Linkage between Catecholate Siderophores and the Multicopper Oxidase CueO in *Escherichia coli*

Gregor Grass,[1,2] Keshari Thakali,[1] Phillip E. Klebba,[3] Daniel Thieme,[2] Axel Müller,[4]
Günter F. Wildner,[4] and Christopher Rensing[1]*

*Department of Soil, Water, and Environmental Science, University of Arizona, Tucson, Arizona[1];
Institute for Microbiology, Martin Luther University, Halle,[2] and Department of Biology,
Ruhr University, Bochum,[4] Germany; and Department of Chemistry
and Biochemistry, University of Oklahoma, Norman, Oklahoma[3]*

The multicopper oxidase CueO had previously been demonstrated to exhibit phenoloxidase activity and was implicated in intrinsic copper resistance in *Escherichia coli*. Catecholates can potentially reduce Cu(II) to the prooxidant Cu(I). In this report we provide evidence that CueO protects *E. coli* cells by oxidizing enterobactin, the catechol iron siderophore of *E. coli*, in the presence of copper. In vitro, a mixture of enterobactin and copper was toxic for *E. coli* cells, but the addition of purified CueO led to their survival. Deletion of *fur* resulted in copper hypersensitivity that was alleviated by additional deletion of *entC*, preventing synthesis of enterobactin. In addition, copper added together with 2,3-dihydroxybenzoic acid or enterobactin was able to induce a Φ(*cueO-lacZ*) operon fusion more efficiently than copper alone. The reaction product of the 2,3-dihydroxybenzoic acid oxidation by CueO that can complex Cu(II) ions was determined by gas chromatography-mass spectroscopy and identified as 2-carboxymuconate.

Recently, two copper efflux exporters were described in *Escherichia coli*, CopA, a copper/silver P-type ATPase (27), and CusCFBA, a protein complex encompassing both the cytoplasmic and outer membranes (10, 21). The multicopper oxidase CueO was identified as an additional component involved in copper homeostasis in *E. coli* (12, 13, 22), and the three-dimensional structure of this protein was solved at a resolution of 1.4 Å (29). It was shown that CueO is a laccase-like enzyme (EC 1.10.3.2), as proposed by Alexandre and Zhulin (2). CueO is a periplasmic protein and translocated into the periplasm via the TAT pathway (7). Four copper ions are bound in the CueO monomer, forming an active center typical of multicopper oxidases such as ascorbate oxidase, fungal laccases, *Saccharomyces cerevisiae* Fet3, and human ceruloplasmin (29). The enzyme is activated by binding of a labile Cu(II) at a novel copper-binding site (30).

The mechanism of protection from copper-mediated toxicity by CueO was postulated to be the oxidation of cuprous copper (12, 23). This hypothesis was recently strengthened by the demonstration that the related multicopper oxidases Fet3 and human ceruloplasmin are able to oxidize Cu(I) to Cu(II), maintaining the cuprous-cupric redox balance in aerobic organisms and preventing copper-mediated toxicity (33, 34).

CueO was responsible for the oxidation of cuprous copper and the potential Cu(II) reductant enterobactin. Enterobactin is the indigenous catecholate siderophore of *E. coli* secreted to sequester iron from the environment. Recently it was demonstrated that enterobactin is a substrate of CueO in vitro (16). Copper in combination with the catecholate siderophore is much more toxic than copper alone because enterobactin and other catecholates can act as a Cu(II) reductants (15, 18). We showed that CueO oxidized the siderophore enterobactin and its precursor 2,3-dihydroxybenzoic acid and thus protected *E. coli* cells against copper-induced killing. The product of 2,3-dihydroxybenzoic acid oxidation was able to bind copper. In addition, the global iron-dependent repressor Fur was identified as an important determinant of copper tolerance, since a *fur* mutant overproduced enterobactin. Reduction of Cu(II) by enterobactin facilitated Cu(I) uptake into cells. These results give insight into the complex mode of CueO-mediated protection from copper toxicity.

MATERIALS AND METHODS

Bacterial strains and growth media. The strains used in this work are listed in Table 1. *E. coli* was grown in Luria-Bertani (LB) medium or Tris-buffered mineral salts medium (19) containing 2 ml of glycerol and 1 g of yeast extract or 3 g of Casamino Acids per liter. Antibiotics [chloramphenicol (15 to 20 μg/ml) pr kanamycin (25 μg/ml)] and CuCl₂ were added where appropriate.

Gene disruptions, deletions, and operon fusions. Genes were disrupted by the insertion of a chloramphenicol resistance cassettes with a protocol developed in the laboratory of B. Wanner which is based on the λ Red recombinase system as described previously (6).

CAS liquid assay and plates. *E. coli* strains GR1 (ΔcueO- cat) and W3110 were grown overnight in Luria-Bertani medium with shaking at 37°C, diluted 1:500 into Tris-buffered minimal medium (19) supplemented with 0.2% glycerol and 0.3% Casamino Acids. Cultures were grown overnight, diluted 1:500 into fresh minimal medium without iron but with 0.3% deferrated Casamino Acids (25) and different concentrations of CuCl₂ and grown for 16 h to an optical density at 600 nm of 1.0. The cell density was adjusted, spent medium was centrifuged at 12,000 rpm for 2 min, and cleared spent medium was stored at 4°C.

The chrome azurol S (CAS) assay solution was prepared as described by Payne (25). In short, 0.0219 g of hexadecyltrimethylammonium bromide was dissolved in 50 ml of water; 1.5 ml of 1 mM FeCl₃ · 6H₂O in 10 mM HCl was mixed with 7.5 ml of 2 mM CAS solution. This Fe-CAS solution and also piperazine buffer (4.307 g of piperazine dissolved in 30 ml of water with 6.75 ml of concentrated HCl to bring the pH to 6.5) was added to the hexadecyltrimethylammonium bromide solution, and the volume was brought up to 100 ml. CAS agar plates

* Corresponding author. Mailing address: Department of Soil, Water, and Environmental Science, University of Arizona, Shantz Bldg. #38, Rm. 424, Tucson, AZ 85721. Phone: (520) 626-8482. Fax: (520) 621-1647. E-mail: rensingc@ag.arizona.edu.

