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The Multi-Copper-Ion Oxidase CueO of *Salmonella enterica* Serovar Typhimurium Is Required for Systemic Virulence▽

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***Salmonella enterica* serovar Typhimurium possesses a multi-copper-ion oxidase (multicopper oxidase), CueO (also known as CuiD), a periplasmic enzyme known to be required for resistance to copper ions. CueO from *S.* Typhimurium was expressed as a recombinant protein in *Escherichia coli*, and the purified protein exhibited a high cuprous oxidase activity. We have characterized an *S.* Typhimurium *cueO* mutant and confirmed that it is more sensitive to copper ions. Using a murine model of infection, it was observed that the *cueO* mutant was significantly attenuated, as indicated by reduced recovery of bacteria from liver and spleen, although there was no significant difference in recovery from Peyer's patches and mesenteric lymph nodes. However, the intracellular survival of the *cueO* mutant in unprimed or gamma-interferon-primed murine macrophages was not statistically different from that of wild-type *Salmonella*, suggesting that additional host factors are involved in clearance of the *cueO* mutant. Unlike a *cueO* mutant from *E. coli*, the *S.* Typhimurium *cueO* mutant did not show greater sensitivity to hydrogen peroxide and its sensitivity to copper ions was not affected by siderophores. Similarly, the *S.* Typhimurium *cueO* mutant was not rescued from copper ion toxicity by addition of the branched-chain amino acids and leucine.**

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) infections usually cause self-limiting gastroenteritis, but they can also lead to systemic disease and mortality in immunocompromised individuals, the elderly, and infants. In the mouse, *S.* Typhimurium is also widely used as a model of typhoid fever (27), and for this reason, this organism is widely studied in the murine system (18). Following initial attachment to M cells of Peyer's patches in the gastrointestinal tract, *S.* Typhimurium cells migrate from its apical side to the basolateral membrane of epithelial cells and into the reticuloendothelial system, where they are engulfed by macrophages (18, 26).

The bactericidal activity of the macrophage is dependent on multiple factors, including NADPH oxidase, which generates reactive oxygen species (ROS) during the respiratory burst, and iNOS, which generates nitric oxide (NO). The ability of *S.* Typhimurium to resist ROS and reactive nitrogen species (RNS) is essential for survival *in vivo*, and specific mechanisms involved in evasion of these host defense systems have been described in some detail (1, 7). Transition metal ions are intimately linked to the biological effects of ROS and RNS. This view is supported by the observation that chelation of intracellular iron significantly reduces the toxic effects of hydrogen peroxide (21). Copper can also exert its toxicity by promoting the Fenton reaction as well as superoxide production by reaction with molecular oxygen (16). Certainly, the bactericidal effects of copper are well known, and two regulons involved in protection against copper toxicity have been described for *Escherichia coli*, where the multi-copper-ion oxidase (multicopper oxidase) CueO from the CueR regulon has been extensively studied (10, 15, 24, 29). CueO is a periplasmic enzyme that catalyzes the oxidation of cuprous copper, ferrous iron, and a variety of diphenolic compounds, including the catecholate siderophore enterobactin. The precise role of CueO in bacterial copper resistance has been somewhat enigmatic, but Grass and coworkers (10) proposed that, in *E. coli*, the enzyme protects cells against copper toxicity by catalyzing the oxidation of copper from cuprous (Cu^{+}) to cupric (Cu^{2+}), thereby protecting the cell by preventing the reduced species from participating in the Fenton reaction (29). In addition, by catalyzing the oxidation of catecholates, CueO generates an insoluble metal-binding polymer in the periplasm, and it was proposed that movement of metal ions (such as copper and iron) into the cell cytoplasm is thus restricted.

S. Typhimurium possesses a CueR regulon composed of genes encoding a copper-sensing transcriptional regulator CueR (also known as SctR), a copper efflux pump (CopA), and a multicopper oxidase, CueO (also known as CuiD) (6, 23). Although CueO is required for copper tolerance in *S.* Typhimurium, the potential role of copper in host defense, as well as copper detoxification systems in host evasion strategies, does

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not seem to have been explored. Here we describe the phenotype of a *cueO* mutant of *S.* Typhimurium, including an assessment of the effect of this mutation on virulence. In so doing, we provide new insights into the role of metal ions in the interaction between the host and intracellular pathogens.

MATERIALS AND METHODS

Materials. Except when stated otherwise, all chemicals were purchased from Sigma (Castle Hill, NSW, Australia). Restriction enzymes were from New England Biolabs and used as in the manufacturer's instructions.

Strains and culture conditions. *S.* Typhimurium SL1344 (35) was used in this study. *E. coli* DH5α was employed for cloning the constructed plasmids. *S.* Typhimurium LB5010 (3) was used to increase the transformation efficiency in SL1344; LB5010 was transformed with the plasmid of interest, which was then extracted from that strain and used to transform SL1344. All bacteria were grown at 37°C in Luria-Bertani (LB) medium at 180 rpm unless stated otherwise. The concentrations of antibiotic used in this study were 100 μg/ml for ampicillin (Amp), 50 μg/ml for kanamycin (Kan), and 25 μg/ml for chlorampenicol (Cm).

