APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2005, p. 5650–5653 Vol. 71, No. 9
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.9.5650–5653.2005

Characterization of a Multicopper Oxidase Gene from *Staphylococcus aureus*

S. Sitthisak, K. Howieson, C. Amezola, and R. K. Jayaswal*

Department of Biological Sciences, Illinois State University, Normal, Illinois 61790-4120

Received 17 December 2004/Accepted 11 April 2005

A multicopper oxidase gene from *Staphylococcus aureus* was cloned and overexpressed. Purified recombinant multicopper oxidase oxidized the substrate 3,3′-dimethoxybenzidine in the presence of copper. Disruption of *mco* showed copper sensitivity and H_2O_2 resistance, suggesting roles for *mco* in copper homeostasis and oxidative stress response. Northern blot analysis showed copper-induced *mco* transcription.

Multicopper oxidases (MCO) are enzymes that are critically involved in copper homeostasis. These enzymes show enhanced oxidase activity for a wide range of substrates (1, 2, 4, 9, 22, 24) and play a role in iron transport in eukaryotes and prokaryotes (9, 23). *mco* genes have been cloned and identified from animals, plants, insects, fungi, and bacteria (3, 4, 8, 12, 16). However, there is no report of *mco* in *Staphylococcus aureus*.

We have been investigating the regulation of metal transport and homeostasis in *S. aureus* (25, 26). To identify new loci involved in metal homeostasis in *S. aureus*, we selected streptonigrin-resistant clones from a transposon Tn*917*-induced mutant library of *S. aureus* strain ATCC 12600 and characterized mutants by cloning and sequencing. In this study, we identified one of the mutations in the gene encoding multicopper oxidase.

Staphylococcus aureus strains were grown in tryptic soy broth (TSB) or defined medium. *Escherichia coli* strains were grown in Luria-Bertani broth with the appropriate antibiotic. DNA manipulation, sequencing, cloning, transformation, and overexpression were performed as described earlier (7, 21, 26). DNA sequences were determined with an ABI Prism 310 automated sequencer (Perkin Elmer, Foster City, CA).

The Tn*917*-flanking sequence of the mutant was cloned in *E. coli* as described earlier (21). Nucleotide analysis of the flanking region of the Tn*917* insertion of one of the streptonigrin-resistant clones showed sequence homology to putative multicopper oxidases. The complete nucleotide sequence of the parental gene copy of *S. aureus* showed an open reading frame (ORF) comprising 1,389 bp encoding a hypothetical polypeptide of 462 amino acids with a predicted molecular mass of 52 kDa and pI of 9.68. Nucleotide comparison showed a 99% sequence identity with a gene in the *S. aureus* EMRSA-16 database. Amino acid comparisons of the translated ORF showed 84% identity to putative MCO from *Staphylococcus epidermidis* and 26 to 41% identities to laccase, CueO, and ascorbate oxidase, which are members of the MCO family. Therefore, we designated this ORF *mco*. Seventy nucleotides upstream of *mco*, an ORF designated *copA*, a copper ATPase, was also identified (Fig. 1).

The multicopper oxidase gene from *S. aureus* was cloned and overexpressed in *E. coli* BL21(DE3). MCO activity was determined by using 3,3′-dimethoxybenzidine as described earlier (5, 15, 18). This enzymatic activity was copper dependent, and the presence of 0.5 mM $CuSO_4$ is optimum for enzymatic activity. The purified MCO showed a specific activity of 9.7 U/mg, compared to 1.6 U/mg in the crude extract (Table 1). The purified MCO also exhibit low levels of ferroxidase (1.58 U/mg) and phenoloxidase (2.3 U/mg) activities compared to those reported for other organisms (11, 13).

Although multicopper oxidase is an important enzyme in many organisms, analysis of the *S. aureus* database indicated the presence of MCO homologues only in an *S. aureus* EMRSA-16 strain. We used Southern blot analysis to search for homologous sequences in various *S. aureus* strains whose genomes have not been sequenced. The *mco* gene hybridized with a 2.5-kb HindIII DNA fragment of three strains (ATCC 12600, H, and Wood) out of seven laboratory strains. However, the most commonly used *S. aureus* strains, RN450 and COL, did not show any sequence homology with *mco*, suggesting that it plays a dispensable role in staphylococci.