TABLE 1. *E. coli* strains

Strain	Relevant genotype	Source or reference
W3110	Wild type	
GR1	$\Delta cueO$::cat	Grass and Rensing (12)
GR417	$\Delta entC$::cat	This study
GG199	Δfur::cat	This study
GG213	$\Delta entC$::cat Δfur	This study
WOII260B	$\Phi(cueO$-lacZ)	Outten et al. (22)

were prepared as described by Schwyn and Neilands (33); 0.5 ml of cleared spent culture medium was added to 0.5 ml of CAS assay solution and mixed, and 10 μl of shuttle solution (0.2 M 5-sulfosalicylic acid, stored in the dark) was added to facilitate transfer of iron from the CAS complex to enterobactin, and the sample was mixed. After 5 min, the absorbance at 655 nm was measured.

Enterobactin concentration determination. The reaction between the CAS solution, Fe(III), and enterobactin proceeds as Fe(III)-CAS + enterobactin → Fe(III)-enterobactin + CAS. The decrease in absorbance at 655 nm can be used to calculate the concentration of enterobactin-bound iron with Fe(III)-CAS having a molar extinction coefficient of $105,000$ M^{-1} cm^{-1}. It is assumed that the Fe(III) and enterobactin form 1:1 complexes (14). Enterobactin concentrations were calculated from desferrioxamine units via a calibration curve.

Ferric enterobactin oxidation by CueO. Purified CueO was suspended at 1 μg/ml in 0.05 M morpholinepropanesulfonic acid (MOPS) buffer, pH 6.5, containing 0.5 mM CuCl$_2$. Enterobactin was purified from culture supernatants of *E. coli* K-12 (17), complexed with iron, and chromatographically purified (33). Various concentrations of ferric enterobactin were added to reaction mixtures at 25°C, and the oxidation of ferric enterobactin was monitored by the change in absorption at 393 nm. The data were plotted and analyzed to determine K_m with the enzyme kinetics algorithm of Grafit (version 4.013; Erithacus Ltd.). Visible spectra of the product were collected on a Beckman DU7 spectrophotometer.

Copper-enterobactin toxicity measurement. *E. coli* strains were grown overnight in LB medium with shaking at 37°C, diluted 1:400 into Tris-buffered minimal medium (19) with 0.2% glycerol and 0.3% Casamino Acids, grown overnight, and diluted 1:400 into fresh minimal medium without iron. Cultures were incubated for 16 h and centrifuged, and the spent medium was filter sterilized (20-μm pore size; Nalgene). Cells to be challenged were also subsequently grown in LB medium and regular minimal medium. Copper challenge was carried out as follows: 5 μl of stationary-phase culture was added to 1 ml of sterile spent medium containing 5 mM CuCl$_2$, mixed, and incubated at room temperature for 15 min. The mixture was diluted 1:200 into LB medium, and 25 μl was plated onto LB agar plates.

Precipitate preparation, UV-visible spectrum, and EDX analysis. Purified CueO protein (5 μg) was added to a solution of 100 mM sodium acetate (pH 6.5), 500 μM CuCl$_2$ and 2 mM 2,3-dihydroxybenzoic acid. The reaction was incubated at 50°C for 2 h. For UV-visible analysis, the reaction product was scanned on a Uvikon 922 (Kontron Instruments). For energy-dispersive X-ray (EDX) analysis, the colored oxidation product was harvested by centrifugation, and the resulting pellet was redissolved in water and recentrifuged to remove excess copper. The washed precipitate was subjected to analysis with an EM 912 OMEGA electron transmission microscope (Leo, Oberkochen, Germany) equipped with an EDX system (energy-dispersible X-ray analysis, LINK eXIII; Oxford Instruments, High Wycombe, United Kingdom) in the spot mode (100-nm spot size at 80 keV and 20 μA emission current). For analysis, a computer program considering the net counts and excitation probability, determined with the in-column filter, was used.

2,3-Dihydroxybenzoic acid oxidation by CueO. Purified CueO was incubated with 1 mM 2,3-dihydroxybenzoic acid in the presence of 0.5 mM CuCl$_2$ and 100 mM Tris-HCl buffer, pH 8.0. The reaction was stopped after 1 min by rapid chilling and acidification with HCl to pH 2, and the reaction mixture was extracted twice with ethyl acetate. The combined organic phase was dried with sodium sulfate and filtered, and the solvent was removed by evaporation in a SpeedVac concentrator (Eppendorf). The sample was redissolved in 20 μl of methanol treated with 100 μl of ethereal diazomethane which was prepared from *N*-nitrosomethyl urea and transferred to an autosampler vial (Chromacol 05-CTV[A]116; Fisher Scientific, Schwerte, Germany). Excessive diazomethane and solvent were removed in a gentle stream of nitrogen, and the methylated compounds were taken up in 20 μl of chloroform.

An aliquot of 1 μl was applied to the gas chromatography-mass spectroscopy (GC-MS) system for mass fragment analysis of main signals. All spectra were
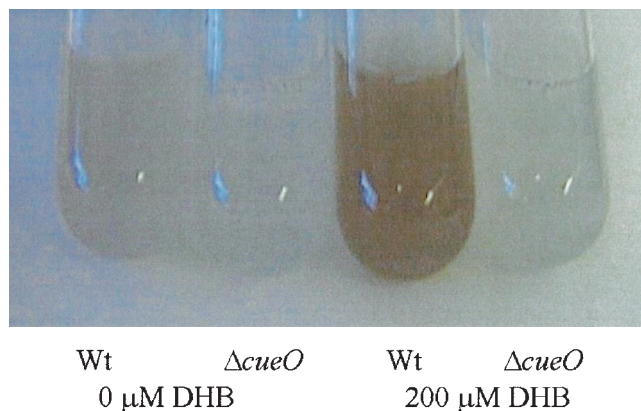

FIG. 1. Effect of 2,3-dihydroxybenzoic acid (DHB) on different *E. coli* strains. Colored precipitate formation in *E. coli* by CueO, CuCl$_2$ and 2,3-dihydroxybenzoic acid in strains GR1 ($\Delta cueO$::cat) and wild-type (Wt) W3110. Overnight cultures grown in Luria-Bertani medium were diluted 1:500 into minimal medium, and grown overnight, diluted 1:500 into fresh minimal medium with 500 μM CuCl$_2$ added to all cultures, 2,3-dihydroxybenzoic acid was added where indicated, and growth at 37°C was continued for 16 h.

recorded with a Finnigan Magnum ion-trap mass spectrometer connected to a Varian GC 3400 gas chromatograph (Varian, Walnut Creek, Calif.). Injections were made with a CTC A200S autoinjector. The following conditions were chosen for GC: splitless injector temperature 260°C, transfer line temperature 260°C, capillary column Zebron ZB-5 (Phenomenex, Aschaffenburg, Germany), film thickness 30 m by 0.25 mm by 0.25 μm, and helium as the carrier gas. The temperature program was constant for 1 min at 60°C and a linear increase was chosen (30°C per min) to 280°C. The mass spectrometer was operated in full scan mode (*m/z* 50 to 400) with chemical ionization and methanol as the reactant gas.

