Molecular characterization of the copper transport system in *Staphylococcus aureus*

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The *Staphylococcus aureus* *copA* gene codes for a putative copper-translocating P-type ATPase and the downstream *copZ* gene codes for a copper chaperone. Genome database analyses demonstrate that these copper transport genes are highly conserved in *S. aureus*. The expression of *copA* and *copZ* was inducible by copper and to some extent by ferric and lead ions. A mutant strain containing a partially deleted *copA* gene was more sensitive than the parent strain to copper, ferric and lead ions. The copper-sensitive phenotype was due to the accumulation of intracellular copper and thus the *copA* product is involved in the export of copper ions. The metal-sensitive phenotype of the mutant was complemented *in trans* by a 2.7 kbp DNA containing *copA*. We have cloned and overexpressed the metal-binding domains of CopA and CopZ and have shown by site-directed mutagenesis that the cysteine residues in the CXXC metal-binding motif in CopA are involved in copper binding and thus play an important role in copper transport in *S. aureus*.

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INTRODUCTION

Staphylococcus aureus is a Gram-positive bacterium that is the causative agent of a wide variety of human infections, ranging from superficial skin and wound infections to deep abscesses and more serious sequelae such as septicaemia, urinary tract infections, osteomyelitis and endocarditis (Archer, 1998). *S. aureus* is capable of growing in a wide range of adverse environmental conditions, including media containing heavy metals. Understanding of metal resistance in staphylococci has progressed rapidly in the past 20 years. It has been well established that some of the genes for resistance to metal ions, such as cadmium, mercury, antimony and arsenic, are encoded by plasmids (Silver & Phung, 1996). However, staphylococcal strains without plasmids also show resistance to heavy metal ions, such as zinc, cobalt and copper. We have previously identified genes involved in iron, zinc and cobalt transport in *S. aureus* (Xiong & Jayaswal, 1998; Singh *et al.*, 1999; Xiong *et al.*, 2000; Cabrera *et al.*, 2001). Some of these heavy metals, such as copper, iron and zinc, are necessary for life. However, little is known about genes involved in copper transport of *S. aureus*.

Copper is an essential trace element required by most organisms as a cofactor for numerous catabolic pathways and electron transport (Mason, 1976; Nicholas *et al.*, 2002; Massaro, 2002). However, copper is toxic to cells at

concentrations higher than physiological levels (Gaetke & Chow, 2003). Therefore, the intracellular concentration of copper is controlled by copper transport systems encoded by *cop* operons, which maintain copper homeostasis (Cooksey, 1993). Copper transport systems have been studied in several micro-organisms. Among bacteria the best-characterized system is that of *Enterococcus hirae*, where the *cop* operon consists of four genes: *copY*, *copZ*, *copA* and *copB*. *copA* and *copB* encode P-type ATPases involved in copper uptake and efflux, respectively. The expression of *copA* and *copB* is induced by copper and further regulated by CopY, a repressor and CopZ, an activator (Solioz & Stoyanov, 2003).

Recently we have identified genes involved in copper transport in *S. aureus* ATCC 12600 (Sitthisak *et al.*, 2005). This strain contained a *mco* gene which encoded a multicopper oxidase and a copper-ATPase (*cop*) gene with 57 % sequence similarity to *copB* from *Ent. hirae*. However, these *mco* and *cop* genes were found only in *S. aureus* MRSA252 and ATCC 12600 but not in the other strains examined. Most of the sequenced *S. aureus* genomes contained another gene designated *copA* which encodes a copper-translocating P-type ATPase followed by a copper metallochaperone (*copZ*).

Copper-ATPase belongs to a large superfamily of CPx-P-type ATPases (Lutsenko & Kaplan, 1995; Solioz & Vulpe, 1996; Rensing *et al.*, 2000; Gatti *et al.*, 2000). Translocation of cations across the cell membrane by copper-ATPases is achieved by utilizing the energy of hydrolysis of the

Abbreviations: BCA, bicinchoninic acid; IAA, iminodiacetic acid-agarose; MBD, metal-binding domain.

terminal phosphate bound in ATP (Lutsenko & Kaplan, 1995). The structure of CPx-type ATPase contains transmembrane helices and conserved structural elements. These elements are the phosphorylation domain (DKTGS/T), the phosphatase domain (TGES/A), the ATP-binding domain (MXGDGXNDXP), and the conserved putative metal-binding cysteine-proline-x sequence (CPx) in the intramembrane domain. The hydrophilic N-terminal part of CPx-type ATPases encompasses one to six heavy-metal-binding domains (MBDs) containing a GMTCXXC motif (Lutsenko *et al.*, 1997; Deigweier *et al.*, 2004). The paired cysteine residues in this domain play an important role in heavy metal binding (Walker *et al.*, 2002, 2004).