The insertion of Tn*917* in the multicopper oxidase gene resulted in streptonigrin tolerance and copper sensitivity. As shown in Fig. 2A and B, the mutant strain grows slowly in medium containing more than 1.5 mM $CuSO_4$. We also checked the mutant's sensitivities to iron, nickel, cobalt, and other metallic ions. So far, we found the *mco* mutant sensitive only to copper and cobalt (data not shown).

A role of *mco* in the oxidative stress response has been

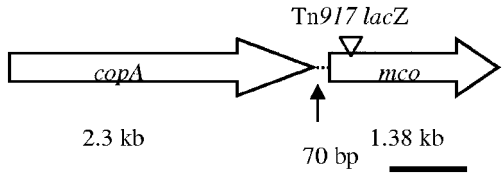

FIG. 1. Schematic representation of the multicopper oxidase and copper ATPase genes. The arrow indicates predicted gene and orientation. The transposon insertion site within the gene is indicated by an inverted triangle. The solid bar indicates the DNA fragment which was used as a probe for the Northern analysis.

* Corresponding author. Mailing address: Microbiology Group, Department of Biological Sciences, Illinois State University, Normal, IL 61790-4120. Phone: (309) 438-5128. Fax: (309) 438-3722. E-mail: drjay@ilstu.edu.

TABLE 1. Overexpression and purification of MCO from *E. coli* BL21(DE3)(pLysS)[a]

Step	Protein (mg)	Total activity (U)[b]	Sp act[c] (U/mg)
Crude extract	21	19.9 ± 1.3	1.6 ± 0.2
Ni column elute	0.6	42.0 ± 2.0	9.7 ± 0.3

[a] Enzyme activity was measured using 3,3′-dimethoxybenzidine as a substrate. The reaction was initiated by the addition of 0.472 μmol of 3,3′-dimethoxybenzidine (*o*-dianisidine) and terminated after 5 and 120 min.
[b] One unit of activity catalyzed the oxidation of 1 μmol of substrate per min.
[c] Specific activity is defined as the μmol of substrate oxidized per min per mg of total protein.

proposed previously (10, 16). To test whether a mutation in *mco* has any impact on the oxidative tolerance of *S. aureus*, the *mco* mutant and the parent cells were grown in TSB containing various concentrations of H_2O_2 and methyl viologen (paraquat). The *mco* mutant cells were able to grow in the medium containing 5 mM H_2O_2, whereas the parent cells were unable to grow (Fig. 2C). However, the tolerance levels of the *mco* mutant and the parent strains to paraquat, another oxidative agent, were similar. Additional experiments using catalase assay activity gels (7) showed that the higher hydrogen peroxide tolerance of the *mco* mutant was not due to the induced expression of the *kat* gene, which encodes the catalase (data not shown).

In the presence of heavy metals, MCO catalyzes the formation of H_2O_2. It has been shown that cupric ions and ceruloplasmin, a multicopper oxidase family protein in human serum, have the capacity to oxidize the substrate with the production of superoxide anions and H_2O_2 (17). Moreover, the production of H_2O_2 in the oxidation of heavy metals by laccase, an MCO in *Stropharia rugosoannulata*, has been reported previously (20). However, the precise role of multicopper oxidase in the hydrogen peroxide tolerance phenotype of the *mco* mutant is unknown.