Generation of *S.* Typhimurium *cueO*, *entC*, and *cueO entC* mutants. In order to delete the *cueO* and *entC* genes, respectively, a three-step PCR procedure was employed to generate an amplification product that contained the kanamycin cassette from pKD4 (5) flanked on both sides by approximately 500 bp of DNA sequence homologous to the target gene to be modified. For *cueO*, the following sets of primers were used: St_cueOP7 (5′-GGCCGGAATTCTAGGGATAACAGGGTAATCGTAGGCTAACAGGCTCAGAG), St_cueOP2 (5′-GAAGCAGCTCCAGCCTACACACATTTCCTTATAATGAACAGGC), St_cueOP5 (5′-GAACTAAGGAGGATATTCATATGGTCTGACCGATGTCTGTGACG), and St_cueOP8 (5′-CGCGCGAATTCTAGGGATAACAGGGTAATGGTATTCAGCCACAGTACGG). For *entC*, the following sets of primers were used: St_entCP7 (5′-GGCCGGAATTCTAGGGATAACAGGGTAATGCCTTTGCTACGTCGCTC), St_entCP2 (5′-GAAGCAGCTCCAGCCTACACACTTCATCTTTCAAGCTGCCTCT), St_entCP5 (5′-GAACTAAGGAGGATATTCATATGCGTATACCTTTCACCCGTTG), and St_entCP8 (5′-CGCGCGAATTCTAGGGATAACAGGGTAATCCTGAAAGTAGGCCACTTCG). These primers were used in combination with the kanamycin cassette amplification primers KanP3 (5′-TGTGTAGGCTGGAGCTGCTTC) and KanP4 (5′-CATATGAATATCCTCCTTAGTTC). The final three-step PCR product for each target gene was gel purified and cloned into pT7Blue (Novagen) according to the manufacturer's instructions to generate a donor plasmid. SL1344 single- and double-gene-deletion mutants were constructed using the "gene gorging method" described by Herring et al. (12). Briefly, SL1344 cells were electrotransformed with a donor plasmid (pT7Blue::*cueO*::Kan or pT7Blue::*entC*::Kan) and a mutagenesis plasmid (pACBSR [12]). Cells containing both plasmids were grown for 9 h in LB medium supplemented with Cm and 0.2% L-arabinose to induce the λ Red and I-SceI genes on pACBSR. Induction of these genes leads to linearization of the donor plasmid by I-SceI and a double recombination with the regions flanking *cueO* and *entC*, resulting in disruption of the respective gene. Clones sensitive to ampicillin and chloramphenicol (but resistant to kanamycin) were selected, and deletion of *cueO* and *entC* was confirmed by sequencing. For the construction of double-gene-deletion mutants, the kanamycin cassette was removed using plasmid pCP20 as described by Datsenko and Wanner (5). All deletion mutants were confirmed by PCR and subsequent DNA sequencing.

Complementation of the *cueO* mutant. The plasmid pCueO was used to complement the *S.* Typhimurium SL1344 *cueO* mutant. It was generated by PCR amplification of the *cueO* (primers St_cueO11 and St_cueO12: 5′-GGAATTCTTAGACACCCGCCTGTTCAT and 5′-CCTCGAGCGTCACAGACATCGGTCAGA, respectively) gene from the chromosome of SL1344 and subsequent cloning into EcoRI-XhoI of pWSK29 (34). Plasmid pCueO was highly stable in *S.* Typhimurium SL1344 during laboratory growth in the absence of ampicillin selection.

Copper sensitivity assay on LB agar media. Serial 10-fold dilutions of cultures of the same density were performed, and 5 μl of each dilution was spotted on LB agar medium containing the indicated amount of copper. Pictures were taken after an overnight incubation at 37°C. Anaerobic cultures were grown using hermetic plastic jars in which oxygen was removed by CO_2 production by AnaerocultP powder (Merck). The data presented are representative of results of three independent experiments.

Mouse model of *S.* Typhimurium colonization. Three groups of five C57BL/6 female 8-week-old mice were anesthetized with Penthrane (methoxyflurane), and stomach acids were neutralized by oral gavage of 100 μl 10% Na_2CO_3 diluted in phosphate-buffered saline (PBS). Ten minutes later, mice were anesthetized again and infected with approximately 10^7 CFU bacteria by a 200-μl volume oral gavage. Mice were monitored daily and weighed on days 0, 1, 4, and 5. On day 5 postinfection, mice were euthanized by CO_2 asphyxiation. Spleen, liver, mesenteric lymph nodes, and Peyer's patches were removed and homogenized by stomaching in 5 ml PBS for 5 min. Organs were plated on LB agar for viable bacterial cell counts.