The *copZ*-encoded copper chaperone is a small protein involved in the intracellular delivery of copper to copper-utilizing enzymes. It functions by protecting the intracellular milieu from copper toxicity and releasing copper to its partner proteins. In *Ent. hirae*, CopZ delivers copper to the CopY repressor. In a number of bacteria, the *copZ* gene is located adjacent to the genes encoding copper-ATPases (Pufahl *et al.*, 1997). CopZ has also been shown to interact with CopA, which may be the site of copper loading of CopZ (Multhaup *et al.*, 2001). Sequence similarities with the N-terminal metal-binding (CXXC) motif from copper-ATPases and metal chaperones have been identified in both eukaryotes and prokaryotes (Harrison *et al.*, 2000).

In this study, we have characterized CopA, a copper-ATPase, and CopZ, a copper chaperone, with respect to their roles in copper transport, their metal-binding domains, and their expression in response to various heavy metals. We have shown that CopA is involved in copper

efflux. Disruption of *copA* resulted in the accumulation of intracellular copper and a copper-sensitive phenotype compared to the parent *S. aureus* strain. Northern blot analysis showed induction of *copA* and *copZ* transcription by copper and, to some extent, by iron and lead. We have cloned and overexpressed the MBDs of CopA and CopZ and have shown by mutagenesis that the cysteine residues in the CXXC metal-binding motif in CopA are involved in copper binding and thus play an important role in copper transport in *S. aureus*.

METHODS

Strains and culture conditions. *S. aureus*, *Escherichia coli* and plasmids used in this study are described in Table 1. *S. aureus* was grown in defined medium (DM) (Townsend & Wilkinson, 1992) or tryptic soy broth (TSB) and *E. coli* was grown in Luria-Bertani broth (LB). When needed, ampicillin (50 µg ml⁻¹), carbenicillin (50 µg ml⁻¹), chloramphenicol (10 µg ml⁻¹), erythromycin (20 µg ml⁻¹) and tetracycline (10 µg ml⁻¹) were added to the growth medium.

Construction of *copA* deletion mutant and complemented *S. aureus* strains. Mutation in *copA* was achieved by an in-frame deletion technique using plasmid pKOR1 as described by Bae & Schneewind (2006). The upstream and downstream regions of *copA* were amplified by PCR using primers *copA*-N1/*copA*-N2 and *copA*-C1/*copA*-C2 (Table 2), with *S. aureus* SH1000 DNA as a template and *Taq* polymerase enzyme (Promega). The two amplified fragments digested at the *Bam*HI site were ligated. The ligated DNA was PCR amplified using primers N1 and C2, which contained *att* sites, and integrated into pKOR1 using site-specific recombination. The recombinant plasmid was transformed into *E. coli* DH5α. Plasmids were purified and verified by sequencing. The resulting plasmids were transformed into *S. aureus* RN4220 and then SH1000. Co-integration

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>S. aureus</i>		
SH1000	NCTC 8325-4 with <i>rsbU</i> mutation repair	Horsburgh <i>et al.</i> (2002)
RN4220	NCTC 8325-4 r ⁻ ; capable of stably maintaining recombinant plasmids	Kreiswirth <i>et al.</i> (1983)
Δ <i>copA</i> mutant	SH1000 with <i>copA</i> partial deletion mutation	This study
<i>copA</i> complement	SH1000-Δ <i>copA</i> mutant containing pLI50- <i>copA</i>	This study
<i>E. coli</i>		
DH5α	<i>supE44 ΔlacU169 (φ80lacΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Invitrogen
BL21(DE3)(pLyS)	F ⁻ <i>ompT hsdSB(r_B⁻ m_B⁻) gal dcm</i>	Novagen
BL21(DE3)(pLyS)/pSA-MBD	BL21(DE3)(pLyS) containing pSA-MBD, Amp ^r Cam ^r	This study
BL21(DE3)(pLyS)/pSA-MBD mutant	BL21(DE3)(pLyS) containing pSA-MBD mutant, Amp ^r Cam ^r	This study
Plasmids		
pCR2.1	PCR cloning vector, Amp ^r Kan ^r	Invitrogen
pKOR1	<i>E. coli</i> / <i>S. aureus</i> shuttle vector; permits rapid cloning via lambda recombination and <i>ccdB</i> selection	Bae & Schneewind (2006)
pLI50	<i>S. aureus</i> shuttle vector	Lee <i>et al.</i> (1987)
pRSETa	Overexpression vector, Amp ^r	Invitrogen
pSA-MBD	pRSETa with MBD from <i>copA</i>	This study
pSA-MBD mutant	pRSETa with mutated MBD from <i>copA</i>	This study
pLI50- <i>copA</i>	pLI50 containing <i>copA</i> promoter and <i>copA</i>	This study