Miscellaneous. Standard molecular genetic techniques were used (31). PCR was performed in the presence of *Pwo* or *Taq* DNA polymerase (Roche, Fermentas). CueO was purified as described previously (13). CueO was concentrated where applicable with Midi centrifuge filters (Nalgene). The protein concentration of purified CueO was determined at 280 nm (ε_{CueO} = 63063 M^{-1} cm^{-1}). The β-galactosidase activity in permeabilized cells was determined as published previously (11, 20).

RESULTS

Oxidation of catecholate siderophores by CueO and copper. When *E. coli* strain W3110 was grown in mineral salt medium under iron-restricted conditions and in the presence of elevated copper concentrations, spent medium and cells turned brownish gray (data not shown). Neither *E. coli* strain GR1 ($\Delta cueO$::cat) nor GR417 ($\Delta entC$::cat) (defective in enterobactin synthesis) was able to produce this colored compound (data not shown). The necessity for the presence of both CueO and EntC clearly indicated that colored-compound formation is strictly dependent on both the oxidase activity of CueO and the presence of enterobactin.

A similar finding was obtained with the enterobactin precursor 2,3-dihydroxybenzoic acid. 2,3-Dihydroxybenzoic acid and copper added to wild-type cultures resulted in enhanced production of a colored precipitate (Fig. 1). No colored precipitate was formed when 2,3-dihydroxybenzoic acid was added to *E. coli* strain GR1 ($\Delta cueO$::cat) (Fig. 1).

Deletion of *cueO*, encoding the multicopper oxidase of *E. coli*, leads to elevated biosynthesis of enterobactin under conditions of iron scarcity when copper is present. Determination of the enterobactin concentrations of spent medium from
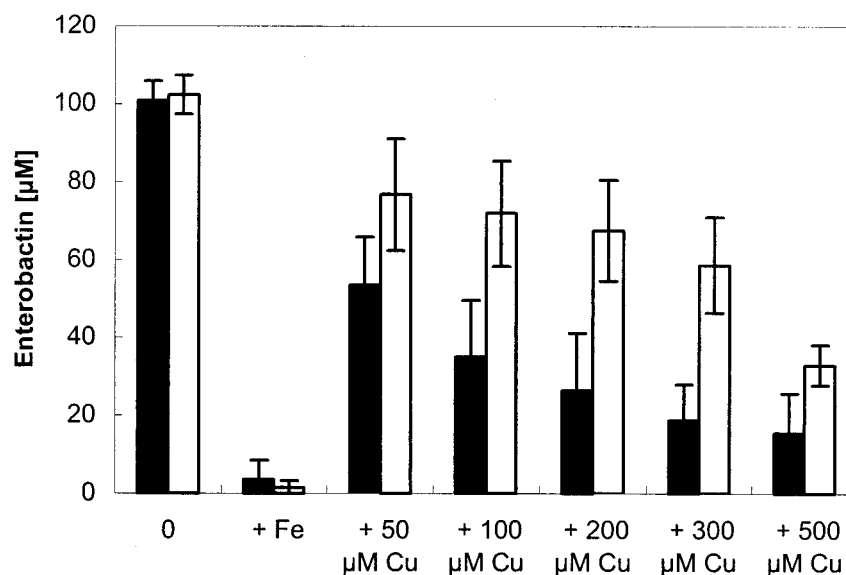
FIG. 2. Enterobactin production in *E. coli* strains in the presence of copper. Overnight cultures grown in Luria-Bertani medium were diluted 1:500 into minimal medium, grown overnight, and diluted 1:500 into fresh minimal medium, and after 2 h of growth at 37°C the cells were diluted 1:500 into fresh minimal medium with the indicated concentrations of $CuCl_2$ and 50 μM $FeCl_3$. The strains were W3110 (solid bars) and GR1 (Δ*cueO*::*cat*) (open bars). Cell growth was continued for 16 h at 37°C with shaking before the enterobactin content was determined as described in Materials and Methods.

strain GR1 (Δ*cueO*::*cat*) and wild-type W3110 was performed in the presence of different concentrations of $CuCl_2$ under iron deprivation (Fig. 2). We clearly demonstrated that in the absence of copper and thus no expression of *cueO*, both wild-type strain W3110 and GR1 (Δ*cueO*::*cat*) produced enterobactin at very similar concentrations. In contrast, under copper stress, i.e., when *cueO* is expressed in the wild-type strain, strain GR1 (Δ*cueO*::*cat*) exhibited a significant increase in enterobactin compared to the wild-type W3110 (Fig. 2). This difference is probably due to the oxidation of enterobactin by CueO (16) in the presence of copper rather than decreased enterobactin biosynthesis in the *cueO* deletion strain GR1 (Δ*cueO*::*cat*), since enterobactin levels were very much the same in the wild-type strain and the *cueO* mutant when copper was not present.

Enterobactin oxidation prevents copper-induced killing of *E. coli*. We started out with the hypothesis that enterobactin can act as a Cu(II) reductant, shifting the balance from Cu(II) to Cu(I), increasing both uptake of Cu(I) and generation of reactive oxygen species by the prooxidant Cu(I). CueO could prevent this in two ways, oxidation of Cu(I) and oxidation of enterobactin.

