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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 (Cu²⁺), 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...
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 where 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 grown in LB medium supplemented with Cm and 0.2% L-arabinose to allow bacterial growth. Pictures were taken of the same density were performed, and 5 replicate colony counts were performed for each plasmid, and the induction phase was conducted overnight. The cells 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 S. Typhimurium genomic DNA using the primers ScueONcoIF (5'-GGCGCGAATTCTAGGGATAACAGGGTAATGCCTTTACACACATTTCCTTATAATGAACAGGCATAAGGTCGCGTATACCTTTCACCCGTTG), and ScueOP5 (5'-GAATATCCTCCTAGTTC). 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 goring method” described by Herring et al. (12). Briefly, SL1344 cells were electrotransformed with the donor plasmid (pT7Blue::cueO::Kan or pT7Blue::entC::Kan) and a mutagenesis plasmid (pCA6606) [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 pCA6606. Induction of these genes leads to linearization of the donor plasmid by I-sceI and a double recombination with the chromosomal integrase, resulting in a double deletion. 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 independent enzyme preparations. 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 × 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 10,000 × g for 30 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 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(II)-complex ([Cu(II)(MeCN)]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 Oxyscan 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(II)-oxidized by O_2, Cu(II) was added last to the reaction mixture and the basal level of Cu(II) 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; ΔcueO, SL1344 cueO::kan (cueO mutant); ΔcueO*, 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 MgSO4, 0.1 mM CaCl2, 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 CuSO4 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. A530 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 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...
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 CuSO₄ (1 mM) (data not shown). Cu²⁺ oxidase activity was measured using the caged Cu²⁺ complex \([\text{Cu}^2+\text{(MeCN)}_4]\text{PF}_6 \) (29). This complex releases free Cu²⁺ 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 Fe²⁺ 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 Fe²⁺ 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...
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 Cu²⁺ 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 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 iso-propylmalate 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

**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).

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

<table>
<thead>
<tr>
<th>Multicopper oxidase</th>
<th>Fe²⁺</th>
<th>Cu⁺</th>
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<tr>
<td><strong>Result</strong> with substrate</td>
<td><strong>Fe²⁺</strong></td>
<td><strong>Cu⁺</strong></td>
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<tr>
<td><strong>Kₘ (μM)</strong></td>
<td><strong>kₘ (μM⁻¹·min⁻¹)</strong></td>
<td><strong>Kₘ (μM)</strong></td>
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<td></td>
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<tr>
<td>S. Typhimurium CueO</td>
<td>52.6 ± 18.1</td>
<td>967 ± 112</td>
</tr>
<tr>
<td>E. coli CueO (29)</td>
<td>129 ± 15</td>
<td>215 ± 9</td>
</tr>
<tr>
<td>S. cerevisiae Fet3 (30)</td>
<td>5.4 ± 0.8</td>
<td>63.9 ± 2.5</td>
</tr>
<tr>
<td>H. sapiens hCp (30)</td>
<td>8.3 ± 1.5</td>
<td>30.3 ± 1.6</td>
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* Ferroxidase and cuprous oxidase activities of purified recombinant CueO from SL1344 were measured in the presence of 1 mM CuSO₄ using the substrates FeSO₄ and [Cu⁺(MeCN₄)PF₆]⁺, respectively.

* The values given are averages ± standard errors.
Furthermore, IFN-α in macrophages is compromised, but we found no evidence of this (17). The implication is that survival of the bacterium within the unfused phagolysosome with intraphagocyte survival and is necessary to support replication in mesenteric lymph nodes. Spi-2 is a virulence locus associated with growth is restricted to the Peyer’s patch (4). In contrast to cueO mutants, however, the phenotype of the cueO mutant partially resembles that of a Spi-2 mutant, where attenuation of the SL1344 cueO mutant to invade intestinal epithelial cells and translocate through the reticuloendothelial system. Rather, attenuation of the SL1344 cueO double mutant to CuSO₄. Five microliters of 1 mM CuSO₄ 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; ΔentC, SL1344 entC::kan; ΔcueO, SL1344 cueO::kan; ΔcueO ΔentC, SL1344 cueO entC::kan; ΔcueO*, SL1344 cueO::kan (pCueO),

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 Cu²⁺ to Cu³⁺ in the periplasm. Our data confirm that in contrast to their eukaryotic counterparts, which appear to be specific for Fe³⁺, the S. Typhimurium and E. coli multicopper oxidases have a higher activity toward Cu⁺ relative to Fe³⁺, and this would be consistent with a role in copper oxidation.

Maintaining copper in a Cu²⁺ 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 dihydroxy acid 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-phosphogluconate dehydratase and aconitate) as targets for copper in S. Typhimurium. However, the differences in resistance to copper be-

FIG. 4. The S. Typhimurium cueO mutant in a siderophore mutant background is still sensitive to copper. Disc diffusion assays were employed to compare the sensitivities of the SL1344 cueO mutant and the SL1344 cueO entC double mutant to CuSO₄. Five microliters of 1 mM CuSO₄ 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; ΔentC, SL1344 entC::kan; ΔcueO, SL1344 cueO::kan; ΔcueO ΔentC, SL1344 cueO entC::kan; ΔcueO*, 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; △, ▲) were grown in minimal medium at 37°C with 1.5 mM alanine (Ala) (open symbols; ◊, △) or 0.5 mM (each) isoleucine (I), leucine (L), and valine (V) (closed symbols; ◇, ▲) and the indicated concentrations of CuSO₄. A₅₀₀ was measured at the end of the exponential phase. The data are representative of results of three independent experiments.
between 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 GofT (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 sxs gene cluster (11) was able to suppress the copper-sensitive phenotype of cutD 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 after 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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REFERENCES

30. Touati, D., M. Jacques, B. Tardat, L. Bouchard, and S. Desipri. 1995. Lethal oxidative damage and mutagenesis are generated by iron in Δfur mutants of

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