Table 2. Primers used in this study

Name of gene/region	Primer	Sequence (5'–3')*
MBD of <i>copA</i>	MBD-N1	GATATCACTGGTATGACT GCGGCCGCAGCG TCAAATCGTATCGAAAAG
	MBD-N2	CATTACAGGTATGACT GCGGCTGCAGCG TCAAGCCGTATTGAAAAAG
	MBD-C1	CTTTTCGATACGATTTGAC CGCTGCGGCCGCAGTC CATACCAGTGATATC
	MBD-C2	CTTTTTCGAATACGGCTTGAC CGCTGCAGCCGCAGTC CATACCTGTAATG
	MBD-F	GGATCCGTGGCTAATACGAAAAAACAACATT
	MBD-B	AAGCTTGCTTAATCAATT TATGTTTCGCG
<i>copA</i>	<i>copA</i> -N1	GGGGACAAGTTTGTACAAAAAAGCAGGCTG TGGCTAATACGAAAAAACAACATT
	<i>copA</i> -N2	GGGGGATCCCACTTGTACCAACAGCAACAAGTA
	<i>copA</i> -C1	GGGGGATCCGGTTGGTAACCGTAAATTAATGGC
	<i>copA</i> -C2	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAG GCATCTTTACGGCGTG
	Com-F	GGAAGCTTGGTTGGTAACCGTAAATTAATGGC
<i>copZ</i>	Com-B	GGGAATTCTAGGCATCTTTACGGCGTG
	<i>copZ</i> -F	CGGATCCATGTCACAAGAAATTTTAA
	<i>copZ</i> -B	GAAGCTTTTAAACGACATCGTAACCT

*Modified bases are indicated by bold letters. The site-specific recombination *attB1* and *attB2* sites are underlined. Restriction sites are in italic.

was induced by growing the bacteria at 43 °C. The integration mutants were subcultured at 30 °C and screened for the loss of tetracycline resistance. The partially deleted *copA* mutant ($\Delta copA$) was further confirmed by PCR and DNA sequencing using *copA*-N1 and *copA*-C2 primers. As shown in Fig. 1, there was a deletion of 1009 bp from the *copA* gene.

To complement the mutation of *copA*, a 2.7 kb fragment containing *copA* was cloned into the shuttle vector pLI50 (Lee *et al.*, 1987). Two primers, Com-F and Com-B (Table 2) were designed and the PCR was performed using *S. aureus* SH1000 DNA as a template. The amplified fragment was cloned into pCR2.1 vector (Invitrogen) and subsequently into plasmid pLI50. The resulting plasmid was transformed into *S. aureus* RN4220. The plasmid was reisolated from *S. aureus* RN4220 and then transformed into the SH1000 $\Delta copA$

mutant. The transformants were tested for their ability to grow in TSB containing 1.5 mM CuSO₄.

Northern blot analysis. RNA was isolated using a Qiagen kit. Ten micrograms of total RNA was electrophoresed in a 1% agarose/0.66 M formaldehyde gel. RNA was transferred to nylon membrane (Millipore) and the blot was probed with radiolabelled *copA* (700 bp) or *copZ* probe (200 bp) under aqueous phase conditions at 65 °C. The probe was prepared using the Prime-a-Gene labelling system (Promega) in the presence of [α -³²P]dCTP.

Determination of intracellular copper, iron and lead concentration. To measure the copper, iron and lead content of the cells, overnight-grown cultures were diluted 1:100 in TSB supplemented

Fig. 1. Schematic representation of *copA* and *copZ* on the chromosome of *S. aureus* SH1000. The first nucleotide of *copA* is used as the starting point for base pair numeration. The arrow indicates the gene and orientation. The thick black line indicates the region (700–1709 bp) that was deleted in the *copA* mutant. Hatched lines indicate the DNA fragments used as probes. A schematic representation of the copper-ATPase, with two heavy-metal-binding CXXC motifs (open circles) and conserved transmembrane domains (filled cylinders), is shown above the *copA* gene.

with 0.5 mM CuSO₄, 0.5 mM FeCl₃ or 0.5 mM PbSO₄ and incubated at 37 °C until the OD₆₀₀ reached 0.6. Cells were harvested and washed three times with 10 mM Tris/HCl (pH 7.4) containing 1 mM EDTA and once with MilliQ H₂O. Cells were dried overnight at 80 °C then dissolved in 30 % nitric acid and the copper, iron and lead contents were measured by inductively coupled argon plasma atom emission spectrometry (Radford *et al.*, 2003).

