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Intracellular Copper Does Not Catalyze the Formation of Oxidative DNA Damage in *Escherichia coli*[▽]

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Because copper catalyzes the conversion of H_2O_2 to hydroxyl radicals in vitro, it has been proposed that oxidative DNA damage may be an important component of copper toxicity. Elimination of the copper export genes, *copA*, *cueO*, and *cusCFBA*, rendered *Escherichia coli* sensitive to growth inhibition by copper and provided forcing circumstances in which this hypothesis could be tested. When the cells were grown in medium supplemented with copper, the intracellular copper content increased 20-fold. However, the copper-loaded mutants were actually less sensitive to killing by H_2O_2 than cells grown without copper supplementation. The kinetics of cell death showed that excessive intracellular copper eliminated iron-mediated oxidative killing without contributing a copper-mediated component. Measurements of mutagenesis and quantitative PCR analysis confirmed that copper decreased the rate at which H_2O_2 damaged DNA. Electron paramagnetic resonance (EPR) spin trapping showed that the copper-dependent H_2O_2 resistance was not caused by inhibition of the Fenton reaction, for copper-supplemented cells exhibited substantial hydroxyl radical formation. However, copper EPR spectroscopy suggested that the majority of H_2O_2-oxidizable copper is located in the periplasm; therefore, most of the copper-mediated hydroxyl radical formation occurs in this compartment and away from the DNA. Indeed, while *E. coli* responds to H_2O_2 stress by inducing iron sequestration proteins, H_2O_2-stressed cells do not induce proteins that control copper levels. These observations do not explain how copper suppresses iron-mediated damage. However, it is clear that copper does not catalyze significant oxidative DNA damage in vivo; therefore, copper toxicity must occur by a different mechanism.

High concentrations of intracellular copper are toxic for both eukaryotic and prokaryotic cells. The human diseases Indian childhood cirrhosis and endemic Tyrolean infantile cirrhosis both result from high dietary levels of copper (44, 62), whereas Wilson disease occurs due to a genetic mutation that prevents the liver from pumping copper into the bile. Wilson disease patients have a defective copy of ATP7b, an ATP-driven copper efflux pump, and hepatocytes are damaged by the high copper levels that result from this defect (7, 58, 63, 64).

Escherichia coli contains a homolog of human ATP7b, CopA, with 31% protein identity (54). CopA pumps excess copper out of the cytosol into the periplasm (54). Once in the periplasm, copper is subject to two other systems, CueO and CusCFBA, that assist CopA in controlling intracellular copper levels (23, 33, 45). CueO is a multicopper oxidase that coverts Cu(I) to Cu(II), a less-toxic form (23, 33, 60). The CusCBA system is a cation diffusion pump that is believed to pump periplasmic copper across the outer membrane and out of the cell (17, 45). CusF is a periplasmic Cu(I) and Ag(I) binding protein that may deliver metals to the CusCBA system (4, 34, 40). *E. coli* mutants that lack CopA, CueO, and CusCFBA cannot grow in medium that contains large amounts of copper (24).

The mechanisms by which copper inhibits or kills overloaded cells are not known. In studies of eukaryotic cells treated with excess copper, workers detected elevated levels of DNA lesions, protein oxidation, lipid peroxidation, and reactive oxygen species generation (3, 36, 53, 55, 59). In vitro studies showed that copper is capable of generating hydroxyl radicals from H_2O_2 and thereby facilitates oxidative DNA damage (26):

$$X_{red} + Cu(II) \rightarrow X_{ox} + Cu(I)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + OH^- + HO^{\cdot}$$

$$HO^{\cdot} + DNA \rightarrow damage$$

In fact, the rate constants for Cu(II) reduction to Cu(I) by sulfhydryls (21) and for oxidation of Cu(I) by H_2O_2 (27) indicate that this Fenton-like process could occur at a physiologically relevant rate. These observations have led to the theory that DNA damage may be an important component of copper toxicity.

Various approaches have demonstrated that in *E. coli* most DNA damage is catalyzed by iron. For example, addition of H_2O_2 to growing cells creates DNA lesions, but these lesions can be prevented by prior addition of cell-permeable iron chelators, as in mammalian cells (29, 42). Furthermore, mutations that disrupt iron homeostatic mechanisms result in high intracellular levels of unincorporated iron and commensurate acceleration of DNA damage (32, 38, 65). The goal of this study was to determine whether a similar phenomenon occurred when copper homeostatic controls were eliminated.

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MATERIALS AND METHODS

Chemicals, enzymes, and media. EDTA disodium salt (EDTA) and potassium cyanide were obtained from Fisher. Cupric sulfate, Chelex 100, bovine liver catalase, hydrogen peroxide, phenol, horseradish peroxidase, amino acids, thiamine, β-mercaptoethanol, *o*-nitrophenyl-β-D-galactopyranoside, diethylenetriaminepentaacetic acid (DTPA), deferoxamine mesylate, 2,2′-dipyridyl, ferric chloride, and trimethoprim (TMP) were obtained from Sigma. α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) was purchased from Aldrich, and 4-aminoantipyrine was obtained from Acros. Hanks balanced salt solution without $CaCl_2$, $MgCl_2$, $MgSO_4$, and phenol red (HBSS) was obtained from Invitrogen.

Luria broth (LB) contained (per liter) 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl along with 8 g of agar for top agar and 16 g of agar for agar plates. M9 glucose minimal medium (M9G) consists of 0.2% glucose, 42 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 20 mM NH_4Cl, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, and 5 μg/ml thiamine (43).

Bacterial growth. Cells were cultured in LB or M9G. Where indicated below, amino acids were added to the latter medium at a final concentration of 0.5 mM. Media were supplemented with 100 μg/ml ampicillin, 20 μg/ml chloramphenicol, 30 μg/ml kanamycin, 100 μg/ml spectinomycin, or 12 μg/ml tetracycline when antibiotic selection was needed.

To ensure that all studies were conducted with exponentially growing cells, aerobic overnight cultures were diluted to an optical density at 500 nm (OD_{500}) of 0.005, and cells were grown aerobically at 37°C to an OD_{500} between 0.1 and 0.2. They were then subcultured again to obtain an OD_{500} of 0.005 in medium with or without copper sulfate and grown aerobically at 37°C to an OD_{500} of 0.1 to 0.2. Absorbance was determined at 500 nm to minimize interference by the absorbance of dissolved copper.

Strain construction. All strains used are listed in Table 1. Mutations were introduced into strains using P1 transduction. The chloramphenicol acetyltransferase gene was removed from the Δ*cusCFBA*::*cm* allele using the temperature-sensitive plasmid pCP20, which was then cured from the strain (11). LEM122 and LEM124 were constructed by P1 transduction of *gshA*::*kan* linked to *srlC300*::Tn*10* into W3110 and GR17, respectively.