Cell culture and *Salmonella* infection *in vitro*. RAW264.7 cells (murine macrophages) were obtained from ATCC and were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 5% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Invitrogen, Australia), 2 mM L-glutamine (Glutamax-1; Invitrogen), 20 U/ml penicillin (Invitrogen), and 20 μg/ml streptomycin (Invitrogen) at 37°C in humidified air containing 5% CO_2. A day before infection, RAW264.7 macrophages were harvested and replated in 24-well plates at a density of 1×10^5 cells/well, with streptomycin and penicillin omitted from the culture medium. Cells were pretreated with 1 ng/ml murine gamma interferon (IFN-γ) (R&D Systems, Minneapolis, MN) 18 h before infection or were left untreated. Macrophages were infected with *S.* Typhimurium at a multiplicity of infection of 10 for 1 h, then washed three times and incubated for 1 h with medium supplemented with 200 μg/ml of gentamicin in order to kill extracellular bacteria. Subsequently, RAW264.7 macrophages were cultured in medium containing 20 μg/ml gentamicin for a further 2 to 24 h. To determine the number of intracellular bacteria, macrophages were lysed in 1 ml phosphate-buffered saline–0.01% Triton X-100 at 2, 8, or 24 h postinfection and the lysate was plated onto LB medium supplemented with 1.5% agar. The numbers of intracellular bacteria were calculated by colony counts after an overnight incubation of the LB agar plates at 37°C.

Expression and purification of CueO. *cueO* was amplified by PCR from SL1344 genomic DNA using the primers ScueONcoIF (5′-CATGCC**ATGG**ATCGCCGTGATTTCTTAAAATATTC) and ScueOStrpR (5′-TTATTTTTCGAACTGCGGGTGGCTCCAAGCGCTGACCGTAAATCCTAACATCA). The NcoI restriction site and a StrepTagII sequence followed by a stop codon (underlined) were introduced at the 5′ and 3′ ends, respectively. The start codon (bold) was introduced through the NcoI restriction site. This PCR product was cloned into pGEM-T Easy according to the manufacturer's instructions. The resulting plasmid was then digested with NcoI and EcoRI restriction enzymes, and the fragment containing the *cueO* coding sequence was fused with the StrepTagII subcloned into the NcoI and EcoRI sites of the pBAD *myc* His A expression vector. The construction of pBAD::*cueO*-StrepTagII changed the second amino acid in the *cueO*-encoded sequence from leucine to aspartic acid; however, comparison with the consensus sequence for the twin arginine translocation pathway (TAT) indicated that aspartic acid may also be recognized (2).

For expression of *cueO*, *E. coli* Top10 cells harboring pBAD::*cueO*StrepTagII were grown in LB medium supplemented with Amp. When cells reached an A_{600} of 0.3, the temperature was lowered from 37°C to 20°C, 0.002% L-arabinose was added, and the induction phase was conducted overnight. The cells were harvested by centrifugation at $6,000 \times g$ for 15 min at 4°C, resuspended in Tris-HCl (pH 8), and lysed by a French press. Insoluble material was removed by centrifugation at 15,000 rpm for 50 min. The cleared lysate was filtered, and CueO purification was pursued using a 1-ml prepacked streptavidin column (GE Healthcare). The concentration of purified protein was determined by measuring A_{280} values and using a calculated molar absorption coefficient (ε_{280} = 65,430 $M^{-1} \cdot cm^{-1}$) (8).

Ferroxidase and cuprous oxidase activity measurements. Ferroxidase and cuprous oxidase activity were determined essentially as described by Stoj and Kosman (30) and Singh et al. (29). In short, oxygen consumption in the presence of ferrous iron or cuprous copper was determined using an oxygen electrode (Hansatech). Reaction mixtures were buffered with 100 mM sodium acetate buffer (pH 5.0). Cuprous copper was added as the caged Cu^+ complex $[Cu^+(MeCN_4)]PF_6$ dissolved in nitrogen-purged 5% acetonitrile. All cuprous copper dilutions were performed using gas-tight syringes. Oxygen consumption measurements with cuprous copper were performed in 100 mM sodium acetate buffer (pH 5.0) with 5% acetonitrile. Reaction mixtures were supplemented with 1 mM $CuSO_4$ to load the labile copper site of CueO. Reactions were started by the addition of 885 ng of recombinant CueO. Oxygen consumption data were recorded using the Oxygraph version 1.01 software. All measurements were performed in quadruplicate, and experiments were repeated with at least three independent enzyme preparations. K_m and V_{max} values were determined using SigmaPlot 2000 software. To minimize the amount of Cu^+ oxidized by O_2, Cu^+ was added last to the reaction mixture and the basal level of Cu^+ auto-oxidation was measured independently and subtracted from the total cuprous oxidase activity.

Bacterial growth experiments. All glassware was washed in 6 N HCl and rinsed in double-distilled water prior to use. Bacteria were grown in MM9 low-iron