Cloning, expression and site-directed mutagenesis of the MBD encoded by *copA*. Two oligonucleotide primers, MBD-F and MBD-B (Table 2), were designed with a *Bam*HI site at the 5' end and a *Hind*III site at the 3' end of the DNA fragment. The PCR was performed using *S. aureus* genomic DNA as template and the PCR product was first cloned into pCR2.1 vector (Invitrogen) and subsequently into the *Bam*HI and *Hind*III sites of pRSETa (Invitrogen). The resulting plasmid, pSA-MBD, was transformed into *E. coli* BL21(DE3)(pLysS) (Novagen) by electroporation. To overexpress the cloned gene product, the transformants were grown in LB containing ampicillin and chloramphenicol at 37 °C until the OD₆₀₀ reached 0.5 and then the cells were induced for the expression of protein by the addition of 2.0 mM IPTG for 3 h. The induced cells were harvested, washed, and resuspended in lysis buffer (20 mM Tris/HCl, pH 7.4, containing 145 mM NaCl). Pellets were chilled on ice and homogenized by sonicator (Branson Sonifier 450) and cell debris was removed by centrifugation 10 000 g at 4 °C. The supernatants were applied to nickel-charged agarose-affinity columns (Novagen) and eluted with 200–400 mM imidazole. Eluted fractions were subjected to 12.5 % SDS-PAGE analysis. Fractions containing the overexpressed His-tag protein were pooled and dialysed against 25 mM Tris/HCl, pH 8.0, containing 100 mM sucrose, 50 mM NaCl and 1 mM DTT.

PCR-based site-directed mutagenesis was performed in order to replace Cys residues by Ala in the MBD of CopA as described by Allemandou *et al.* (2003). The four primers MBD-N1, MBD-N2, MBD-C1 and MBD-C2 were used to exchange Cys at positions 16, 19, 83 and 86 for Ala residues. Mutations were confirmed by DNA sequencing. The fragment corresponding to the mutated MBD was gel purified and subcloned into the *Bam*HI and *Hind*III sites of pRSETa, and the resulting plasmid, pSA-MBD mutant, was overexpressed in *E. coli* BL21(DE3)(pLysS) as described above. His-tag fragments from both the recombinant proteins were removed by using an enterokinase kit (Invitrogen) before performing the binding assay.

Bicinchoninic acid (BCA)-based copper-binding assay. The stoichiometry of copper binding by the putative wild-type MBD or the mutated MBD was determined by a BCA-based assay (Brenner & Harris, 1995; Lutsenko *et al.*, 1997). To determine the amount of copper bound to the protein *in vitro*, purified copper-free MBD or mutated MBD proteins at a concentration of 100 µg ml⁻¹ were mixed with CuCl₂ (20 µmol copper per µmol protein). After 10 min incubation at room temperature, and bound copper was removed by dialysis at 4 °C overnight and bound copper was measured by the BCA assay as described by Lutsenko *et al.* (1997).

Cation-binding specificity of the MBD by iminodiacetic acid-agarose (IAA) chromatography. IAA columns equilibrated with different heavy metals were used to determine the cation-binding specificity as described by Lutsenko *et al.* (1997). Columns containing 100 µl IAA (Sigma) were extensively washed with 50 mM sodium phosphate buffer (pH 7.5) and then separately equilibrated with 10 volumes of the same buffer containing one of several heavy metal chloride compounds (CdCl₂, CuCl₂, CoCl₂, MnCl₂ and FeCl₃) at a final concentration of 1 mM. Excess metal ions were removed by extensive washing with sodium phosphate buffer and then 100 µg purified MBD protein or mutated MBD protein (ΔMBD) was added to the resin and incubated for 10 min at room temperature. Columns

were centrifuged to remove unbound proteins. Columns were washed with 500 µl sodium phosphate buffer and bound proteins were eluted from the column with 50 mM EDTA in sodium phosphate buffer. Both eluted and unbound proteins were concentrated and analysed by 12.5 % SDS-PAGE.

Involvement of cysteine residues in the MBD. Involvement of the cysteine residues was demonstrated by the ability of copper to protect the cysteine residues in the MBDs against labelling with the cysteine-directed fluorescent reagent 7-diethylamino-3-(4'-maleimidyl-phenyl)-4-methylcoumarin (CPM), as described by Lutsenko *et al.* (1997) and Walker *et al.* (2002). Briefly, 50 µg of purified MBD or ΔMBD protein was incubated in the presence of different concentrations of copper for 10 min, and then pulse-labelled with a 20 molar excess of CPM for 1 min in the dark. Proteins were separated by 15 % SDS-PAGE. The CPM-labelled proteins were then monitored under UV light.