Complementation studies were performed to provide genetic evidence that copper sensitivity and H_2O_2 tolerance are due to the transposon insertion within the *mco*. A 1.8-kb BamHI-HindIII fragment containing *mco* was cloned in the shuttle vector pLI50 (14) and transformed into an *mco* mutant

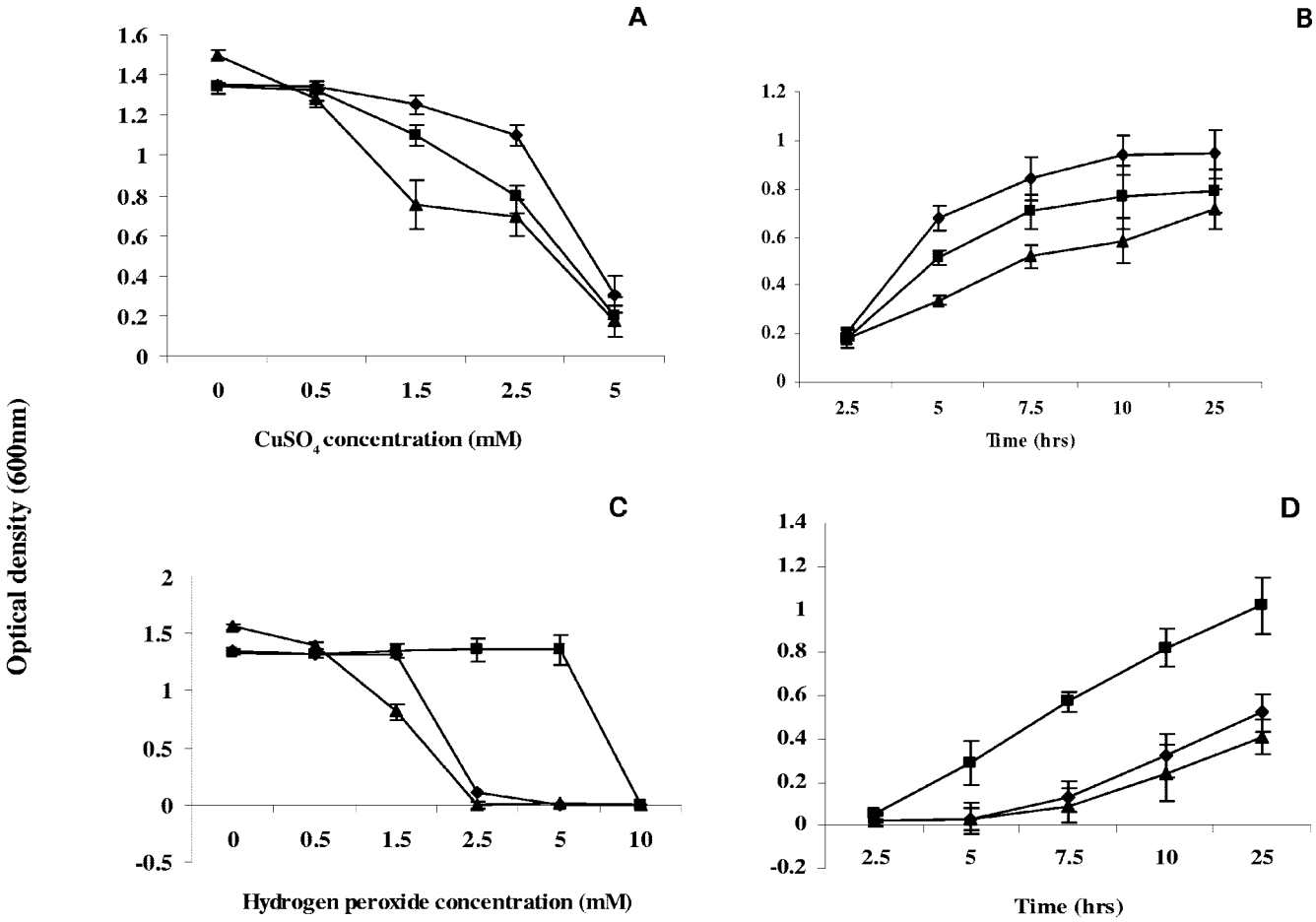

FIG. 2. Effects of copper and hydrogen peroxide on growth. Overnight cultures were diluted 1:500 in TSB with different concentrations of $CuSO_4$ (A) or H_2O_2 (C) and incubated at 37°C with shaking. Cell growth was monitored by measuring optical density at 600 nm for 18 h. Growth curves with 2.5 mM $CuSO_4$ (B) and 1.5 mM H_2O_2 (D) are shown. Overnight cultures were diluted 1:500 in TSB with 1.5 mM H_2O_2 or 2.5 mM $CuSO_4$. Cell growth was monitored by measuring absorbance at 600 nm at various intervals of incubation at 37°C with shaking. Symbols: ◆, wild type; ■, *mco* mutant; ▲, *mco* complemented strain. Each point represents the mean value ± standard deviation (represented by bar) of three experiments.

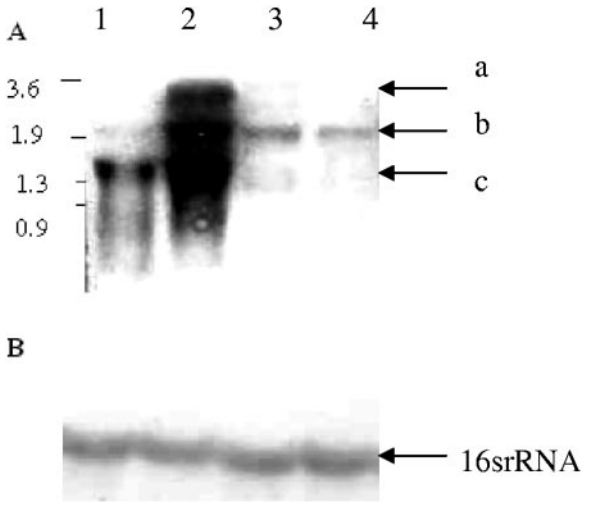

FIG. 3. Northern blot analysis. (A) Ten micrograms of total RNA was electrophoresed on formaldehyde agarose gels (1.2%) and transferred to a nitrocellulose membrane. The blot was probed with radiolabeled *mco*. Lane 1, RNA extract from the wild type grown in defined medium (DM); lane 2, RNA extract from the wild type grown in DM with 200 μM $CuSO_4$; lane 3, RNA extract from mutant grown in DM; and lane 4, RNA extract from the mutant grown in DM with 200 μM $CuSO_4$. Arrows indicate the multiple transcripts of approximately 1.3 (a), 2.0 (b), and 3.5 (c) kb. Molecular size markers (in kilobases) are indicated on the left. (B) Same blot, probed with radiolabeled 16S rRNA gene.