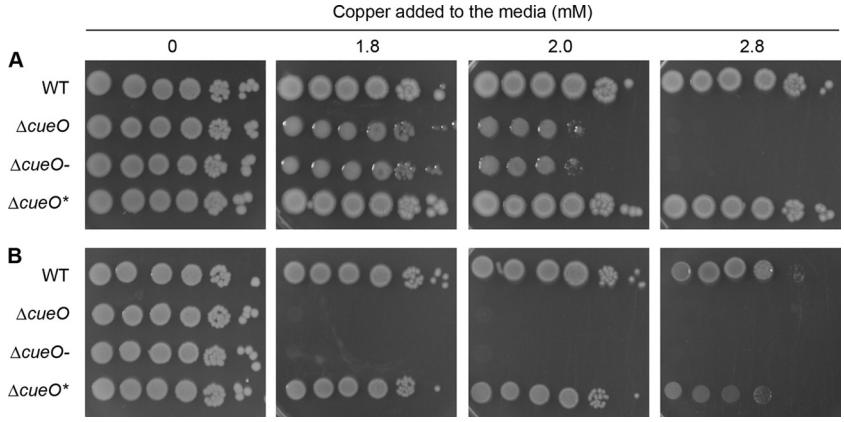

FIG. 1. CueO is essential for *S.* Typhimurium copper resistance *in vitro*. Copper sensitivity and complementation of the *S*. Typhimurium SL1344 *cueO* mutant were tested by spotting serial 10-fold dilutions of cultures of the same density onto LB agar plates containing increasing amounts of copper. Pictures were taken after an overnight incubation at 37°C in the presence (A) or absence (B) of oxygen. The data are representative of results of three independent experiments. WT, SL1344; Δ*cueO*, SL1344 *cueO*::*kan* (*cueO* mutant); Δ*cueO*-, SL1344 *cueO*::*kan* (pWSK29); Δ*cueO**, SL1344 *cueO*::*kan* (pCueO) (complemented mutant).

glycerol medium (MM9 medium) (28) supplemented with 26.2 mM morpholinepropanesulfonic acid (MOPS) free acid, 22.1 mM MOPS sodium salt, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% glycerol, 0.3% deferrated Casamino Acids, 0.002% thiamine, and 0.2% succinate and adjusted to pH 7. Prior to plating, a 1-ml aliquot of each culture (with the same cell density) was centrifuged; the supernatant was collected to check that the *entC* and *cueO entC* mutants did not produce any siderophores using the CAS assay (22) (data not shown). Disk diffusion assays were performed on MM9 low-iron glycerol medium supplemented with 1.5% agar seeded with cultures of the same cell density. Five microliters of 1 M $CuSO_4$ was added to the center of each plate and allowed to diffuse through the agar. The zones of inhibition were measured after overnight incubation at 37°C. The data presented are representative of results of three independent experiments.

To test if isoleucine, leucine, and valine could restore growth in the presence of copper, *S.* Typhimurium SL1344 and the *cueO* strain were grown at 37°C in aerobic minimal medium containing glucose as the carbon source essentially as described by Macomber and Imlay (19). Histidine (0.0021%) was also added to the medium, since SL1344 is a histidine auxotroph. Cultures were supplemented with either 1.5 mM alanine (control) or 0.5 mM L-isoleucine/L-leucine/L-valine and the indicated concentration of copper. A_{500} was measured at the end of the exponential phase. The data presented are representative of results of three independent experiments.

RESULTS

Deletion of *cueO* in *S.* Typhimurium results in enhanced sensitivity to copper. We deleted the *cueO* gene in *S.* Typhimurium SL1344 and tested copper sensitivity by spotting serial 10-fold dilutions of cultures of the same density onto agar plates containing increasing amounts of copper. Figure 1A shows that the growth of the *cueO* mutant was reduced at copper concentrations higher than 1.8 mM and that this mutant does not survive in the presence of 2.8 mM copper. In contrast, no toxic effect of copper on the wild-type strain SL1344 was observed at any concentration of copper tested. Figure 1A also shows that introduction of the *cueO*-containing plasmid pCueO into the *cueO* mutant restored the pattern of susceptibility to copper to that of the wild-type strain, confirming functional complementation of the *cueO* mutant. Thus, *cueO* in *S.* Typhimurium SL1344 is required for copper resistance. Figure 1B shows that when the agar plates were incubated under anaerobic conditions, the survival of the *cueO* mutant was also impaired by the presence of copper. Interestingly, the *cueO* mutant was even more sensitive to copper under anaerobic conditions.

A mutation in *cueO* partially attenuates *Salmonella* in a mouse model of colonization. We tested the abilities of strain SL1344, the *cueO* mutant, and the complemented strain to colonize the Peyer's patches, mesenteric lymph nodes, spleen, and liver in a murine model system. Three groups of five C57BL/6 female 8-week-old mice were inoculated with the three strains, and recovery of bacteria by viable count, from different organs, was used to determine virulence. Deletion of *cueO* significantly attenuated *S.* Typhimurium colonization of liver and spleen (Fig. 2A and B), with an approximately 100-fold decrease in recovery of *S*. typhimurium from these sites compared to that of the wild-type strain. In contrast, there was no significant difference between the *cueO* mutant and the wild-type strain in the colonization of the mesenteric lymph nodes and Peyer's patches (Fig. 2C and D). Figure 2 also shows that the complemented *cueO* strain was recovered from liver and spleen at levels similar to those of the wild-type strain, SL1344. This confirms that the partial attenuation of the *cueO* mutant is directly linked to the mutation in *cueO*.

Macrophage survival assay. In view of the differences in colonization of the liver and spleen between the wild-type strain and the *cueO* mutant and the importance of macrophages in dissemination of *S.* Typhimurium to these organs (33), we determined whether there was a difference in the abilities of these strains to survive within macrophages. Surprisingly, there was no significant difference in the numbers of cells recovered from RAW264.7 during an infection with strain SL1344 and during an infection with the isogenic *cueO* mutant (Fig. 3). As expected, overnight priming of macrophages with IFN-γ decreased intramacrophage survival of *S.* Typhimurium, but even under these conditions, there was no difference in the relative survival of the two strains. Similarly, there was no difference between wild-type SL1344 and the *cueO* mutant in intracellular survival in mouse bone marrow-derived macrophages (BMM) (data not shown).