Inductively coupled plasma measurement of intracellular copper. One-liter cultures were grown aerobically at 37°C to an OD_{500} of 0.1 to 0.2 in LB with or without 2.0 mM $CuSO_4$. The cells were then centrifuged, washed twice in 200 ml of 20 mM Tris-HCl–1 mM EDTA (pH 7.4), and then washed once and resuspended in 0.5 ml of 20 mM Tris-HCl (pH 7.4). Cells were lysed with a French press. Debris was pelleted by centrifugation at 22,000 × *g* for 20 min. The metal content of the supernatant was determined with an OES Optima 2000 DV (Perkin-Elmer).

H_2O_2 killing assay. Cells were grown aerobically at 37°C to an OD_{500} of 0.1 to 0.2 in LB with or without 2.0 mM $CuSO_4$. Cultures were diluted to an OD_{500} of 0.025 in LB at 37°C. H_2O_2 was added, and at different times aliquots were removed and diluted with LB containing catalase. Further dilutions of the sample were added to LB top agar and plated on LB agar. Colonies were enumerated after overnight incubation at 37°C. The level of survival was normalized to the number of viable cells prior to the addition of H_2O_2.

Similar experiments were performed with cells whose sulfur source had been switched from sulfate to cystine (49). Cells were grown aerobically at 37°C to an OD_{500} of 0.1 to 0.2 in M9G containing each of the 20 standard amino acids except Met and Cys at a concentration of 0.5 mM. $CuSO_4$ (400 μM) was added to some samples. Cultures were diluted to an OD_{500} of 0.025, and either cystine or Na_2SO_4 was added to a concentration of 0.5 mM. The cultures were incubated at 37°C for 5 min, and 2.5 mM H_2O_2 was then added. At each time, aliquots were diluted with LB containing catalase and plated on LB agar. The plates were incubated overnight at 37°C.

***thyA* forward mutagenesis assay.** To determine the rate of mutagenesis, Thy^- mutants were selected using TMP (43). TMP is a dihydrofolate reductase inhibitor that suppresses the growth of Thy^+ cells but not the growth of *thyA* mutants if thymine is present in the medium. Cells were grown aerobically at 37°C to an OD_{500} of 0.1 to 0.2 in LB with or without 2.0 mM $CuSO_4$. Cultures were diluted to an OD_{500} of 0.025 in 37°C LB with or without 2.0 mM $CuSO_4$, and 2.5 mM H_2O_2 was added. At intervals aliquots were removed and diluted with LB containing catalase. Cells were then plated for survival analysis as described above and for mutagenesis. To determine the number of Thy^- cells, 250 μl of cells diluted to an OD_{500} of 0.01 was added to F-top agar containing 1 mg/ml thymine and 0.1 mg/ml TMP. The top agar was then spread onto LB agar plates. The plates were incubated overnight at 37°C.

Phage inactivation. Oxidative inactivation of phage results from DNA damage (29). Bacteriophage λ_{vir} (10^7 PFU/ml) was inactivated in 0.8% NaCl (pH 7) (without buffer) by addition of copper or iron plus H_2O_2. Bacteriophage were incubated for 30 s with the metal prior to H_2O_2 addition. At different times, aliquots were removed, diluted with 0.8% NaCl containing catalase, mixed with the indicator strain W3110 in LB top agar, and then plated on LB agar. The number of plaques was determined after overnight growth at 37°C. The level of survival was normalized to the number of PFU prior to the addition of H_2O_2.

Quantitative PCR (qPCR) for DNA lesions. Cells were grown aerobically at 37°C to an OD_{500} of 0.1 to 0.2 in LB with or without 2.0 mM $CuSO_4$ and then diluted to an OD_{500} of 0.025 in 37°C LB. KCN was added to a concentration of 3.3 mM in order to accelerate DNA damage (70), and cells were incubated at 37°C for 5 min. H_2O_2 (2.5 mM) was then added. After 5 min, catalase was added, and the cells were placed on ice. Aliquots (20 μl) were removed, diluted with LB, and plated onto LB to determine cell survival. The remaining culture was centrifuged at 8,000 × *g* for 5 min at 4°C. Cells were lysed, and genomic DNA was isolated with a DNeasy tissue kit (QIAGEN). The genomic DNA was quantified using the Quant-iT PicoGreen double-stranded DNA reagent protocol of Invitrogen. The primer sequences are as follows: 5′-GGCGTGAACTCGCAAAA TATTACGATTCAGCC for the forward primer and 5′-AGGGCAACGGAAC ACCCGCCCAGAGCATAACC for the reverse primer. The primers encompassed a 10-kb region around *fumC*. PCR was performed using the Expand Long Template PCR system (Roche). The 25-μl reaction mixture contained 5 to 10 ng of genomic DNA for the template, each deoxynucleoside triphosphate at a concentration of 500 μM, each primer at a concentration of 300 nM, PCR buffer with 27.5 mM $MgCl_2$, and 3.75 U of DNA polymerase. PCR products were electrophoresed and then visualized and quantified with a phosphorimager.

EPR measurement of unincorporated intracellular iron and copper. One-liter cultures were grown aerobically at 37°C in LB with or without 2.0 mM $CuSO_4$. For measurement of free iron (69), cells were centrifuged and resuspended in 8 ml of LB at 37°C. One milliliter of 100 mM DTPA was added to block further iron import, followed by 1 ml of 200 mM deferoxamine mesylate. The latter compound penetrates cells, binds unincorporated ferrous iron, and in the presence of oxygen triggers the oxidation of the iron to electron paramagnetic resonance (EPR)-detectable ferric iron. Cells were incubated aerobically at 37°C for 15 min and then centrifuged. The cell pellet was washed twice with 5 ml of ice-cold 20 mM Tris-HCl (pH 7.4) and finally resuspended in 200 μl of ice-cold 20 mM Tris-HCl–10% glycerol (pH 7.4). An aliquot of the cell suspension (200 μl) was loaded into a quartz EPR tube, frozen on dry ice, and stored at −80°C. The EPR standard was $FeCl_3$ dissolved in 20 mM Tris-HCl–10% glycerol-20 mM deferoxamine mesylate; the concentration in the standard was determined using a ε at 420 nm of 2.865 mM^{-1} cm^{-1}. EPR signals were measured with a Varian Century E-112 X-band spectrophotometer equipped with a Varian TE102 cavity and temperature controller. The spectra were measured at a temperature of 15 K with the following settings: field center, 1,570 G; field sweep, 400 G; modulation frequency, 100 kHz; modulation amplitude, 12.5 G; time constant, 0.032; receiver gain, 4,000; and power, 10 mW.