strain. As shown in Fig. 2C and D, the complemented strain became H_2O_2 sensitive as predicted; however, it unexpectedly remained copper sensitive (Fig. 2A and B). To explain this result, we hypothesize that the mutation within the *mco* gene had an additional unanticipated negative effect upon *copA* expression, resulting in higher intracellular copper levels, which would cause copper sensitivity. In order to test this hypothesis, we measured the intracellular copper contents of the mutant, wild-type, and complemented strains by using an atomic absorption spectrophotometer as described previously (19). The intracellular copper contents in the mutant and complemented strains were 5 pmol and 10 pmol/10^8 cells, respectively, while the copper content of the wild type was less than 1 pmol/10^8 cells. These results suggest that the failure to complement the copper-sensitive phenotype may be due to an inability of the cell to efflux copper out of the cell.

Northern blot analysis was performed to examine the effect of copper on *mco* transcription. As shown in Fig. 3 (lane 2), three transcripts (1.3, 2.0, and 3.5 kb) were induced by elevated concentrations of copper ions (200 μM) in the parent strain, but no induction occurred in the mutant strain (Fig. 3, lane 4). The three different transcripts might represent the products from different genes or of specific degradation. The 3.5-kb transcript likely corresponds to a transcript containing both *mco* and *copA*. The nucleotide sequence analysis indicated that *mco* and *copA* genes in *S. aureus* are separated by 70 bp and have no significant sequence identity. Therefore, hybridization of all three transcripts with *mco* genes is puzzling and possibly indicative of a complex transcriptional regulation that needs further investigation.