Properties of recombinant CueO from *S.* Typhimurium strain SL1344. CueO from *E. coli* has been shown to possess

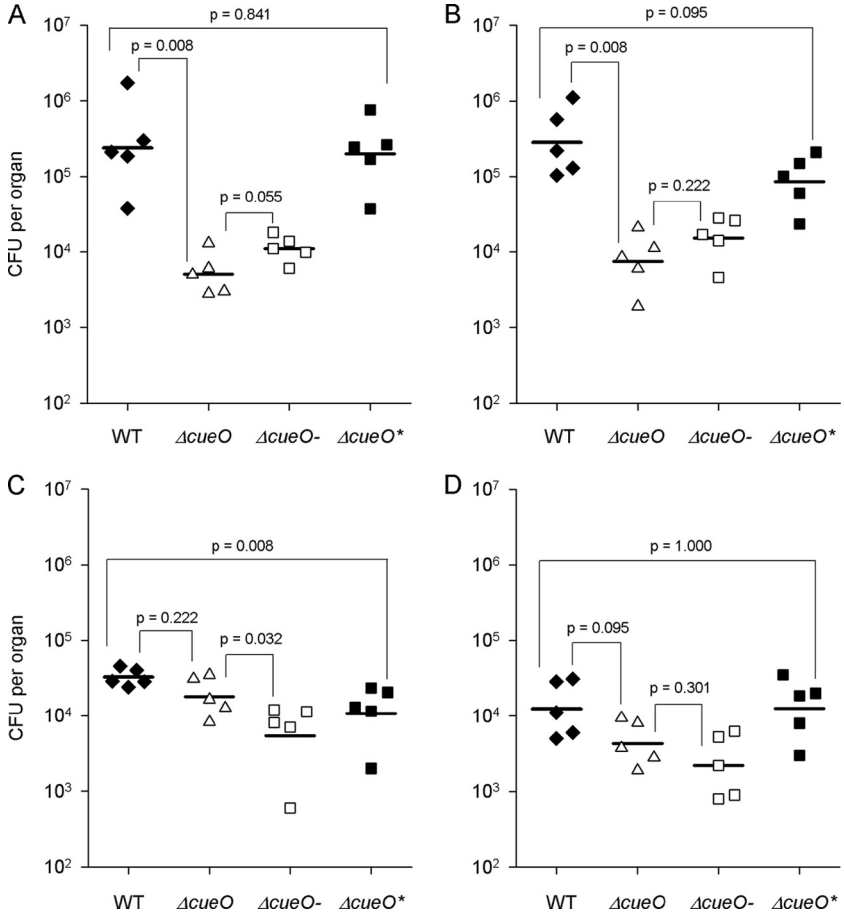

FIG. 2. The *S.* Typhimurium *cueO* mutant is partially attenuated in a mouse model of colonization. Colonization of C57BL/6 mice was used to assess the fitness of the SL1344 *cueO* mutant *in vivo*. Data points represent CFU isolated from the spleen (A), liver (B), mesenteric lymph nodes (C), and Peyer's patches (D) of 8-week-old female C57BL/6 mice 5 days postinfection. WT (◆), SL1344; Δ*cueO* (Δ), SL1344 *cueO*::*kan* (*cueO* mutant); Δ*cueO*- (□), SL1344 *cueO*::*kan* (pWSK29); Δ*cueO** (■), SL1344 *cueO*::*kan* (pCueO) (complemented mutant). Significant differences were analyzed by nonparametric Mann-Whitney U test.

ferroxidase, cuprous oxidase, and laccase activity (oxidation of phenolic compounds), and it is proposed that oxidation of copper and enterobactin in the periplasm by CueO protects the cell from copper stress (10, 14). CueO in *S.* Typhimurium SL1344 has 80% identity at the amino acid level to *E. coli* CueO and possesses all of the residues required for coordination of the types 1, 2, and 3 copper centers (24). CueO from *S.* Typhimurium also possesses all of the identified residues for coordinating the labile copper center and a 20-amino-acid extension of the methionine-rich α-helix (25).

To characterize the functional properties of the *S.* Typhimurium CueO protein, recombinant CueO protein was purified and examined for ferroxidase and cuprous oxidase activity (Table 1). No significant ferroxidase activity was observed in the absence of excess $CuSO_4$ (1 mM) (data not shown). Cuprous oxidase activity was measured using the caged Cu^+ complex $[Cu^+(MeCN_4)]PF_6$ (29). This complex releases free Cu^+ into the reaction mixture. The K_m values determined for ferroxidase and cuprous oxidase activities were less than those previously reported for CueO from *E. coli* (2.5-fold and 3-fold, respectively) (29). Cuprous oxidase activity was comparable to those demonstrated for *Saccharomyces cerevisiae* Fet3 and *Homo sapiens* hCp, although the K_m for ferroxidase activity of CueO from SL1344 was 10-fold and 6.5-fold greater than those determined for Fet3 and hCp, respectively (Table 1). The calculated turnover numbers (k_{cat}/K_m) for Fe^{2+} and Cu^+ were significantly higher for CueO from SL1344 than for CueO from *E. coli*, Fet3, and hCp. Hence, CueO from *S.* Typhimurium is a cuprous oxidase and ferroxidase with a high affinity for both Fe^{2+} and Cu^+ and significantly faster catalysis than CueO from *E. coli*.