Molecular genetic procedures. Plasmid and chromosomal DNA isolation, DNA manipulation, digestion of DNA with restriction enzymes, DNA ligation, Northern blot analysis and PCRs were performed as described by Sambrook & Russell (2001).

RESULTS AND DISCUSSION

Analysis of copper transport genes in *S. aureus*

The genome analysis of seven strains of *S. aureus* showed the presence of similar copper transport systems consisting of two genes, designated *copA* (2408 bp) and *copZ* (206 bp), separated by approximately 200–800 bp. The *copA* gene is monocistronic, with no genes for potential regulatory proteins immediately upstream or downstream of the *copA* operon (Fig. 1). Upstream of *copA*, potential –35 (TTGCAC) and –10 (TAAAT) sequences, and a putative Shine–Dalgarno (GGGAGG) sequence, were identified. The molecular masses of the putative proteins encoded by *copA* and *copZ* were predicted to be 86.7 and 7.2 kDa, respectively. The *copA* gene encodes a copper-translocating P-type ATPase protein exhibiting features of a CPx-type ATPase that pumps copper out of the cytoplasm (Huffman & O'Halloran, 2001). CopA is a transmembrane protein that contains two heavy-metal-binding sites with CXXC motifs (MBD) in the N-terminal region, six conserved intramembrane helices, a phosphorylation domain, phosphatase domains and two additional transmembrane helices at the N-terminus. *copZ* encodes a copper-binding chaperone protein which contains only an MBD at the N-terminus. The putative characterization of CopA protein was achieved by using a computer program provided by San Diego Super Computer Center (<http://workbench.sdsc.edu/>).

The genome database analyses suggested that the genes involved in copper transport are conserved in *S. aureus*. *copA* and *copZ* showed almost 100 % sequence identity among *S. aureus* strains and about 75 % with other staphylococcal species. We also performed PCR using primers from *copA* and *copZ*, confirming that these genes are also present in other *S. aureus* strains such as ATCC 12600, H and Wood 46 whose genomes have not been

Fig. 2. Effect of copper on growth of *S. aureus* strain SH1000. Overnight grown cultures were diluted 1 : 200 in DM (○) and TSB (●), each containing various concentrations of CuSO₄. Cultures were incubated at 37 °C with shaking. Growth was monitored by measuring the OD₆₀₀ after 12 h. Each point represents the mean ± SD of three experiments.

sequenced (data not shown). The CopA from *S. aureus* showed 49 % sequence identity with CopA of *Ent. hirae*. In addition, the CopA of *S. aureus* showed a similar CPC motif and two metal-binding motifs (CXXC). CopA, which is responsible for the copper influx in *Ent. hirae*, has a similar CXXC domain (Solioz & Vulpe, 1996).

Copper tolerance in *S. aureus*

To determine the tolerance level of *S. aureus* SH1000 to copper, cells were grown in either chemically defined

medium (DM) or TSB containing various concentrations of CuSO₄. As shown in Fig. 2, the MIC of CuSO₄ in TSB was around 2.5 mM whereas in DM it was about 0.2 mM. The higher level of copper tolerance in TSB may be due to the faster growth rate and some factor(s) not available in DM.

copA and *copZ* transcriptions are induced by copper

Northern blot analysis was performed to evaluate the expression of *copA* and *copZ* during the growth of *S. aureus* in the presence of various metal ions. Total RNA was isolated from cultures exposed to various concentrations of copper, cobalt, cadmium, lead, iron and zinc. The expression of *copA* and *copZ* was induced when *S. aureus* SH1000 was grown in DM containing CuSO₄. Northern blot analysis revealed the presence of two transcripts, of ~2.4 kb and ~2.0 kb, when *copA* was used as probe (Fig. 3a). The 2.4 kb band represents the *copA* transcript whereas the 2.0 kb transcript might represent multiple transcripts from the same gene, a degradation product of *copA* or the transcript of some homologous gene. The expression of *copZ* was also dependent on copper ion concentration and it was induced by as low as 5 µM copper (Fig. 3b). A 0.2 kb transcript was detected when *copZ* was used as probe, which seemed to be in good agreement with that predicted from the sequence data.