Cuprous ion cannot be directly detected by EPR; however, redox-active cuprous ion can be detected if it is first oxidized to cupric ion by reaction with H_2O_2. Cells were centrifuged, washed twice in 5 ml of ice-cold 20 mM Tris-HCl (pH 7.4), and then resuspended in 200 μl of ice-cold 20 mM Tris-HCl–10% glycerol (pH 7.4). H_2O_2 was added to the cells to a concentration of 10 mM, and the cell suspension was immediately added to a quartz EPR tube and frozen on dry ice. The spectra were measured at a temperature of 50 K with the following settings: field center, 2,900 G; field sweep, 2,000 G; modulation frequency, 100 kHz; modulation amplitude, 5 G; time constant, 0.032; receiver gain, 10,000; and power, 0.6 mW.

H_2O_2 scavenging by whole cells. Fifty-milliliter cultures were grown aerobically at 37°C to an OD_{500} of 0.1 in LB. Copper was added to a concentration of 2.0 mM, and cells were incubated aerobically at 37°C for 30 min. The cells were centrifuged, washed twice in 25 ml ice-cold LB (pH 7.0), and then resuspended in 1 ml of ice-cold LB (pH 7.0). The cells were diluted to an OD_{500} of 0.1 in 37°C LB (pH 7.0) containing 2.5 mM H_2O_2. At intervals, aliquots were centrifuged to remove cells, and the supernatant was diluted 1/10 in 50 mM potassium phosphate (KP_i) (pH 7.0). The amount of residual H_2O_2 was determined by addition of 400 μl of a sample to 800 μl of a reaction mixture containing 500 nM H_2O_2, 2.5 mM phenol, 0.5 mM 4-aminoantipyrine, 40 μg horseradish peroxidase, and 1 mM KP_i (pH 7.0) (25). The reaction was allowed to proceed to completion, and the final absorbance at 505 nm was determined.

Spin trapping of hydroxyl radicals. One-liter cultures were grown aerobically at 37°C to an OD_{500} of 0.1 to 0.2 in LB with or without 2.0 mM $CuSO_4$. Cells were centrifuged, washed twice with 25 ml of ice-cold Chelex-treated HBSS, and resuspended in 0.5 ml of ice-cold HBSS. Approximately 100 μl of the cell

TABLE 1. Strains, plasmid, and phage used in this study

Strain, plasmid, or phage	Genotype	Reference or source
Strains		
AB1157	*thr-1 leuB6 proA2 his-4 thi-1 argE2 lacY1 galK2 rpsL supE44 ara-14 xyl-15 mtl-1 tsx-33*	46
AS421	As GC4468 *rpoS*::Tn*10*	22
AS430	As GC4468 Δ*oxyR*::*spc*	Lab strain
BW6165	Hfr PO120 *ara-41 lacY1 xyl-7 mel-2 argE86*::Tn*10*	68
BW831	*soxS3*::Tn*10* Δ(*argF-lac*) *rpsL sup*(am)	66
BW25113	$lacI^q$ $rrnB_{T14}$ $lacZ_{WJ16}$ *hsdR514* $araBAD_{AH33}$ $rhaBAD_{LD78}$	11
CSH7	*lacY rpsL thi-1*	39
DW3110	As W3110 plus *copA*::*kan*	54
GC4468	Δ*lacU169 rpsL*	15
GR17	As W3110 plus *copA*::*kan* Δ*cueO* Δ*cusCFBA*::*cm*	24
JI360	As MG1655 plus *katE12*::Tn*10*	56
JI364	As MG1655 plus Δ(*katG17*::Tn*10*)*1*	56
KK158	As CSH7 plus Δ(*xthA-pncA*) *zdh201*::Tn*10*	Lab strain
LEM1	As W3110 plus *recA56 srlC300*::Tn*10*	This study
LEM2	As W3110 plus *recA56 srlC300*::Tn*10 copA*::*kan* Δ*cueO* Δ*cusCFBA*::*cm*	This study
LEM25	As W3110 plus Δ(*katG17*::Tn*10*)*1 copA*::*kan* Δ*cueO* Δ*cusCFBA*::*cm*	This study
LEM27	As W3110 plus Δ(*katG17*::Tn*10*)*1 katE12*::Tn*10*	This study
LEM29	As W3110 plus Δ(*katG17*::Tn*10*)*1 katE12*::Tn*10 copA*::*kan* Δ*cueO* Δ*cusCFBA*::*cm*	This study
LEM33	As W3110 plus *copA*::*kan* Δ*cueO* Δ*cusCFBA*	This study
LEM37	As W3110 plus *katE12*::Tn*10 copA*::*kan* Δ*cueO* Δ*cusCFBA*	This study
LEM56	As W3110 plus *dps*::*cm recA56 srlC300*::Tn*10*	This study
LEM58	As W3110 plus *dps*::*cm recA56 srlC300*::Tn*10 copA*::*kan* Δ*cueO* Δ*cusCFBA*	This study
LEM63	As W3110 plus *recA938*::*cm copA*::*kan* Δ*cueO* Δ*cusCFBA*	This study
LEM65	As W3110 plus *rpoS*::Tn*10 recA938*::*cm*	This study
LEM67	As W3110 plus *rpoS*::Tn*10 recA938*::*cm copA*::*kan* Δ*cueO* Δ*cusCFBA*	This study
LEM80	As W3110 plus *recA938*::*cm* Δ(*katG17*::Tn*10*)*1 katE12*::Tn*10 copA*::*kan* Δ*cueO* Δ*cusCFBA*	This study
LEM88	As W3110 plus *recA938*::*cm gshA*::*kan srlC300*::Tn*10 copA*::*kan* Δ*cueO* Δ*cusCFBA*	This study
LEM107	As W3110 plus *recA938*::*cm gshA*::*kan srlC300*::Tn*10*	This study
LEM112	As W3110 plus *soxS3*::Tn*10 recA938*::*cm*	This study
LEM114	As W3110 plus *soxS3*::Tn*10 recA938*::*cm copA*::*kan* Δ*cueO* Δ*cusCFBA*	This study
LEM116	As W3110 plus Δ*oxyR*::*spc recA938*::*cm*	This study
LEM118	As W3110 plus Δ*oxyR*::*spc recA938*::*cm copA*::*kan* Δ*cueO* Δ*cusCFBA*	This study
LEM120	As W3110 plus *copA*::*kan* Δ(*katG17*::Tn*10*)*1 katE12*::Tn*10*	This study
LEM122	As W3110 plus *gshA*::*kan srlC300*::Tn*10*	This study
LEM124	As W3110 plus *gshA*::*kan srlC300*::Tn*10 copA*::*kan* Δ*cueO* Δ*cusCFBA*::*cm*	This study
LEM129	As W3110 plus Δ(*xthA-pncA*) *zdh201*::Tn*10*	This study
LEM130	As W3110 plus Δ(*xthA-pncA*) *zdh201*::Tn*10 copA*::*kan* Δ*cueO* Δ*cusCFBA*	This study
MG1655	Wild type	46
OD120	As AB1157 plus *gshA*::*kan srlC300*::Tn*10*	13
SM5	As AB1157 plus *recA938*::*cm*	70
W3110	IN(*rrnD-rrnE*)*1 rph-1*	46
WOII260B	As BW25113 plus λ(*cueO-lacZ*)	48
WOII260E	As BW25113 plus λ(*copA-lacZ*)	47
WOIII1A	As BW25113 plus λ(*cusC-lacZ*)	47
ZM12	As AB1157 plus *recA56 srlC300*::Tn*10*	Lab strain
Plasmid		
pCP20		8
Phage		
λ_{vir}		Jeff Gardner