In order to validate this hypothesis, we investigated whether enterobactin oxidation by CueO reduces the toxicity of the interaction of enterobactin with copper. Therefore, *E. coli* strain W3110 was challenged with spent medium supplemented with copper from either a *cueO* deletion strain, which is rich in enterobactin, or from strain GR417 (Δ*entC*::*cat*), which is unable to produce enterobactin (Table 2). No cells survived with spent medium of strain GR1 (Δ*cueO*::*cat*) (Table 2). Conversely, spent medium of strain GR417 (Δ*entC*::*cat*), which contained no enterobactin at all, was not toxic for *E. coli* cells in the presence of copper. This indicated that copper alone was lethal only in combination with enterobactin under the conditions used. Moreover, addition of purified CueO protein to the

survival assay with spent medium rich in enterobactin resulted in survival of challenged cells (Table 2). This again demonstrated that oxidation of enterobactin by CueO rescued *E. coli* cells from copper- and enterobactin-induced killing. Conversely, when CueO was heat inactivated (10 min of boiling) prior to copper challenge, *E. coli* cells did not survive (data not shown). Thus, protection against copper toxicity could only be accomplished when CueO oxidized enterobactin.

Oxidation of ferric enterobactin. The ability of CueO to act on catecholate compounds, especially its oxidation of the native *E. coli* siderophore enterobactin, led us to test its recognition and oxidation of the iron complex ferric enterobactin. When suspended with ferric enterobactin at slightly acidic pH in the presence of copper ions, CueO rapidly oxidized the ferric siderophore (16). We reevaluated these experiments with slightly different conditions that better mimicked the

TABLE 2. Enterobactin toxicity to *E. coli* in the presence of Cu and the protective activity of CueO

Source of spent medium[a]	CFU[b]	Enterobactin[c] (μM)
Δ*entC* strain	>10³	0
Δ*cueO* strain	0	57.2
Δ*cueO* strain + CueO[d]	>10³	57.2

[a] Strains Δ*entC*, Δ*cueO*, and W3110 (wild type) were grown to stationary phase in iron-depleted minimal medium with subtoxic concentrations of $CuCl_2$, and the supernatant filter was sterilized.

[b] Stationary-phase cultures of *E. coli* strain W3110 were diluted into fresh minimal medium and, after growth for 2 h, diluted into the respective spent medium, challenged with 5 mM $CuCl_2$ for 15 min, diluted into LB medium, and plated on LB plates.

[c] Enterobactin content was determined from the spent supernatant as described in Materials and Methods.

[d] Before addition of cells, CueO was added (2 μM final concentration) for 10 min at 37°C.
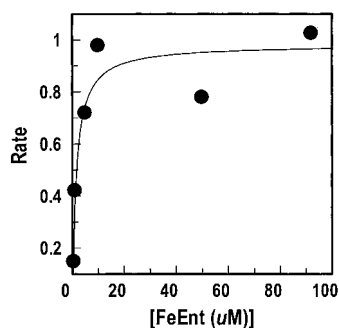
FIG. 3. Oxidation of ferric enterobactin by purified CueO. The initial rates of ferric enterobactin (FeEnt) oxidation by CueO (1 μg/ml) were spectrophotometrically monitored at 393 nm; the data were analyzed and plotted with the enzyme kinetics algorithm of Grafit 4.013 (Erithacus Ltd., Middlesex, United Kingdom). The apparent K_m of the plotted data for the enzymatic reaction was 1.5 μM.
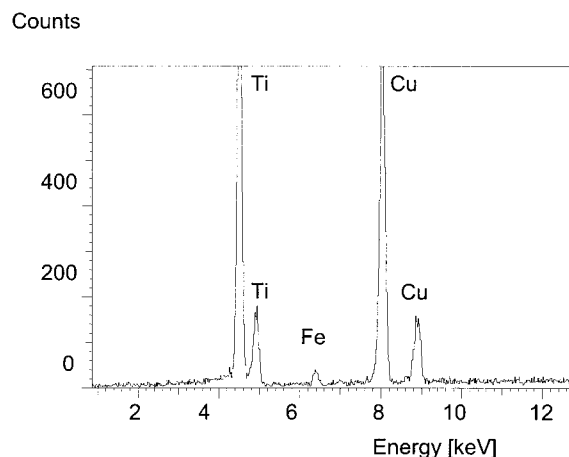

FIG. 4. Spectrum of CueO oxidation product. 2,3-Dihydroxybenzoic acid was oxidized by CueO, and the oxidation product was analyzed. For EDX analysis, the colored oxidation product was harvested by centrifugation, and the resulting pellet was redissolved in water and recentrifuged to remove excess copper. The washed precipitate was subjected to EDX analysis with an EM 912 Omega electron transmission microscope (Leo, Oberkochen, Germany) equipped with an EDX system (energy-dispersible X-ray analysis Link eXIII; Oxford Instruments, High Wycombe, United Kingdom) in the spot mode (100-nm spot size at 80 keV and 20 μA emission current).

physiological, periplasmic environment of CueO. Under these conditions, we determined a K_m of 1.5 μM for ferric enterobactin oxidation by CueO (Fig. 3) compared to the 40 μM described by Kim et al. (16). This high affinity of ferric enterobactin indicates that it is a natural substrate of CueO in vivo.

Oxidation-product of 2,3-dihydroxybenzoic acid and CueO sequesters copper. Incubation of 2,3-dihydroxybenzoic acid with CueO in the presence of copper resulted in the formation of an insoluble colored precipitate. The precipitate was found predominantly in the growth medium, indicating that, after oxidation, 2,3-dihydroxybenzoic acid is translocated to the outside of the cell. This oxidation product might act as a metal-chelating compound, and therefore the precipitate was examined by EDX spectral analysis. It was demonstrated that copper was bound to this polymeric 2,3-dihydroxybenzoic acid oxidation product (Fig. 4). Since 2,3-dihydroxybenzoic acid is also an excellent iron chelator, iron was also detected in the analysis. This suggests that oxidized 2,3-dihydroxybenzoic acid did not lose its ability to bind iron but gained the ability to complex copper. This could create a sink for copper outside the cell, thereby decreasing the overall solubilized copper concentration.