In order to determine the metal ion specificity of *copA* and *copZ* induction, *S. aureus* was grown in the presence of various heavy metal ions such as cadmium, cobalt, ferric, lead, manganese and zinc ions. In addition to copper, the transcription of *copA* was to some extent induced by ferric

Fig. 3. Northern blot analysis to measure the expression of *cop* genes in response to various metal ions. *S. aureus* cells grown in DM under various conditions were collected at an OD₆₀₀ of 0.4 and total RNA was isolated as described in Methods. (a, b) Ten micrograms of RNA was electrophoresed on formaldehyde gel (1.2 %) and transferred to nitrocellulose membrane, and the blots were probed with radiolabelled *copA* (a) or *copZ* (b). Response to copper. Lane 1, RNA from *S. aureus* grown in DM; lanes 2–5, RNA from *S. aureus* grown in DM with CuSO₄ at 5 µM (lane 2), 50 µM (lane 3), 75 µM (lane 4) and 100 µM (lane 5). Positions of RNA molecular size markers (kb) are shown on the left. (c) Induction of *copA* by other metal ions. RNA was extracted from *S. aureus* grown in DM with 75 µM CuSO₄ (lane 1); 50 µM CoCl₂ (lane 2); 75 µM PbSO₄ (lane 3); 75 µM FeCl₃ (lane 4); 50 µM ZnCl₂ (lane 5) or 50 µM MnCl₂ (lane 6). The bottom panels of (a–c) show ethidium-bromide-stained gels indicating that equivalent amounts of RNA were loaded to each lane.

and lead ions (Fig. 3c). There was no effect of cobalt, cadmium, manganese, silver and zinc (Fig. 3c and data not shown). The induction of *copA* gene by other metal ions such as silver has been reported (Rensing *et al.*, 2000). We also measured the expression of *copA* and *copZ* during the growth cycle. The expression of *copA* and *copZ* was growth phase dependent: both genes were expressed to a higher level in the exponential phase than in the stationary phase (data not shown). As described above we have not found either any potential regulatory gene or possible regulatory sequences upstream of *copA*. Therefore, it is difficult to predict the mechanism by which copper regulates the expression of *copA* and *copZ* in *S. aureus*.

copA* mutation induces copper, iron and lead sensitivity in *S. aureus

A partial deletion in the *copA* gene was constructed as described in Methods. To examine the effect of mutation in *copA*, *S. aureus* strains SH1000 (parent) and the *copA* partial deletion mutant ($\Delta copA$) strains were grown in DM with or without copper. There was no difference in the growth rate between parent and $\Delta copA$ strains in DM without copper (data not shown). However, in the presence of 75 μ M copper sulfate the $\Delta copA$ strain showed

increased copper sensitivity (Fig. 4a). There was a significant difference in growth rate between the wild-type and the mutant strain ($P=0.004$). The $\Delta copA$ mutant was also tested for ferric iron and lead sensitivity, as the Northern analysis suggested that the expression of *copA* is also induced by ferric and lead ions. As shown in Fig. 4(b, c), the $\Delta copA$ mutant was also sensitive to ferric and lead ions. There was a significant difference in growth rate between the wild-type and the mutant strain ($P=0.004$ and 0.023 respectively). In *B. subtilis*, mutation in *copA* sensitized the cells to copper but not to other metal ions (Gaballa & Helmann, 2003).

Complementation experiments were performed to provide genetic evidence that the copper, ferric and lead sensitivity is due to the mutation in the *copA* gene. A 2.7 kb fragment containing the *copA* gene with promoter sequences was cloned in the shuttle vector pLI50 and transformed into the $\Delta copA$ mutant. The complemented strain was tested for growth in the presence of copper, ferric ions and lead. As shown in Fig. 4(a, b), the complemented strain was able to grow at the same rate as the parent strain in the presence of copper and iron but it grew slightly slower in the presence of lead (Fig. 4c). These results suggested that the copper-, ferric- and lead-sensitive phenotype of the mutant was due to the mutation in the *copA* gene. The capacity to transport

Fig. 4. Effect of copper (a), iron (b), lead (c) and hydrogen peroxide (d) on growth of *S. aureus* strains. Overnight cultures were diluted 1 : 200 in DM with 75 μ M CuSO₄ (a), 500 μ M FeCl₃ (b), 50 μ M PbSO₄ (c) or 1.5 mM hydrogen peroxide (d), and incubated at 37 °C with shaking. Cell growth was monitored by measuring OD₆₀₀. ◆, Wild-type; ■, *copA* mutant ; ▲, *copA* complemented strain. Each point represents the mean $\pm$ SD of three experiments.

diverse metals by CPX-ATPase has been shown in *Archaeoglobus fulgidus*, *B. subtilis* and other micro-organisms (Arguello *et al.*, 2003; Gaballa & Helmann, 2003). In addition, association between genes for copper transport and genes for iron transport has been found in *Saccharomyces cerevisiae* (van Bakel *et al.*, 2004, 2005). Since both lead and iron can induce the transcription of the *copA*, this further suggests that *copA* may function as a co-transporter for these ions.