suspension (the concentrations were adjusted so that all reaction mixtures had the same cell density) was added to a room temperature mixture (final volume, 1 ml) containing 100 μM DTPA, 10 mM 4-POBN, 170 mM ethanol, 1 mM H_2O_2, and HBSS (51). The reaction mixture was added to an EPR flat cell. The spectra were measured after incubation for 12 min at room temperature with the following settings: field center, 3,393 G; field sweep, 100 G; modulation frequency, 100 kHz; modulation amplitude, 1 G; time constant, 0.064; receiver gain, 63,000; and power, 20 mW.

Enzyme assays. Total catalase activity was determined by a modified assay for H_2O_2 clearance. Cells were washed twice in ice-cold 50 mM KP_i–0.1 mM EDTA (pH 7.0) and then resuspended in ice-cold 50 mM KP_i (pH 7.0). The cells were lysed using a French press, and the debris was removed by centrifugation at 22,000 × *g* for 20 min. The extracts were dialyzed overnight against 500 volumes of 50 mM KP_i–0.1 mM EDTA (pH 7.0) at 4°C. The dialyzed extracts were then diluted with 50 mM KP_i (pH 7.0) containing 10 mM H_2O_2. At intervals, aliquots were diluted 1/50 with 50 mM KP_i (pH 7.0). The amount of residual H_2O_2 was determined by the 4-aminoantipyrine–horseradish peroxidase method (see above).

Hydroperoxidase I (HPI), the KatG catalase, was specifically assayed on the basis of its ability to act as a peroxidase. Cell extracts were added to a solution containing 300 μM *o*-dianisidine, 900 μM H_2O_2, and 10 mM KP_i (pH 6.4), and the reaction was monitored at 460 nm.

β-Galactosidase activity was assayed using extracts prepared in ice-cold 50 mM Tris-HCl (pH 8.0) and a French press. After the debris was removed by centrifugation, cell extracts were added to a solution containing 0.7 mg/ml *o*-nitrophenyl-β-D-galactopyranoside, 10 mM KCl, 1 mM $MgSO_4$, 40 mM β-mercaptoeth-

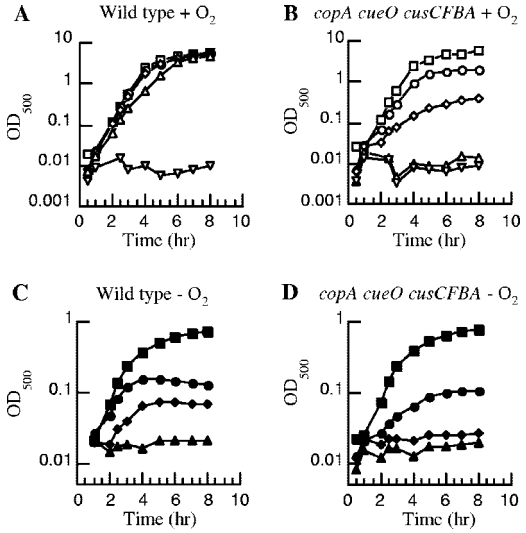

FIG. 1. Copper supplements are more toxic during anaerobic growth than during aerobic growth. (A and B) *E. coli* cultures were grown aerobically in LB, and $CuSO_4$ was added to a final concentration of 0 mM (□), 1 mM (○), 2 mM (◇), 3 mM (△), or 4 mM (▽). (A) Wild-type strain W3110. (B) GR17 (*copA cueO cusCFBA* mutant). (C and D) Cultures were grown anaerobically in LB, and $CuSO_4$ was added to a final concentration of 0 mM (■), 0.25 mM (●), 0.5 mM (♦), or 1 mM (▲). (C) Wild-type strain W3110. (D) GR17 (*copA cueO cusCFBA* mutant). The data are representative of three independent experiments.

anol, and 150 mM sodium phosphate buffer (pH 7.0), and the reaction was monitored at 420 nm.

Protein concentrations were determined using the Coomassie protein assay reagent (Pierce) with bovine serum albumin as the standard.

RESULTS

Copper inhibits the growth of cells. Our goal was to test whether copper can catalyze oxidative DNA damage. To do this, we tried to create forcing circumstances in which the intracellular copper levels were high and their contribution to damage was most pronounced. The *copA* gene encodes an exporter that pumps copper out of the cytoplasm, and consequently *copA* mutants grew more poorly than wild-type cells in LB that was supplemented with millimolar concentrations of copper (54) (data not shown). The growth defect was augmented by elimination of periplasmic copper oxidase (CueO) and an efflux (Cus) system, which removed copper(I) from the periplasm and thereby indirectly decreased its entry into the cytosol (Fig. 1). These results are consistent with those of previous studies (24).

The *copA cueO cusCFBA* copper resistance mutant was inhibited by micromolar concentrations of copper in defined media, perhaps because excess copper inhibited catabolic or biosynthetic processes essential for growth under those conditions (data not shown). We used LB for our study because the cells could tolerate higher copper levels. Using inductively coupled plasma analysis, we determined that supplementation with 2.0 mM copper increased the amount of total cellular copper more than 20-fold compared to the levels in cells grown on LB alone (data not shown).

It has been speculated that copper toxicity may be mediated by oxidative DNA damage. However, under anaerobic conditions copper suppressed the growth rate of *E. coli* even more strongly than it suppressed the growth rate under aerobic conditions (Fig. 1) (5, 47). This result demonstrated that copper toxicity cannot be mediated exclusively by oxidative DNA damage.

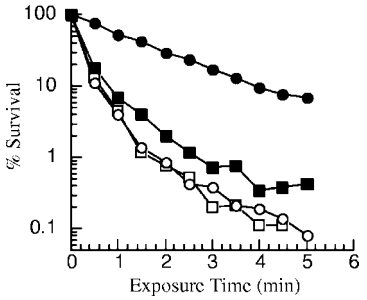

FIG. 2. Copper protected DNA-repair-deficient mutants from killing by H_2O_2. The LEM1 (*recA* $CopA^+$ $CueO^+$ Cus^+) (squares) and LEM2 (*recA copA cueO cusCFBA*) (circles) mutants were grown to the early log phase in LB without $CuSO_4$ (open symbols) or with 2.0 mM $CuSO_4$ (solid symbols). The cultures were then challenged with 2.5 mM H_2O_2. At each time, aliquots were removed and viability was determined. Recombination-proficient strains ($RecA^+$) exhibited >80% survival during the same treatment (data not shown). The data are representative of three independent experiments.