In conclusion, we have identified an *mco* gene which encodes an enzyme that shows oxidase activity in *S. aureus.* The expression of this gene can be induced by copper. A transposon-induced mutant showed copper and cobalt sensitivity and H_2O_2 tolerance. Since *mco* was not found in all strains of *S. aureus*, this suggests that it plays a dispensable role in staphylococci. It has been suggested that the *mco* role in copper homeostasis may be compensated for by other gene systems (6). Further studies are required to determine the role of *mco* in heavy metal homeostasis and oxidative response.

Nucleotide sequence accession number. The complete nucleotide sequence of the parental gene copy of *S. aureus* has been deposited to the GenBank database under accession no. AY259130 gi 33391259.

We are grateful to James Webb, Illinois State University, for his help in copper measurement and Chia Lee, University of Kansas, for providing plasmid pLI50. We thank Anthony Otsuka for critical reading of the manuscript.

This work has been supported by a Grant-in-Aid from the American Heart Association—Greater Midwest Affiliate to R.K.J.

REFERENCES

1. **Askwith, C. C., and J. Kaplan.** 1998. Site-directed mutagenesis of the yeast multicopper oxidase Fet3p. J. Biol. Chem. **273:**22415–22419.
2. **Brouwers, G. J., J. P. de Vrind, P. L. Corstjens, P. Cornelis, C. Baysse, and E. W. de Vrind-de Jong.** 1999. *cumA*, a gene encoding a multicopper oxidase, is involved in Mn^{2+} oxidation in *Pseudomonas putida* GB-1. Appl. Environ. Microbiol. **65:**1762–1768.
3. **de Silva, D., S. Davis-Kaplan, J. Fergestad, and J. Kaplan.** 1977. Purification and characterization of Fet3 protein, a yeast homologue of ceruloplasmin. J. Biol. Chem. **272:**14208–14213.
4. **Eck, R., S. Hundt, A. Hartl, E. Roemer, and W. Kunkel.** 1999. A multicopper oxidase gene from *Candida albicans*: cloning, characterization and disruption. Microbiology **145:**2415–2422.
5. **Feldman, S. L., J. S. V. Hunter, A. Zgirski, V. M. Chidambaram, and E. Frieden.** 1982. Comparison of the catalytic oxidation of cysteine and o-dianisidine by cupric ion and ceruloplasmin. J. Inorg. Biochem. **17:**51–60.
6. **Grass, G., and C. Rensing.** 2001. Genes involved in copper homeostasis in *Escherichia coli.* J. Bacteriol. **183:**2145–2147.
7. **Horsburgh, M. A., M. O. Clements, H. Crossley, E. Ingham, and S. J. Foster.** 2001. PerR control oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus.* Infect. Immun. **69:**3744–3754.
8. **Hullo, M.-F., I. Moszer, A. Danchin, and I. Martin-Verstraete.** 2001. CotA of *Bacillus subtilis* is a copper-dependent laccase. J. Bacteriol. **183:**5426–5430.
9. **Huston, W. M., M. P. Jennings, and A. G. McEwan.** 2002. The multicopper oxidase of *Pseudomonas aeruginosa* is a ferroxidase with a central role in iron acquisition. Mol. Microbiol. **45:**1741–1750.
10. **Inoue, K., T. Akaike, Y. Miyamoto, T. Okamoto, T. Sawa, M. Otagiri, S. Suzuki, T. Yoshimura, and H. Maeda.** 1999. Nitrosothiol formation catalyzed by ceruloplasmin. Implication for cytoprotective mechanism in vivo. J. Biol. Chem. **274:**27069–27075.
11. **Kim, C., W. W. Lorenz, J. T. Hoopes, and J. F. Dean.** 2001. Oxidation of phenolate siderophores by the multicopper oxidase encoded by the *Escherichia coli yacK* gene. J. Bacteriol. **183:**4866–4875.
12. **Kim, J. S., M. H. Kim, M. H. Joe, S. S. Song, I. S. Lee, and S. Y. Choi.** 2002. The *sctR* of *Salmonella enterica* serovar *Typhimurium* encoding a homologue of MerR protein is involved in the copper-responsive regulation of *cuiD.* FEMS Microbiol. Lett. **23:**99–103.
13. **Larrondo, L. F., L. Salas, F. Melo, R. Vicuña, and D. Cullen.** 2003. A novel extracellular multicopper oxidase from *Phanerochaete chrysosporium* with ferroxidase activity. Appl. Environ. Microbiol. **69:**6257–6263.
14. **Lee, C. Y., J. J. Schmidt, A. D. Johnson-Winegar, L. Spero, and J. J. Iandolo.** 1987. Sequence determination and comparison of the exfoliative toxin A and toxin B genes from *Staphylococcus aureus.* J. Bacteriol. **169:**3904–3909.
15. **Lehmann, H. P., K. H. Schosinsky, and M. F. Beeler.** 1974. Standardization of serum ceruloplasmin concentrations in international enzyme units with *o*-dianisidine dihydrochloride as substrate. Clin. Chem. **20:**1564–1567.
16. **Lim, S. Y., M. H. Joe, S. S. Song, M. H. Lee, J. W. Foster, Y. K. Park, S. Y. Choi, and I. S. Lee.** 2002. *cuiD* is a crucial gene for survival at high copper environment in *Salmonella enterica* serovar *Typhimurium.* Mol. Cells **31:**177–184.
17. **Lipsky, P. E.** 1984. Immunosuppression by D-penicillamine in vitro: inhibition of human T lymphocyte proliferation by copper- or ceruloplasmin-dependent generation of hydrogen peroxide and protection by monocytes. J. Clin. Investig. **73:**53–65.
18. **Outten, F. W., D. L. Huffman, J. A. Hale, and T. V. O'Halloran.** 2001. The