Phenotypic characterization of the *cueO* mutant. In view of the partial attenuation of the *cueO* mutant in the mouse infection assay, we attempted to understand the molecular basis of this phenotype. Loss of multicopper oxidase activity might be expected to alter iron and copper homeostasis, and since ferrous and cuprous ions can react with hydrogen peroxide to generate hydroxyl radicals via the Fenton reaction, this might increase the susceptibility of the *cueO* mutant to hydrogen peroxide. However, there was no apparent difference in the susceptibilities of strain SL1344 and the *cueO* mutant to killing by hydrogen peroxide or the superoxide generator paraquat (data not shown). Although CueO is active toward cuprous and ferrous ions, it has been suggested that in *E. coli* it affords

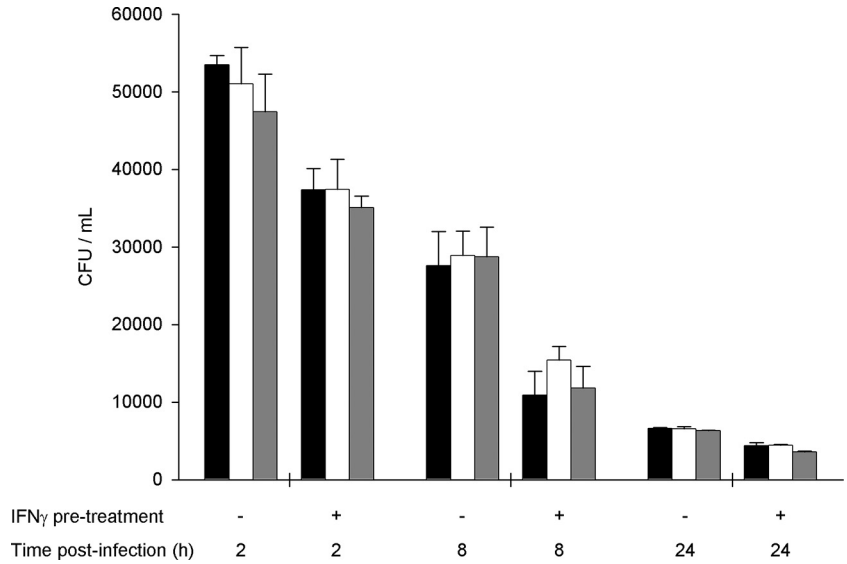

FIG. 3. The survival of the *S.* Typhimurium *cueO* mutant in RAW264.7 macrophages is not affected *in vitro*. Macrophages were untreated or treated with IFN-γ (1 ng/ml) 18 h before infection. RAW264.7 cells were then infected with SL1344 (black bars), the SL1344 *cueO* mutant (white bars), or the *cueO* complemented mutant (gray bars) at a multiplicity of infection (MOI) of 10. At 2, 8, and 24 h postinfection, macrophages were lysed and CFU were enumerated. Data are shown as means ± standard deviations of results of experiments performed in triplicate. The bacterial load in macrophages prestimulated with IFN-γ was significantly lower than that in the unstimulated ones at any of the time points (analysis of variance [ANOVA]; $P < 0.001$), whereas no differences in the survival of the *cueO* mutant and that of the wild type or the complemented mutant were observed (*t* tests; $P > 0.05$).

protection by acting as a phenol oxidase under iron-limiting conditions (10). In this model, oxidation of a catecholate siderophore by CueO would lead to generation of a polyphenol complex that would chelate metal ions and also inhibit the catechol-dependent reduction of Cu^{2+} back to the toxic Cu^{+} ion. We tested this model in *S.* Typhimurium by generating a *cueO entC* double mutant that lacked CueO and the ability to synthesize the catecholate siderophore enterobactin. Figure 4 shows that the zone of killing in the disk diffusion assay was greater for the *cueO* mutant than for strain SL1344. However, there was no significant difference between results for the *cueO entC* double mutant and the *cueO* mutant, indicating that that there was no apparent synergy between the effect of loss of multicopper oxidase activity and the production of enterobactin.

Recently, Macomber and Imlay (19) showed that a deficiency in copper homeostasis in *E. coli* causes inactivation of key dehydrate enzymes that contain an iron-sulfur cluster. It has been hypothesized that copper ions directly inactivated such enzymes. Susceptible enzymes include 6-phosphogluconate dehydratase, a key enzyme of the Entner-Doudoroff (ED) pathway, as well as dihydroxy acid dehydratase and isopropylmalate dehydratase, key enzymes in the synthesis of the branched amino acids isoleucine and valine and of leucine, respectively. Using minimal media, we tested whether copper induced isoleucine-leucine-valine (ILV) auxotrophy in the *S.* Typhimurium *cueO* mutant (Fig. 5). It was observed that addition of ILV did not rescue the mutant from killing by copper ions. In contrast, ILV addition was able to rescue growth of *E. coli* K-12 following treatment with copper (data not shown). This result is consistent with the observations of the effect of copper in *E. coli* (19). Thus, in contrast to the situation in *E. coli*, we were not able to clearly identify targets for copper toxicity in the *S.* Typhimurium *cueO* mutant.