Copper protects against H_2O_2. DNA recombination provides an important repair pathway, and the rate at which *recA* mutants die during H_2O_2 exposure reflects the rate of DNA damage. To determine whether copper accelerates oxidative DNA damage, we created congenic $CopA^+$ $CueO^+$ Cus^+ *recA* and *copA cueO cusCFBA recA* mutant strains. Cells were grown to the early log phase and challenged with H_2O_2 for 5 min. Surprisingly, addition of 2 mM copper to the culture medium slightly protected the $CopA^+$ $CueO^+$ Cus^+ *recA* strain (Fig. 2). The protective effect was even greater for the *copA cueO cusCFBA recA* mutant (Fig. 2). Copper-dependent H_2O_2 resistance also occurred in base excision repair (*xthA*) mutants. Cells were grown to the early log phase and challenged with H_2O_2. After an H_2O_2 challenge for 6 min, 11% ± 2% of *xthA* mutant cells and 7.6% ± 2% of *copA cueO cusCFBA xthA* mutant cells survived. In contrast, after copper supplementation 37% ± 19% of *xthA* mutant cells and 77% ± 25% of *copA cueO cusCFBA xthA* mutant cells survived. Thus, rather than exacerbating toxicity, excessive copper diminished it.

This unexpected phenomenon was further tested by measurement of H_2O_2-induced mutagenesis. DNA repair-proficient cells are not killed by the H_2O_2 doses used in these experiments, but they accrue mutations. When *copA cueO cusCFBA* cells growing in standard LB were challenged with H_2O_2, the number of *thyA* mutants increased 10-fold. However, when 2 mM $CuSO_4$ was included in the medium, the H_2O_2 was not mutagenic (Fig. 3).

Copper blocks iron-mediated H_2O_2 killing. In usual circumstances the oxidative DNA damage due to H_2O_2 is mediated by iron (29). The kinetics of iron-mediated H_2O_2 killing are distinctive in that low doses of H_2O_2 result in a higher rate of killing than high doses (30). This phenomenon can be reproduced in vitro when iron and H_2O_2 inactivate phage by dam-

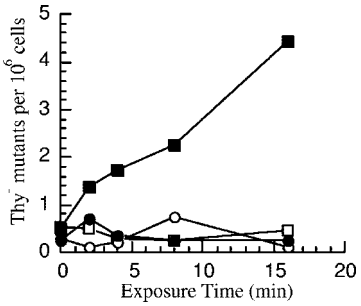

FIG. 3. Copper suppresses mutagenesis by H_2O_2. GR17 (*copA cueO cusCFBA*) mutant cells were grown to the early log phase in LB without $CuSO_4$ (squares) or with 2.0 mM $CuSO_4$ (circles). Cultures were then diluted to an OD_{500} of 0.025, and either no H_2O_2 (open symbols) or 2.5 mM H_2O_2 (solid symbols) was added. At each time, catalase was added to aliquots to scavenge H_2O_2, and both viability and *thyA* mutants were quantified. The data are representative of three independent experiments.

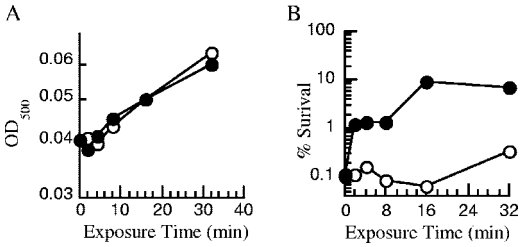

FIG. 5. Copper exposure confers H_2O_2 resistance before growth slows. Mutant LEM63 (*copA cueO cusCFBA recA*) was grown to early log phase in LB. $CuSO_4$ (2.0 mM) (●) or double-distilled H_2O (○) was added. At subsequent times the OD_{500} was determined (A), and an aliquot was removed and challenged with 2.5 mM H_2O_2 for 5 min (B). Catalase was then added, and viability was determined by plating. The data are representative of three independent experiments.

aging their DNA (29) (Fig. 4A). The explanation for this is that high concentrations of H_2O_2 quench the ferryl radical before it can dissociate into ferric iron and the genotoxic hydroxyl radical (29, 35). Both the $CopA^+$ $CueO^+$ Cus^+ *recA* strain and the *copA cueO cusCFBA recA* strain exhibited these kinetics when they were grown in standard LB prior to challenge with H_2O_2; killing was more marked with 2.5 mM H_2O_2 than with 10 mM H_2O_2 (Fig. 4C and D). However, when the *copA cueO cusCFBA*

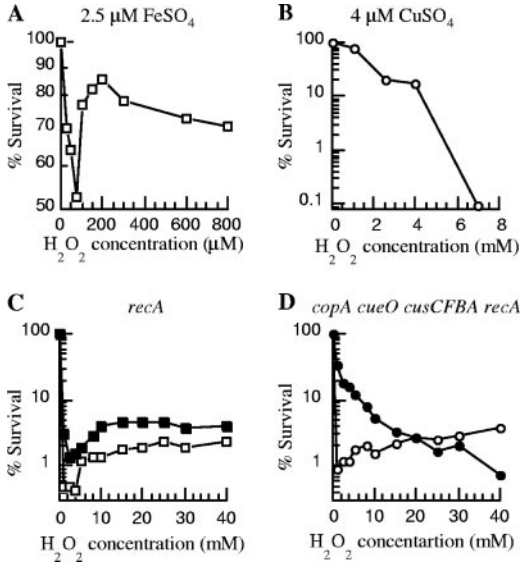

FIG. 4. Kinetic analysis indicates that copper specifically suppresses iron-mediated killing by low doses of H_2O_2. (A and B) λ_{vir} was exposed in vitro to 2.5 μM $FeSO_4$ (A) or 4 μM $CuSO_4$ (B) in the presence of various concentrations of H_2O_2. After a 30-s H_2O_2 challenge for iron-exposed phage and a 5-min challenge for copper-exposed phage, catalase was added to scavenge the H_2O_2, and phage viability was determined by examining plaque formation on W3110. (C and D) Mutants LEM1 (*recA*) (C) and LEM2 (*copA cueO cusCFBA recA*) (D) were grown to early log phase in LB containing 2.0 mM $CuSO_4$ (solid symbols) or no $CuSO_4$ (open symbols). Subsequently, cells were challenged for 2.5 min with various concentrations of H_2O_2. Catalase was added, and viability was determined. The data are representative of three independent experiments.

recA mutant was precultured in copper-rich medium, the rate of killing was markedly suppressed at the lower concentrations of H_2O_2. Only at inordinately high H_2O_2 concentrations were the cells killed. The latter dose-response curve resembles the curve which we observed when DNA was exposed to copper and H_2O_2 in vitro (Fig. 4B).