Short-term incubation of CueO with 2,3-dihydroxybenzoic acid resulted in an oxidation product in which the aromatic ring was opened by oxidation and oxygen was added in a stoichiometry of 1 mol of O_2 per mol of 2,3-dihydroxybenzoic acid, as determined by oxygraph analysis (30). The reaction product was identified as 2-carboxymuconate by GC-MS and was able to complex Cu(II) ions. The product has a molecular weight of 229, indicating that all three carboxylic groups are methylated. The molecular weight of 197 corresponds to a fragment of the reaction product which is expected when one molecule of methanol is lost, as shown in Fig. 5.

Enterobactin may facilitate copper uptake. Previously, Outten et al. (23) had shown that *cueO* expression is induced by the cytoplasmic regulator of transcription CueR in a copper-dependent fashion. Lysogen *E. coli* strain WOII260B (22) expressing a Φ(*cueO-lacZ*) operon fusion was also used to elucidate induction of *cueO* in this study. Compared to copper alone, elevated *lacZ* expression in *E. coli* Φ(*cueO-lacZ*) was observed when 2,3-dihydroxybenzoic acid or enterobactin in

combination with copper was added (Fig. 6). This may be due to increased copper uptake as a result of Cu(II) reduction by enterobactin or 2,3-dihydroxybenzoic acid.

It is thought that copper imposes oxidative stress by catalyzing Fenton-like reactions (26). This raised the question of whether expression of *cueO* was also induced by oxidative stress independent of copper. However, compounds generating oxidative stress, such as hydrogen peroxide, t-butyl peroxide, and paraquat, did not induce the reporter, and neither did
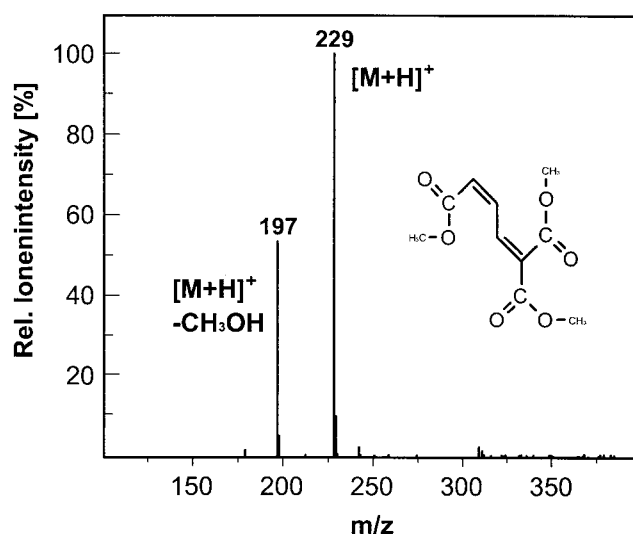

FIG. 5. Chemical ionization-MS spectrum of the 2,3-dihydroxybenzoate oxidation product catalyzed by CueO in the presence of 0.5 mM $CuCl_2$. The *m/z* of 229 represents the protonized trimethylated reaction product 2-carboxymuconate, and the *m/z* of 197 represents a fragment after the loss of one molecule of methanol.
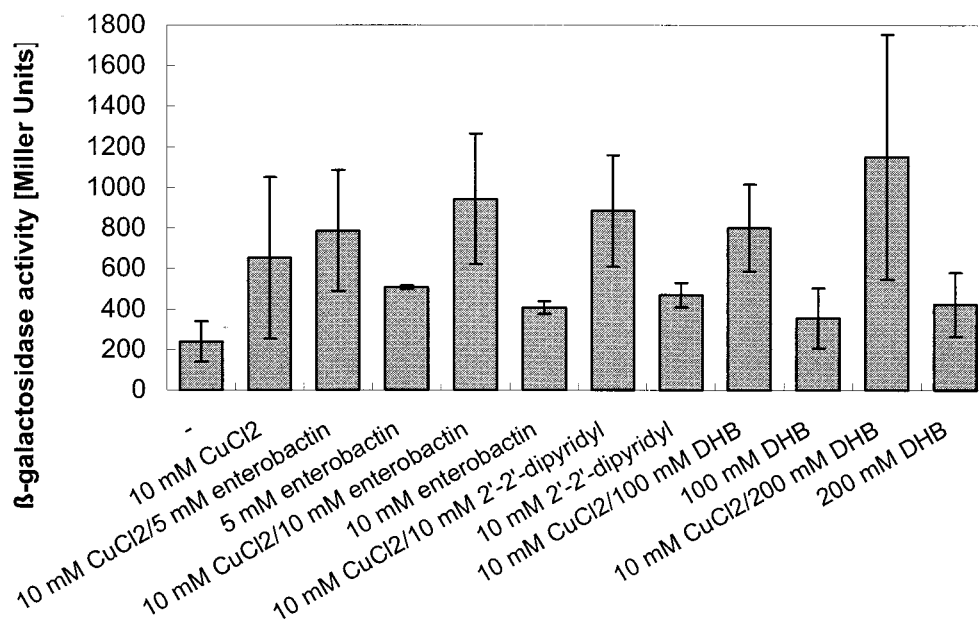
FIG. 6. Induction of *cueO* in *E. coli* strain WOII260B Φ(*cueO-lacZ*) under copper stress. Cells of *E. coli* strain WOII260B Φ(*cueO-lacZ*) containing a *cueO-lacZ* operon fusion on the bacterial chromosome grown in LB medium were diluted 15-fold into fresh minimal medium and induced after 2 h of growth. Incubation was continued with shaking at 37°C for 2 h, and the β-galactosidase activity was determined. Each experiment was performed in triplicate, and the average and standard deviation were calculated.

a radical-generating system comprising hematin and peroxide (data shown). Menadione induced *cueO* only in the presence of copper (data not shown). However, Φ(*cueO-lacZ*) was induced in the presence of low added copper concentrations (10 μM) by 2′,2′-dipyridyl, a strong chelator of iron and copper [Martell's critical stability constants log K = 8.5 for Cu(II), log K = 16.3 for Fe(III)] (Fig. 6). There was also a slight increase in Φ(*cueO-lacZ*) induction in medium without added copper, possibly due to residual copper in the medium. This could indicate that copper bound to 2′,2′-dipyridyl is able to enter the cell and might increase the cytoplasmic copper concentration. This all suggested that expression of *cueO* is directly dependent on copper and not indirectly on copper-generated oxidative stress.