Mutation in *copA* causes hydrogen peroxide sensitivity

Another phenotype of the $\Delta copA$ mutant was found to be its increased sensitivity to hydrogen peroxide. As shown in Fig. 4(d), the growth of $\Delta copA$ mutant in DM containing 1.5 mM H_2O_2 was slower compared to the parent strain. The $\Delta copA$ mutant's hydrogen peroxide tolerance was restored to the normal level, as observed in the parent strain, by complementation. We also checked the tolerance levels of the $\Delta copA$ mutant to another oxidative agent, paraquat, and found no difference (data not shown). The precise mechanism by which the $\Delta copA$ mutant becomes sensitive to hydrogen peroxide is not clear. However, as mentioned above CopA is a Cu(I)-translocating P-type ATPase, and mutation in *copA* causes the accumulation of Cu^+ in the mutant cells. In addition, the $\Delta copA$ mutant showed increased iron sensitivity (Fig. 4b) and a higher concentration of intracellular iron (Table 3). Iron and copper are both capable of catalysing the formation of hydroxyl radicals ($OH^\bullet$) from H_2O_2 via the Haber–Weiss reaction (Bremner, 1998; Pierre & Fontecave, 1999), which may be the cause of the H_2O_2 -sensitive phenotype.

Functional analysis of *copA*

The intracellular concentration of copper was measured to prove that *copA* was involved in the efflux of copper ions and that copper sensitivity was due to the accumulation of copper. *S. aureus* strains grown in TSB medium containing 0.5 mM copper were collected, washed and digested. They were used to determine intracellular copper concentration by inductively coupled argon plasma atom emission spectrometry (see Methods). As shown in Table 3,

intracellular copper concentrations increased over seven-fold in the *copA* mutant when cultures were grown in TSB containing 0.5 mM $CuSO_4$ compared to the parent strain ($P=0.0001$). In the $\Delta copA$ complemented strain the concentrations of intracellular copper were equivalent to the concentrations in the parent strain. We also determined the colony count of cells grown in the presence of various ions. There were slightly fewer c.f.u. ml^{-1} in the mutant compared to wild-type and the complemented strains (data not shown). However, this difference cannot account for a several-fold increase in the concentration of intracellular ions. The accumulation of high concentrations of copper ions in the $\Delta copA$ mutant cells is indicative of their inability to efflux copper. The lower concentration in the complemented strain also supports the idea that CopA functions as a copper transporter. Under normal physiological conditions, *copA* and *copZ* are not transcribed in either the mutant or the parent strain. This suggests that the *copA* is not essential for the growth of *S. aureus* under normal conditions but is required under conditions of high copper.

The intracellular concentrations of iron and lead were also measured (Table 3). The concentrations of these metals were higher in the $\Delta copA$ mutant when cultures were grown in TSB containing 0.5 mM $FeCl_3$ or 0.5 mM $PbSO_4$ ($P=0.001$ and 0.029 respectively). The accumulation of iron and lead ions in the $\Delta copA$ mutant cells may be due to the inability of these cells to efflux both metal ions.

Characterization of the MBD in *copA*

It has been shown that the N-terminal domains of the Wilson's and Menkes disease proteins bind copper selectively *in vivo* as well as *in vitro* (Lutsenko *et al.*, 1997; DiDonato *et al.*, 1997). Here we have investigated the role of the putative MBD in CopA protein from *S. aureus* strain SH1000. The MBD, containing $C^{16}XXC^{19}$ and $C^{83}XXC^{86}$ motifs, was cloned and overexpressed (Fig. 5a) as described in Methods, and its copper-binding ability was determined *in vitro*.

The requirement for cysteine residues in the MBD was investigated by site-directed mutagenesis of the CXXC motifs, in which four cysteine residues in the motifs were replaced by alanine. By using the BCA-based copper-binding assay, the stoichiometry of copper binding to the MBD *in vitro* was 1.54 ± 0.2 μmol copper per μmol protein, whereas 0.52 ± 0.2 μmol copper bound per μmol mutated MBD protein. Our experiments in a BCA-based assay showed that copper binds to the MBDs both *in vitro* and *in vivo* in the presence of the reducing agent DTT. This suggested that MBDs purified in the presence of DTT are in the reduced form, containing free thiol groups in the paired cysteine residues, and bind to copper better than oxidized MBDs (disulfide bound). Since the thiol groups are easily oxidized by oxygen, DTT is required in the buffer to maintain the reduced form of the MBD protein.