DISCUSSION

Although the bactericidal effects of copper are well established, the possibility that enzymes and transporters involved in

TABLE 1. Kinetic properties of *S.* Typhimurium CueO and comparison with *E. coli*, yeast, and human multicopper oxidases[a]

Multicopper oxidase	Result[b] with substrate					
	Fe^{2+}			Cu^{+}		
	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot min^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot min^{-1}$)
S. Typhimurium CueO	52.6 ± 18.1	967 ± 112	18.4	34.4 ± 12.7	558 ± 49.5	16.2
E. coli CueO (29)	129 ± 15	215 ± 9	1.7	169 ± 24	651 ± 37	3.9
S. cerevisiae Fet3 (30)	5.4 ± 0.8	63.9 ± 2.5	11.8	37.9 ± 3.6	79.2 ± 2.7	2.1
H. sapiens hCp (30)	8.3 ± 1.5	30.3 ± 1.6	3.7	36.8 ± 5.1	22.5 ± 0.8	0.6

[a] Ferroxidase and cuprous oxidase activities of purified recombinant CueO from SL1344 were measured in the presence of 1 mM $CuSO_4$ using the substrates $FeSO_4$ and $[Cu^{+}(MeCN_4)]PF_6$, respectively.
[b] The values given are averages ± standard errors.

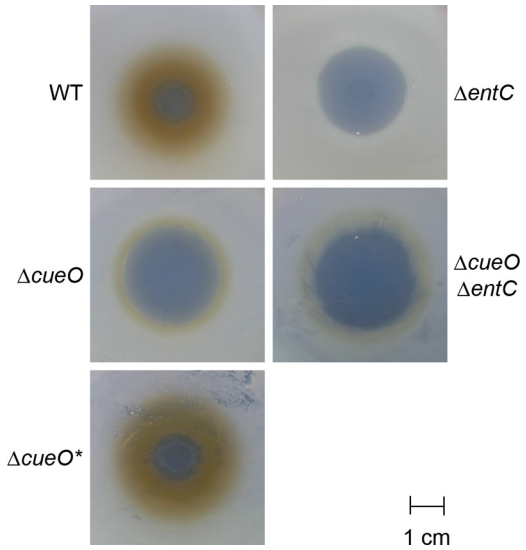

FIG. 4. The *S.* Typhimurium *cueO* mutant in a siderophore mutant background is still sensitive to copper. Disk diffusion assays were employed to compare the sensitivities of the SL1344 *cueO* mutant and the SL1344 *cueO entC* double mutant to $CuSO_4$. Five microliters of 1 M $CuSO_4$ was deposited at the center of each MM9 low-iron glycerol agar plate previously seeded with the indicated bacteria. Pictures were taken after an overnight incubation at 37°C. The data presented are representative of results of three independent experiments. WT, SL1344; Δ*entC*, SL1344 *entC*::*kan*; Δ*cueO*, SL1344 *cueO*::*kan*; Δ*cueO* Δ*entC*, SL1344 *cueO entC*::*kan*; Δ*cueO**, SL1344 *cueO*::*kan* (pCueO).

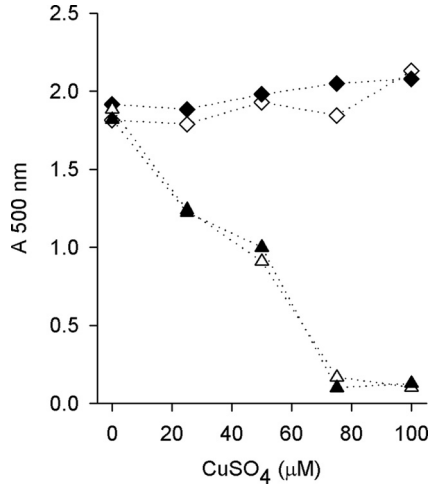

FIG. 5. The copper sensitivity phenotype of *S.* Typhimurium is not rescued by the addition of isoleucine, leucine, and valine. SL1344 (diamonds; ◆, ◇) and SL1344 *cueO* mutant (triangles; ▲, Δ) were grown in minimal medium at 37°C with 1.5 mM alanine (Ala) (open symbols; ◇, Δ) or 0.5 mM (each) isoleucine (I), leucine (L), and valine (V) (closed symbols; ◆, ▲) and the indicated concentrations of $CuSO_4$. A_{500} was measured at the end of the exponential phase. The data are representative of results of three independent experiments.

copper homeostasis would have a role in *Salmonella* pathogenesis has not been explored. Our results indicate that mutation of *cueO* results in a considerable reduction in virulence of *S.* Typhimurium in the mouse model system. Bacterial loads were not reduced in Peyer's patches or mesenteric lymph nodes, implying that there was no defect in the ability of the *cueO* mutant to invade intestinal epithelial cells and translocate through the reticuloendothelial system. Rather, attenuation of the *cueO* mutant seemed to be linked to its failure to survive once disseminated. In this context, the phenotype of the *cueO* mutant partially resembles that of a Spi-2 mutant, where growth is restricted to the Peyer's patch (4). In contrast to Spi-2 mutants, however, the *cueO* mutant was also found in the mesenteric lymph nodes. Spi-2 is a virulence locus associated with intraphagocyte survival and is necessary to support replication of the bacterium within the unfused phagolysosome (17). The implication is that survival of the *cueO* mutant within macrophages is compromised, but we found no evidence of this in *in vitro* assays using RAW264.7 cells or BMM as a model. Furthermore, IFN-γ, which primes macrophage antimicrobial responses, did not alter the survival of the *cueO* mutant relative to that of the wild-type strain. This indicates that the conditions that lead to a reduced survival of the *cueO* mutant in the murine model of infection are not replicated in the *in vitro* infection assay using macrophages; unidentified host factors are likely to be required for effective clearance of the *cueO* mutant.