Deferoxamine, a cell-permeable iron chelator, protected *recA* cells grown in LB with or without $CuSO_4$ and *copA cueO cusCFBA recA* cells grown in LB without copper (data not shown). However, it had no effect on the residual killing observed when the *copA cueO cusCFBA recA* cells had been grown in copper-rich medium. These results are consistent with the idea that copper had already blocked iron-mediated DNA damage in the copper-replete cells.

Thus, the primary result is that even when cells are overloaded with copper, the copper does not generate a significant amount of DNA damage in the presence of low, physiological concentrations of H_2O_2. We are left with the following two new questions. (i) How does copper block iron-mediated H_2O_2 killing? (ii) What prevents copper from causing oxidative DNA damage in vivo?

Copper-mediated H_2O_2 resistance is not due to slow growth. In principle, slow growth per se might protect cells from oxidative DNA damage in at least two ways: a diminution in the metabolic flux can slow the availability of cellular reductants to transfer electrons to iron, an obligatory step in driving the Fenton reaction; and less-frequent replication may allow the cell more time to repair DNA damage before a replication fork passes, thereby lessening the chance of replication fork collapse. Therefore, we tested whether copper-mediated H_2O_2 resistance correlated closely with growth inhibition.

The *copA cueO cusCFBA recA* mutant was grown in LB, and 2 mM $CuSO_4$ was then added. The growth rate was monitored, and at intervals the sensitivity of the cells to H_2O_2 was determined. We observed that the addition of copper rapidly conferred resistance to H_2O_2, whereas the decline in the growth rate developed much more slowly (Fig. 5).

In a second experiment, we used β-thienylalanine, an inhibitor of aromatic amino acid biosynthesis, to decrease the growth rate twofold, an effect comparable to the effect observed after addition of copper when protection was manifest. The β-thienylalanine treatment did not increase the fraction of cells that survived a subsequent H_2O_2 challenge

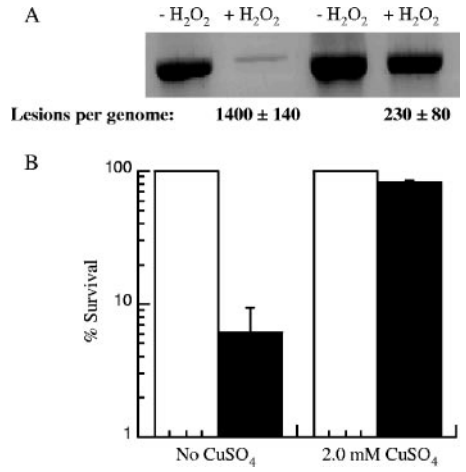

FIG. 6. Copper prevented the formation of oxidative DNA lesions in vivo. GR17 (*copA cueO cusCFBA*) mutant cells were grown to the early log phase in LB with or without 2.0 mM $CuSO_4$. Cells were diluted to an OD_{500} of 0.025 and incubated for 5 min in the presence of 3.3 mM KCN. H_2O_2 (2.5 mM) was then added. After 5 min catalase was added. (A) The total genomic DNA was isolated, and qPCR was performed using equal amounts of template DNA. The qPCR products were stained with ethidium bromide and scanned. Damage to the template DNA reduced the yield of the PCR product, and Poisson analysis allowed us to calculate the number of H_2O_2-induced lesions per genome that blocked the PCR polymerase. (B) After H_2O_2 challenge, cell viability was determined by plating. Solid bars, cells treated with 2.5 mM H_2O_2; open bars, untreated cells. The data are the means of three independent experiments. Error bars represent standard deviations.

(data not shown). Thus, both experiments indicated that the copper-induced resistance to H_2O_2 does not correlate with the growth rate.

Copper blocks oxidative DNA damage. In principle, copper might protect cells from H_2O_2 either by preventing DNA damage or by increasing cellular tolerance of DNA damage. To make this distinction, we employed qPCR to quantify the DNA lesions that were produced during H_2O_2 exposure. Unfortunately, qPCR is not sensitive enough to determine the rate of DNA damage when H_2O_2 alone is added to cells. However, the rate at which H_2O_2 damages DNA is substantially increased by respiratory inhibitors, which force the accumulation of free reduced flavin (reduced flavin adenine dinucleotide [$FADH_2$]), which in turn drives iron-mediated oxidative DNA damage (71). When *copA cueO cusCFBA* cells were exposed to H_2O_2 in the presence of cyanide, substantial lethality occurred, and the number of DNA lesions could be determined. (Neither lethality nor damage occurred if H_2O_2 was not added.) However, if the cells were first cultured in the presence of copper, both the level of lethality and the numbers of lesions were far lower (Fig. 6). Poisson analysis indicated that routinely grown cells suffered 1,400 ± 140 lesions per genome upon exposure to CN^- and H_2O_2, whereas copper treatment reduced the number to 230 ± 80 lesions per genome. Since cellular DNA was harvested immediately after H_2O_2 exposure, this effect was unlikely to be due to the stimulation of DNA repair. Furthermore, as noted above, copper protected mutants lacking either the known recombinational (RecA-dependent) or excisional (exonuclease III-dependent) pathways that repair oxidative DNA damage. These results indicate that copper somehow prevents iron-mediated oxidative DNA damage.

Protective effect of copper is not mediated by changes in ferrous iron or H_2O_2. Three things are needed for iron-mediated oxidative DNA damage to occur: redox-active iron, an electron donor, and H_2O_2. We tested whether copper affected the availability of any of these components inside the cell.

EPR spectroscopy was used with intact cells in order to quantify the unincorporated iron, which catalyzes hydroxyl radical production (see Materials and Methods). The iron pool in copper-treated *copA cueO cusCFBA* mutant cells (68 ± 11 μM) was not significantly different from that in untreated cells (77 ± 7 μM). In both cases essentially all of the iron was in the ferrous form, as the ferric iron signal became visible only when deferoxamine was added to trigger its oxidation.