Deletion of *fur* leads to copper hypersensitivity. The Fur protein of *E. coli* is the global regulator for iron uptake systems, defense against oxidative stress, and iron storage (3). Thus, a strain deleted of *fur* is also derepressed for enterobactin biosynthesis. We demonstrated that deletion of the *fur* gene rendered *E. coli* hypersensitive to copper (Fig. 7). The presence of ascorbate at the initial setup of the experiment was essential for the copper-sensitive phenotype of the *fur* mutants, indicating that the production of the prooxidant Cu(I) is the first step in creating toxic oxygen intermediates.

Because the combination of enterobactin and copper was shown to be detrimental for *E. coli*, the *entC* gene was deleted in addition to *fur*, preventing biosynthesis of enterobactin. The resulting strain tolerated copper much better than the single *fur* mutant (Fig. 7). An *entC* single deletion mutant exhibited copper tolerance comparable to that of the wild type at 100 μM (data not shown). These results support our hypothesis on the interaction of CueO with enterobactin that oxidation of enterobactin in the presence of copper is advantageous for *E.*

coli. Moreover, this clearly indicated that the deregulation of iron uptake might lead to enhanced copper uptake through transporters such as FeoB, MntH, and ZupT, but additional deletion of enterobactin production rendered *E. coli* more copper tolerant than a single *fur* deletion, making enterobactin the major contributor to cytotoxicity under copper stress. These results are consistent with the data obtained for enterobactin- and copper-dependent killing of *E. coli*. Previously, no involvement of the global iron repressor Fur in copper tolerance had been observed. This emphasizes the interrelationship
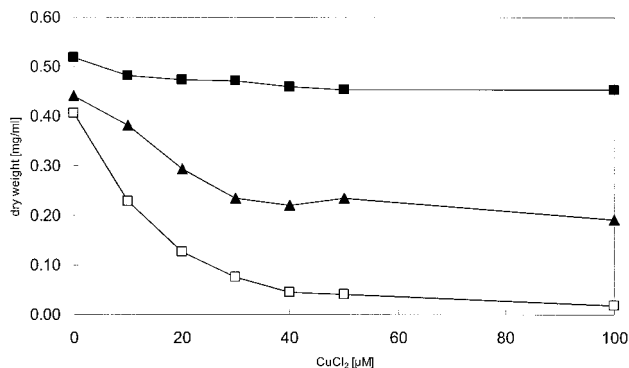

FIG. 7. Copper resistance of different *E. coli* strains. Growth in the presence of different $CuCl_2$ concentrations is shown. Overnight cultures were diluted 1:400 into fresh LB broth and, after 2 h of growth, diluted into fresh LB broth with the indicated concentrations of $CuCl_2$ and 1 mM ascorbate. Cell growth after 6 h of incubation at 37°C with shaking was monitored as the optical density at 600 nm and converted to dry weight. The *E. coli* strains used were W3110 (■), GG199 (Δ*fur::cat*) (□), and GG213 (Δ*fur::cat* Δ*entC*) (▲). Experiments were performed at least in triplicate, and the average is shown.
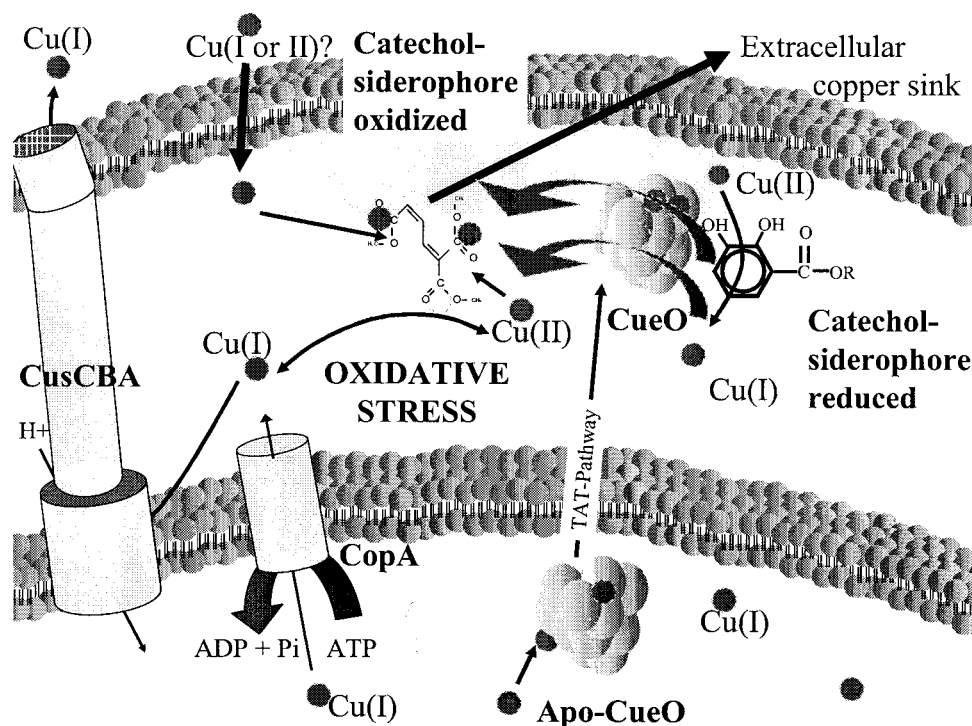
FIG. 8. Model of copper homeostasis in *E. coli* and the central role of CueO. Copper enters the bacterial cell by an unknown mechanism and exerts its toxicity by generating reactive oxygen species via copper-mediated redox cycling. Cytoplasmic detoxification of copper is accomplished by the P-type ATPase CopA, while periplasmic copper is effluxed by the CusCBA complex. The multicopper oxidase CueO probably converts Cu(I) to the less toxic Cu(II). Additionally, when copper is present, CueO oxidizes the catechol siderophore enterobactin, avoiding enterobactin-mediated reduction of Cu(II) to Cu(I). The resulting oxidation product of enterobactin, 2-carboxymuconate, sequesters copper and might constitute a copper sink after export to the outside.

of copper and iron homeostasis in *E. coli*. The point of contact is probably enterobactin and CueO.