Table 3. Intracellular metal content of *S. aureus* strains

Each value represents the mean $\pm$ SD of three experiments.

<i>S. aureus</i> strain	Metal content [p.p.m. (mg dry wt) $^{-1}$]		
	Copper	Iron	Lead
Wild-type	0.0095 ± 0.001	0.0024 ± 0.001	0.036 ± 0.02
Mutant ($\Delta copA$)	0.068 ± 0.02	0.0085 ± 0.002	0.086 ± 0.01
Complemented	0.0078 ± 0.004	0.001 ± 0.001	0.035 ± 0.03

Fig. 5. (a) MBD protein (lane 1) and Cys-mutated MBD protein (lane 2) analysed on a 12.5 % SDS-polyacrylamide gel. (b) Binding of overexpressed MBD of CopA or Δ MBD protein to IAA-resin, equilibrated with different metal ions. IAA-resin was charged with different heavy metal as indicated above the respective lane. Bound and unbound proteins were concentrated and subjected to 12.5 % SDS-PAGE (see Methods for details). Row A, bound MBD protein; row B, unbound MBD protein; row C, bound Δ MBD protein; row D, unbound Δ MBD protein. (c) Fluorescent labelling to examine the involvement of cysteine residues in the MBDs. MBD proteins (50 μ g) were incubated with the fluorescent reagent 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin in the presence of the indicated copper chloride concentrations and then subjected to 12.5 % SDS-PAGE. Fluorescence was detected under UV light. Row A, MBD, row B, Δ MBD; row C, MBD-CopZ.

In addition, we tested the binding by using IAA chromatography, equilibrated with different heavy metals (copper, cobalt, cadmium, iron and manganese and lead). As shown in Fig. 5b, A, binding was detected for copper-, cobalt- and cadmium-equilibrated columns eluted by EDTA but no binding was observed for iron-, manganese- or lead-equilibrated columns (Fig. 5b, B). Based on *in vivo* studies (data presented in Figs 3 and 4) it was expected that iron and lead would bind to the MBD in IAA chromatography. In *in vitro* studies, IAA chromatography showed that cobalt binds to the MBD, but it does not induce the transcription of *copA* in Northern blot analysis (Fig. 3). The discrepancies in these results are difficult to interpret, but it might be that *in vivo* metal ions regulate the transcription of the *copA* through a separate metal-binding regulatory protein. *In vitro* binding of various metals to the MBD of the CopA protein may depend on conditions such as pH, ion concentration, binding constant, redox state of

metals, etc. The MBD of CopA and other copper P-type ATPases is thought to initially bind copper and then deliver it to a distinctly separate copper-transporting site (probably within the transmembrane domains). It is conceivable that iron binds directly to the copper transporting site without first interacting with the MBD. In this study we wanted to examine the role of the MBD of *copA*. We have shown that it binds copper *in vitro*, and a copper-protein complex may play an important role during copper transport. Northern blot data showed that copper regulates *copA* expression. *In vitro* the MBD binds cadmium and cobalt, but these metals do not regulate *copA* expression. It may be that the conformation of the protein when it binds to these non-copper metals is incompatible with gene expression. Of course, metal binding to the MBD and gene expression are two different phenomena and not necessarily correlated. *In vitro* studies suggest the role of the CXXC motifs in metal binding. When the Cys-mutated MBD protein was used in this experiment no binding was observed under any condition (Fig. 5b, C and D).

To further confirm the role of cysteine residues in the MBD, a cysteine-directed fluorescent reagent, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin was used as described in Methods. As shown in Fig. 5(c, A and C), when the MBDs from *copA* and *copZ* were incubated with fluorescent reagent in the presence of various concentrations of copper, a concentration of copper above 50 μ M prevented the fluorescent labelling of cysteine residues. There was no effect on fluorescent labelling when the MBD protein was incubated with other heavy metals. The labelling of the Cys-mutated MBD protein with fluorescent reagent was not observed, whether copper was present or not (Fig. 5c, B). These results confirmed that cysteine binds to copper and the formation of a copper-protein complex may play an important role during copper transport.

Concluding remarks

We have characterized a *copA* gene responsible for the efflux of mainly copper ions and probably also ferric and lead ions in *S. aureus*. The *copZ* gene encodes a copper-binding chaperone most probably involved in the intracellular delivery of copper to copper-utilizing enzymes. The expression of *copA* and *copZ* is inducible by copper and to some extent by ferric and lead ions. *In vitro* metal-binding studies showed that copper, cadmium and cobalt can bind to the MBD of CopA. We are currently investigating the regulation aspects of *copA* and *copZ* genes in *S. aureus* using DNA microarray technology.

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