The rate at which H_2O_2 damages DNA is highest when intracellular reductants are available to return H_2O_2-oxidized iron back to the ferrous form. In this way each iron atom can continuously cycle, thereby catalyzing the formation of multiple hydroxyl radicals. The reductant that drives Fenton reactions in routinely cultured cells is unknown. However, treatment of *E. coli* with respiratory inhibitors and with cystine can accelerate the rate of DNA damage by providing high levels of intracellular $FADH_2$ and cysteine, respectively, as reductants of free iron. The qPCR experiment described above demonstrated that copper interfered with $FADH_2$-driven DNA damage. Cysteine-driven damage is apparent when cells are exposed to H_2O_2 shortly after cystine is added to the medium of sulfate-grown cells (see Materials and Methods). Under these conditions cystine is rapidly imported through CysB-induced transporters, and the intracellular cysteine levels overshoot before subsequently stabilizing. During the overshoot period, the cell is very sensitive to H_2O_2 exposure (49). When this experiment was performed with *copA cueO cusCFBA* mutants, the presence of copper in the medium again strongly decreased the rate of killing (data not shown). Thus, since copper blocked DNA oxidation whether it was driven by $FADH_2$ or cysteine, it is unlikely that copper intervenes by blocking a specific mechanism of iron reduction.

When *copA cueO cusCFBA* mutants were cultured in copper-rich medium, their total catalase activity increased ninefold (Fig. 7). Addition of mutations in *katE* and *katG*, encoding the two catalases, revealed that copper strongly induces the expression of *katE* and moderately induces the expression of *katG* as well. These genes are known to be regulated by RpoS and OxyR, respectively, and the results suggest that these regulons are activated during copper influx. Nevertheless, catalase induction was not responsible for resistance to DNA damage, as the *katE katG copA cueO cusCFBA recA* mutant was still protected from H_2O_2 when it was pretreated with copper. After 2 min of exposure to 2.5 mM H_2O_2, 31% of *katE katG copA cueO cusCFBA recA* mutant cells supplemented with 2.0 mM copper survived, compared to the 0.2% of cells grown without excess copper that survived. In fact, calculations predicted that the elevated catalase titers would be insufficient to substantially diminish the millimolar dose of H_2O_2 during the limited period of cell exposure (57). Furthermore, data obtained during these experiments confirmed that the copper-loaded catalase mutants did not scavenge a significant fraction

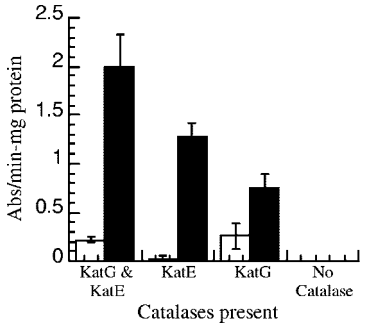

FIG. 7. Copper treatment induces both cell catalases. Strains GR17 (*copA cueO cusCFBA*; expressing KatG and KatE), LEM25 (*copA cueO cusCFBA katG*; expressing KatE), LEM37 (*copA cueO cusCFBA katE*; expressing KatG), and LEM29 (*copA cueO cusCFBA katE katG*; lacking both catalases) were grown in LB without $CuSO_4$ (open bars) or with 2.0 mM $CuSO_4$ (solid bars). Extracts were prepared, and their abilities to scavenge 10 mM H_2O_2 were determined at room temperature. The H_2O_2-scavenging ability of LEM29 (*copA cueO cusCFBA katE katG*) was ≤0.005 unit of absorbance (Abs)/min/mg protein and therefore could not be determined. The data are the means of three independent experiments. Error bars represent standard deviations.

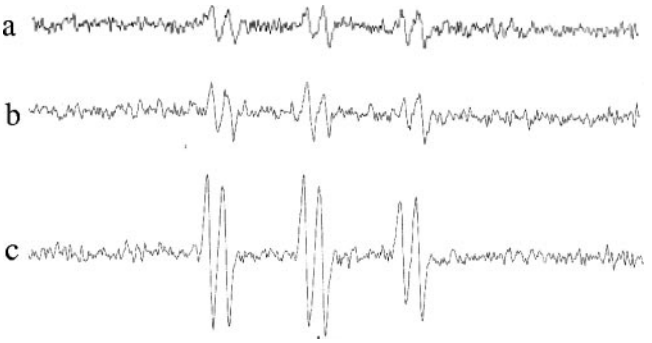

FIG. 8. Copper did not change hydroxyl radical generation inside cells. LEM29 (*copA cueO cusCFBA katE katG*) mutant cells were grown in LB without $CuSO_4$ (a) or with 2.0 mM $CuSO_4$ (b and c). For panel c, 1 mM dipyridyl was added to cells 5 min prior to exposure to 1 mM H_2O_2. Intracellular hydroxyl radicals were trapped and detected by EPR spectroscopy as described in Materials and Methods. No signal was present if H_2O_2 was not added (data not shown). The data are representative of three independent experiments.

of the H_2O_2, indicating that the intracellular copper does not protect cells by chemically degrading H_2O_2.

Under the protection conditions, measurements showed that the *katE katG copA cueO cusCFBA recA* cells did not scavenge a detectable fraction of the 2.5 mM H_2O_2, which ruled out the possibility that intracellular copper might rapidly degrade entering H_2O_2.

Altogether, this investigation established that excess copper blocks iron-mediated DNA oxidation but failed to reveal the mechanism by which it does so. One possibility is that free iron is displaced from the surface of DNA so that hydroxyl radical formation occurs in free solution.

Copper does not change the rate of HO· production. The DNA damage studies summarized above gave no indication that copper could catalyze hydroxyl radical formation inside cells. This issue was directly tested using EPR spectroscopy. In the presence of ethanol and 4-POBN, hydroxyl radicals can be scavenged and detected as stable 4-POBN-CH(CH_3)OH radicals. Both *katG katE* and *copA cueO cusCFBA katG katE* mutants were exposed to 1 mM H_2O_2 in the presence of the spin trapping reagents. (Catalase mutants were used in order to prevent H_2O_2 decomposition by the dense cell suspensions that were needed for this experiment.) The two strains exhibited similar rates of hydroxyl radical generation, and copper supplementation had little effect upon the EPR signal (Fig. 8; data not shown). Thus, when cells were exposed to H_2O_2, intracellular copper decreased the amount of DNA damage without significantly changing the rate of hydroxyl radical formation.

Interestingly, addition of the cell-penetrating metal chelator dipyridyl slightly decreased the rate of hydroxyl radical generation in cultures that were not supplemented with copper (data not shown). In contrast, dipyridyl substantially increased the rate of hydroxyl radical generation in the copper-replete cells (Fig. 8). Dipyridyl can bind both metals, but whereas it suppresses Fenton chemistry by iron (29), it does not block the redox cycling of copper. The stimulation of hydroxyl radical production therefore probably indicated that dipyridyl displaced some copper from biomolecules that were inhibiting its redox activity.

While it is clear that growth in copper-rich medium had no effect on the overall hydroxyl radical generation during H_2O_2 challenge, these experiments did not distinguish whether hydroxyl radical generation occurred in the cytoplasm or in the periplasm.