The biochemical basis for the protection of *S.* Typhimurium by CueO remains to be established, but it seems most probable that it is linked to its copper-detoxifying role. Under aerobic conditions, the cuprous oxidase activity of CueO would oxidize Cu^+ to Cu^{2+} in the periplasm. Our data confirm that in contrast to their eukaryotic counterparts, which appear to be specific for Fe^{2+}, the *S.* Typhimurium and *E. coli* multicopper oxidases have a higher activity (k_{cat}/K_m) toward Cu^+ relative to Fe^{2+}, and this would be consistent with a role in copper oxidation.

Maintaining copper in a Cu^{2+} state would reduce its permeability and entry into the cytoplasm, where it would exert toxic effects. Our observation that the *cueO* mutant was highly sensitive to copper under anaerobic conditions is consistent with previous observations (6, 23) and suggests that binding of copper in the periplasm may also be of importance in protection against this toxic ion. The suggestion that the phenol oxidase activity of CueO might have a role in generating a polyphenolic copper chelator from catecholate siderophores was also tested. However, since we did not observe any discernible difference between the *cueO* mutant and the *cueO entC* double mutant with respect to copper sensitivity, the CueO and catecholate siderophores do not seem to be of importance for copper resistance in *S.* Typhimurium.

Recently, Macomber and Imlay (19) showed that copper toxicity in *E. coli* was linked to its effect on the activity of key dehydratase enzymes. It was established that copper induced ILV amino acid auxotrophy in an *E. coli cueO* mutant, and we made a similar observation for *E. coli* K-12. However, we did not observe that ILV could facilitate the growth of the *S.* Typhimurium *cueO* mutant in the presence of copper. This does not rule out the key enzymes of amino acid biosynthesis, dihydroxy acid dehydratase and isopropylmalate isomerase (IPMI), or other key copper-sensitive dehydratases involved in central carbon metabolism (e.g., 6-phosphgluconate dehydratase and aconitase) as targets for copper in *S.* Typhimurium. However, the differences in resistance to copper be-

tween *E. coli* and *S.* Typhimurium suggest that failures in protection against copper toxicity are linked to additional biochemical factors in *S.* Typhimurium.

It is often the case that the phenotypic effect of a metabolic mutation does not manifest itself unless the mutation is analyzed in a genetic background that contains additional mutations. This is true in the case of metal ion metabolism and oxidative stress. For example, in *E. coli* a mutation in the *fur* gene, which causes increased iron loading, leads to hypersensitivity to oxygen only when the *recA* gene is also deleted (31). We have previously observed that the *cueO* mutant of *E. coli* was more sensitive to peroxide and superoxide stress (32), but such sensitivity was not observed in the *S.* Typhimurium mutant. This suggests that the differences between the oxidative stress defense and metal ion-metabolizing systems in *S.* Typhimurium and *E. coli* may underpin the phenotypic differences between the *cueO* mutants. *S.* Typhimurium lacks the Cus system found in *E. coli* that contributes to resistance at high concentrations of copper (20). However, while both *S.* Typhimurium and *E. coli* possess the copper efflux pump CopA, *S.* Typhimurium also possesses an additional copper efflux pump GolT (6). The complement of periplasmic thioredoxin-like proteins also differs between the two bacterial species. In *E. coli*, the Dsb system is well characterized; interestingly, it has been observed that *dsbC* mutants, deficient in protein disulfide isomerase, are more sensitive to copper ion toxicity (13). Similar experiments have not been performed with *S.* Typhimurium, but it is known that in addition to the Dsb proteins associated with protein disulfide bond formation, *S.* Typhimurium contains a gene cluster encoding thioredoxin-like proteins predicted to operate in the periplasm. This *scs* gene cluster (11) was able to suppress the copper-sensitive phenotype of *cutF* mutants. Thus, the defenses against copper toxicity in *Salmonella* seem to be more diverse than those of *E. coli*, and this may have some significance with regard to virulence.

Recent studies of the elemental composition of the phagosome of *Mycobacterium tuberculosis* (29, 30) demonstrated that iron accumulates in *M. tuberculosis* phagosomes to concentrations up to ~2.5 mM over 24 h. Interestingly, copper concentrations between 10 and 426 μM were reported, although no significant changes in concentration were observed over 24 h. CueO is found in a number of intracellular pathogens, including *M. tuberculosis* and *Legionella pneumophila* (14). Furthermore, *M. tuberculosis* expresses a copper-binding metallothionein protein that partially protects against copper toxicity (9). Therefore, it seems likely that copper has a bactericidal role in innate host defenses and that pathogen copper detoxification systems may represent targets for the development of novel antimicrobial agents.

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