DISCUSSION

In our initial hypothesis (12), we and others proposed that CueO converts periplasmic Cu(I) to the less toxic Cu(II) (8, 13, 23, 28). This mode of copper protection was recently confirmed for the related multicopper oxidase Fet3 in *S. cerevisiae*. Fet3 was shown to possess cuprous oxidase activity (35). This activity was only necessary for protection from copper toxicity if a copper reductase was also present (34). We also believe that CueO-dependent cuprous oxidase activity may be important in conferring copper resistance in *E. coli* and recently demonstrated CueO-dependent Cu(I) oxidase activity (unpublished data). In addition to this activity, CueO appears to protect cells from the interaction of enterobactin with copper by oxidation of enterobactin. The current study suggests that CueO performs a catalytic reaction, oxidizing the iron chelator enterobactin under copper stress in *E. coli*. Figure 8 depicts known factors important for periplasmic copper homeostasis in *E. coli* and presents the most recent model of the central role of CueO as an interface between copper detoxification and iron homeostasis.

CueO is only active aerobically because multicopper oxidases use oxygen as a terminal electron acceptor. Furthermore, copper slowly oxidizes catechols in the presence and/or absence of multicopper oxidases. One electron is transferred to

molecular oxygen in copper-mediated oxidation of catechols, forming superoxide, which is subsequently reduced to hydrogen peroxide and hydroxyl radicals. In other words, enterobactin and other catechols reduce Cu(II) to Cu(I) and thereby change the steady-state level of Cu(I) (15, 18, 32). Increased redox cycling of copper could lead to both increased production of reactive oxygen species and increased Cu(I) uptake. CueO can therefore perform several beneficial reactions. CueO can reduce the amount of Cu(I), reduce the presence of unoxidized enterobactin, and prevent the accumulation of free intermediate reactive oxygen species by coupling the oxidation of substrates with the complete reduction of oxygen to water.

Given the relatively small increase in *cueO* expression in the presence of copper plus siderophore compared to that in the presence of copper alone, the effect of siderophore-mediated reduction and subsequent uptake of copper into the cytoplasm and, thus, increased copper concentration cannot be considered a major stress factor within the cytoplasm. Probably, the critical compartment for copper-siderophore toxicity is the periplasmic space, where CueO exerts its protective oxidase activity.

This situation is reminiscent of mammalian systems, in which ceruloplasmin, in addition to its ferrooxidase activity, is thought to oxidize catecholamines such as 6-hydroxydopamine and thereby prevent the formation of reactive oxygen species (9). For example, copper accelerated the autooxidation of 6-hydroxydopamine 61-fold (5). Copper neurotoxicity was also

found to be dependent on dopamine-dependent copper uptake and may contribute to the death of dopaminergic neurons in Parkinson's disease. Interestingly, copper was implicated in the increased incidence of parkinsonism in subjects exposed to copper in mining operations in Chile (24). Since ceruloplasmin was also shown to have Cu(I) oxidase activity, it might have a bigger role in protection from neurodegenerative diseases than previously anticipated.

CueO might modify enterobactin so that it accumulates with sequestered copper in the periplasm and the extracellular medium. EDX analysis clearly demonstrated that the water-insoluble 2,3-dihydroxybenzoic acid oxidation product was able to bind copper. It was pointed out before that catechol-metal complexes are highly stable and that compounds containing catecholic nuclei can sequester metals from other complexes. This would also result in the prevention of redox cycling of those metals (4, 32).

In order to prevent an unfavorable constitutive oxidation of enterobactin by CueO, expression of *cueO* occurs only in the presence of copper. This control mechanism would ensure that the deleterious interaction between copper and enterobactin is avoided. CueO is regulated at the genetic and enzymatic levels by copper. CueO possesses a methionine-rich region that is involved in copper-dependent enzyme regulation. Recently, we showed that this region is essential for CueO-mediated siderophore oxidation (30).

E. coli strains deleted of *fur* were described as having a higher free iron concentration (3, 36). However, it was determined that such bacteria contain considerably less total iron, presumably by downregulating iron storage (1). Our results demonstrated that deletion of *fur* resulted in a severe copper-sensitive phenotype. Growth of a *fur*-deleted strain was almost completely inhibited at a CuCl$_2$ concentration as low as 100 μM in the presence of ascorbate. The parental wild-type strain *E. coli* W3110 under the same growth conditions is only inhibited at much higher concentrations (12). About 5,000 copies of the Fur protein are usually present in an *E. coli* cell, but under redox stress this level is increased twofold (1). A *fur* mutant also produces an increased amount of enterobactin. That enterobactin with copper is toxic to cells is again supported by the ability of the Δ*fur* Δ*entC* double deletion strain, which is unable to synthesize enterobactin, to tolerate higher copper concentrations than the Δ*fur* single-deletion strain.

The fact that the interaction of copper and the natural iron chelator enterobactin is toxic indicates that CueO and enterobactin constitute a connecting link between copper and iron homeostasis in *E. coli*. These links between iron and copper homeostasis in *E. coli* provide a launching pad from which to tackle the physiological mechanisms of trace metal homeostasis in prokaryotes. It also might initiate studies of CueO-mediated protection from catecholate siderophore-enhanced copper toxicity as a model for copper damage to dopaminergic neurons.

