased sensitivity, suggesting that superoxide dismutase activity is actually contributing to copper toxicity. This can be explained by the Haber-Weiss reaction, as copper can act as a catalyst with H_2O_2 to create toxic hydroxyl radicals, as follows: $Cu^{2+} + O_2^- \rightarrow Cu^{1+} + O_2$ (reaction 1); $Cu^{1+} + H_2O_2 \rightarrow Cu^{2+} + O_2 + OH^- + OH$ (reaction 2). Therefore, in the absence of all cellular SOD activity, there is less H_2O_2 to react with the copper. As a result, reaction 2 does not occur, hence showing increased resistance to copper. Therefore, H_2O_2 scavenging is an important *S. aureus* copper resistance mechanism. This agrees with our previous results which demonstrated that the copper efflux *copA* mutant shows increased sensitivity to H_2O_2 (37).

Previous studies demonstrated that copper toxicity in *Saccharomyces cerevisiae* was not due entirely to excess intracellular copper but was due to surface-produced Cu^{1+} (34), as the increased copper sensitivity observed due to the deletion of a surface-associated oxidase, Fet3p, was suppressed by the codeletion of the surface ferric/cupric reductase but not by the deletion of the major copper uptake transporters. Our results showing the protective effects of extracellular ROS quenchers and the importance of H_2O_2 suggest that copper toxicity is also occurring at the cell surface in *S. aureus*. It is not clear how surface Cu^{1+} is produced on the staphylococcal cell surface, but the oxidative stress enzymes do appear to be required for resistance to this surface toxicity. This would seem unlikely, as the oxidative stress enzymes are supposed to be intracellular; however, they have been found associated with cell surface

extracts in *S. aureus* (35), and therefore, our data suggest that this localization may not just be experimental artifact but may have physiological relevance. This evidence of extracellular copper toxicity may also help explain why copper surfaces are so effective at preventing *S. aureus* survival (28).

As well as inducing stress responses, excess copper also leads to the repression of two major global virulence gene regulators, *sae* and *agr*. Biofilm formation is an extremely important *S. aureus* virulence factor. Copper surface films on catheters show antibacterial activity against *S. aureus* biofilms (23), but it was not known why. Therefore, our observation that copper represses *sae* and *agr*, which results in the repression of proteins involved in biofilm formation, such as Eap, has important implications for the effect of copper on virulence and the antimicrobial uses of copper.

In summary, our data suggest that copper toxicity in *S. aureus* is caused through copper catalyzing the intracellular and extracellular generation of oxidative stress through the Haber-Weiss reaction and by affecting the function/folding of proteins. Hence, excess environmental copper leads to the stimulation of a global response, which includes the induction of the copper, oxidative stress, and misfolded protein responses and the repression of two important global regulators, Agr and Sae, both of which are essential for *S. aureus* virulence (Fig. 2).

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