independent cue and cus systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli.* J. Biol. Chem. **17:**30670–30677.

19. **Radford, D. S., M. A. Kihlken, G. P. Borrelly, C. R. Harwood, N. E. Le Brun, and J. S. Cavet.** 2003. CopZ from *Bacillus subtilis* interacts in vivo with a copper exporting CPx-type ATPase CopA. FEMS Microbiol. Lett. **220:**105–112.
20. **Schlosser, D., and C. Höfer.** 2002. Laccase-catalyzed oxidation of Mn^{2+} in the presence of natural Mn^{3+} chelators as a novel source of extracellular H_2O_2 production and its impact on manganese peroxidase. Appl. Environ. Microbiol. **68:**3514–3521.
21. **Scybert, S., R. Pechous, S. Sitthisak, M. J. Nadakavukaren, B. J. Wilkinson, and R. K. Jayaswal.** 2003. NaCl-sensitive mutant of *Staphylococcus aureus* has a Tn*917-lacZ* insertion in its *ars* operon. FEMS Microbiol. Lett. **222:** 171–176.
22. **Solomon, E. I., U. M. Sundaram, and T. E. Machonkin.** 1996. Multicopper oxidases and oxygenases. Chem. Rev. **7:**2563–2606.
23. **Spizzo, T., C. Byersdorfer, S. Duesterhoeft, and D. Eide.** 1997. The yeast FET5 gene encodes a FET3-related multicopper oxidase implicated in iron transport. Mol. Gen. Genet. **256:**547–556.
24. **Stoj, C., and D. J. Kosman.** 2003. Cuprous oxidase activity of yeast Fet3p and human ceruloplasmin: implication for function. FEBS Lett. **554:**422–426.
25. **Xiong, A., and R. K. Jayaswal.** 1998. Molecular characterization of a chromosomal determinant conferring resistance to zinc and cobalt ions in *Staphylococcus aureus.* J. Bacteriol. **180:**4024–4029.
26. **Xiong, A., V. K. Singh, G. Cabrera, and R. K. Jayaswal.** 2000. Molecular characterization of a ferric uptake regulator, Fur, from *Staphylococcus aureus.* Microbiology **146:**659–668.