ACKNOWLEDGMENTS

REFERENCES

1. **Abdul-Tehrani, H., A. J. Hudson, Y. S. Chang, A. R. Timms, C. Hawkins, J. M. Williams, P. M. Harrison, J. R. Guest, and S. C. Andrews.** 1999. Ferritin mutants of *Escherichia coli* are iron deficient and growth impaired, and *fur* mutants are iron deficient. J. Bacteriol. **181:**1415–1428.
2. **Alexandre, G., and I. B. Zhulin.** 2000. Laccases are widespread in bacteria. Trends Biotechnol. **18:**41–42.
3. **Andrews, S. C., A. K. Robinson, and F. Rodriguez-Quinones.** 2003. Bacterial iron homeostasis. FEMS Microbiol. Rev. **27:**215–237.
4. **Avdeef, A., S. R. Sofen, T. L. Bregante, and K. N. Raymond.** 1978. Coordination chemistry of microbial iron transport compounds. 9. Stability constants for catechol models of enterobactin. J. Am. Chem. Soc. **100:**5362–5370.
5. **Bandy, B., P. B. Walter, J. Moon, and A. J. Davison.** 2001. Reaction of oxygen with 6-hydroxydopamine catalyzed by Cu, Fe, Mn and V complexes: identification of a thermodynamic window for effective metal catalysis. Arch. Biochem. Biophys. **389:**22–30.
6. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
7. **DeLisa, M. P., P. Lee, T. Palmer, and G. Georgiou.** 2004. Phage shock protein PspA of *Escherichia coli* relieves saturation of protein export via the Tat pathway. J. Bacteriol. **186:**366–373.
8. **Finney, L. A., and T. V. O'Halloran.** 2003. Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. Science **300:**931–936.
9. **Floris, G., R. Medda, A. Padiglia, and G. Musci.** 2000. The physiopathological significance of ceruloplasmin. A possible therapeutic approach. Biochem. Pharmacol. **60:**1735–1741.
10. **Franke, S., G. Grass, and D. H. Nies.** 2001. The product of the *ybdE* gene of the *Escherichia coli* chromosome is involved in detoxification of silver ions. Microbiology **147:**965–972.
11. **Grass, G., B. Fan, B. P. Rosen, K. Lemke, H. G. Schlegel, and C. Rensing.** 2001. NreB from *Achromobacter xylosoxidans* 31A is a nickel-induced transporter conferring nickel resistance. J. Bacteriol. **183:**2803–2807.
12. **Grass, G., and C. Rensing.** 2001. Genes involved in copper homeostasis in *Escherichia coli*. J. Bacteriol. **183:**2145–2147.
13. **Grass, G., and C. Rensing.** 2001. CueO is a multicopper oxidase that confers copper tolerance in *Escherichia coli*. Biochem. Biophys. Res. Commun. **286:**902–908.
14. **Hasegawa, H., M. Matsui, M. Suzuki, K. Naito, K. Ueda, and Y. Sohrin.** 2001. The possibility of regulating the species composition of marine phytoplankton using organically complexed iron. Anal. Sci. **17:**209–211.
15. **Kamau, P., and R. B. Jordan.** 2002. Kinetic study of the oxidation of catechol by aqueous copper(II). Inorg. Chem. **41:**3076–3083.
16. **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.
17. **Klebba, P. E., M. A. McIntosh, and J. B. Neilands.** 1982. Kinetics of biosynthesis of iron-regulated membrane proteins in *Escherichia coli*. J. Bacteriol. **149:**880–888.
18. **Li, Y., M. A. Trush, and J. D. Yager.** 1994. DNA damage caused by reactive oxygen species originating from a copper-dependent oxidation of the 2-hydroxy catechol of estradiol. Carcinogenesis **15:**1421–1427.
19. **Mergeay, M., D. Nies, H. G. Schlegel, J. Gerits, P. Charles, and F. van Gijsegem.** 1985. *Alcaligenes eutrophus* is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J. Bacteriol. **162:**328–334.
20. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Munson, G. P., D. L. Lam, F. W. Outten, and T. V. O'Halloran.** 2000. Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. J. Bacteriol. **182:**5864–5871.
22. **Outten, F. W., C. E. Outten, J. Hale, and T. V. O'Halloran.** 2000. Transcriptional activation of an *E. coli* copper efflux regulon by the chromosomal MerR homologue, CueR. J. Biol. Chem. **275:**31024–31029.
23. **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. **276:**30670–30677.
24. **Paris, I., A. Dagnino-Subiabre, K. Marcelain, L. B. Bennett, P. Caviedes, R. Caviedes, C. O. Azar, and J. Segura-Aguilar.** 2001. Copper neurotoxicity is dependent on dopamine-mediated copper uptake and one-electron reduction of aminochrome in a rat substantia nigra neuronal cell line. J. Neurochem. **77:**519–529.
25. **Payne, S. M.** 1994. Detection, isolation, and characterization of siderophores. Methods Enzymol. **235:**329–344.
26. **Pena, M. M. O., J. Lee, and D. J. Thiele.** 1999. A delicate balance: homeostatic control of copper uptake and distribution. J. Nutr. **129:**1251–1260.
27. **Rensing, C., B. Fan, R. Sharma, B. Mitra, and B. P. Rosen.** 2000. CopA: an

Escherichia coli Cu(I)-translocating P-type ATPase. Proc. Natl. Acad. Sci. USA **97:**652–656.

28. **Rensing, C., and G. Grass.** 2003. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. FEMS Microbiol. Rev. **27:**197–213.

29. **Roberts, S. A., A. Weichsel, G. Grass, K. Thakali, J. T. Hazzard, G. Tollin, C. Rensing, and W. R. Montfort.** 2002. Crystal structure and electron transfer kinetics of CueO, a multicopper oxidase required for copper homeostasis in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **99:**2766–2771.

30. **Roberts, S. A., G. F. Wildner, G. Grass, A. Weichsel, A. Ambrus, C. Rensing, and W. R. Montfort.** 2003. A labile regulatory copper ion lies near the T1 copper site in the multicopper oxidase CueO. J. Biol. Chem. **278:**31958–31963.

31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

32. **Schweigert, N., A. J. B. Zehnder, and R. I. L. Eggen.** 2001. Chemical properties of catechols and their molecular mode of toxic action in cells, from microorganisms to mammals. Environ. Microbiol. **3:**81–91.

33. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for detection and determination of siderophores. Anal. Biochem. **160:**47–56.

34. **Shi, X., C. Stoj, A. Romeo, D. J. Kosman, and Z. Zhu.** 2003. Fre1p Cu^{2+} reduction and Fet3p Cu^{1+} oxidation modulate copper toxicity in *Saccharomyces cerevisiae*. J. Biol. Chem. **278:**50309–50315.

35. **Stoj, C., and D. J. Kosman.** 2003. Cuprous oxidase activity of yeast Fet3p and human ceruloplasmin: implication for function. FEBS Lett. **554:**422–426.

36. **Touati, D., M. Jaques, B. Tardat, L. Bouchard, and S. Despied.** 1995. Lethal oxidative damage and mutagenesis are generated by iron in Δ*fur* mutants of *Escherichia coli*: protective role of superoxide dismutase. J. Bacteriol. **177:**2305–2314.