Most H_2O_2-oxidizable copper is in the periplasm. EPR was used to determine the level of H_2O_2-oxidizable copper inside the cells. EPR can readily detect copper in the Cu^{2+} redox state. Both *katG katE* cells and *copA cueO cusCFBA katG katE* cells grown in LB with or without copper supplements contained little or no Cu^{2+}, which matched our expectation for a reducing environment. However, substantial amounts of oxidizable Cu^{1+} were present inside the cells, for when cells were treated with 10 mM H_2O_2 immediately before freezing, a Cu^{2+} signal was observed (Fig. 9A). Growth in the presence of excess copper increased the H_2O_2-oxidizable copper 5-fold for wild-type cells and 27-fold for the copper resistance mutant (Fig. 9B).

Notably, the Cu^{1+} content was not detectably higher in a *copA katG katE* mutant than in the isogenic $CopA^+$ strain, even though the growth defect of the former strain, which lacks the CopA cytosolic exporter, implies that cytosolic copper levels are elevated. Indeed, addition of copper also protected *copA recA* cells from killing by H_2O_2 (data not shown). We inferred that the majority of the H_2O_2-oxidizable copper is located in the periplasm rather than in the cytoplasm, so that in the *copA* mutant the cytosolic copper is relatively scarce or is redox inactive. Consequently, the periplasm may have been the site of the majority of hydroxyl radical generation seen previously, reconciling this result with the lack of DNA damage.

In vitro, ligands block copper-mediated oxidative DNA damage. In vitro results have demonstrated that copper is capable of causing DNA damage; indeed, in vitro DNA damage assays showed that copper readily catalyzed phage inactivation by H_2O_2 (Fig. 4B). However, since copper did not cause oxidative

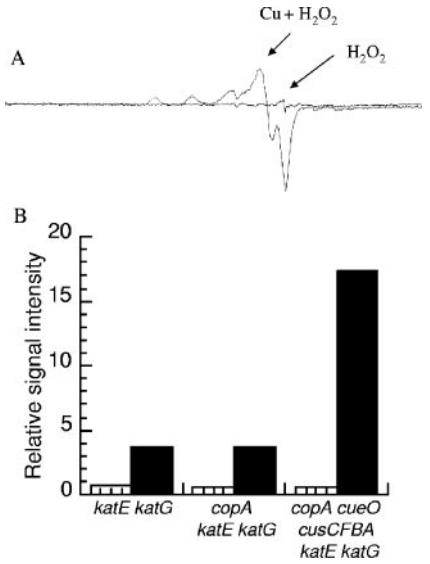

FIG. 9. H_2O_2-oxidizable copper is located in the periplasm. Cells were grown in LB with or without 2.0 mM $CuSO_4$. Cells were concentrated as described in Materials and Methods, and 10 mM H_2O_2 was added immediately before freezing. (A) EPR spectra of LEM29 (*copA cueO cusCFBA katE katG*) cells grown with and without 2.0 mM $CuSO_4$. No signal was evident if H_2O_2 was not added (data not shown). (B) The relative signal intensities of LEM27 (*katE katG*), LEM120 (*copA katE katG*), and LEM29 (*copA cueO cusCFBA katE katG*) mutant cells grown with (solid bars) and without (open bars) 2.0 mM $CuSO_4$ and exposed to H_2O_2 were calculated by dividing the EPR signal intensity by the OD_{600} of the cell paste. The data are representative of three independent experiments.

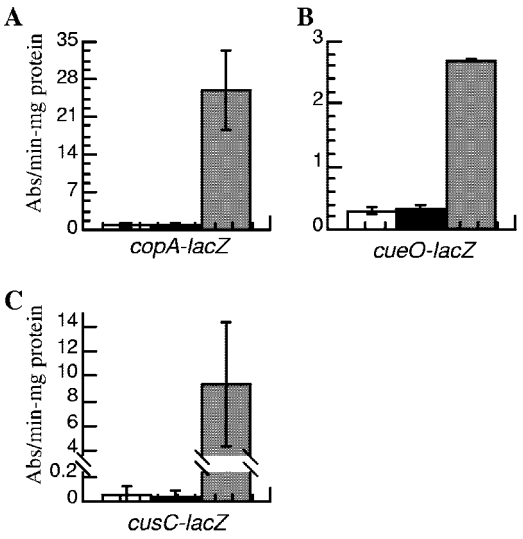

FIG. 10. H_2O_2 does not induce copper efflux genes. WOII260E (*copA-lacZ*) (A), WOII260B (*cueO-lacZ*) (B), and WOIII1A (*cusC-lacZ*) (C) cells were grown to an OD_{500} of 0.1 in LB and then challenged with 60 μM H_2O_2 (solid bars) or 2.0 mM $CuSO_4$ (shaded bars). Control cells were not challenged (open bars). Cells were grown for 60 min at 37°C and then harvested. The data are the means of three independent experiments. Error bars represent standard deviations.

DNA damage in vivo, the cytosolic milieu must either prevent copper-mediated hydroxyl radical generation or ensure that it does not occur near the DNA. Copper can be strongly bound by sulfurous and nitrogenous ligands, which may suppress its redox activity. Indeed, phage inactivation was fully blocked by physiological concentrations of glutathione, cysteine, and histidine (data not shown). This effect did not arise from simple scavenging of hydroxyl radicals, as higher concentrations of ethanol that should have resulted in a greater scavenging effect were not protective (data not shown).

H_2O_2 does not induce copper efflux. The results described above indicate that even high levels of cellular copper do not generate significant DNA damage when cells are exposed to H_2O_2. This result contrasts with what is observed in iron-overloaded cells.

Recent results obtained in several labs indicate that the response of cells to H_2O_2 stress is designed to avoid iron-mediated hydroxyl radical formation. During exposure to H_2O_2, the OxyR transcription factor is activated to stimulate expression of genes that are involved in tolerance to and defense against H_2O_2 (9, 74, 75). Two such genes are *dps* and *fur* (2, 73). Dps is an iron-sequestering protein that protects cells from DNA damage during H_2O_2 stress by binding free iron (1, 72). Fur is a repressor of genes that encode iron import systems (6, 14). Collectively, the effect of expression of these proteins during oxidative stress is to decrease the amount of free iron that might otherwise catalyze Fenton chemistry. Mutations in *oxyR*, *dps*, or *fur* lead to such high levels of free iron that even 1 μM H_2O_2 is sufficient to generate lethal DNA damage (50).

If copper were as capable of catalyzing hydroxyl radical formation as iron is, then one might expect the H_2O_2 response to include induction of the *copA*, *cueO*, and *cusCFBA* operons. To test this idea, we examined gene fusions in which the promoters for *copA*, *cueO*, and *cusCFBA* preceded a promoterless *lacZ* gene on a λ phage (47). When 2 mM copper was added to growth medium (Fig. 10), *copA* expression increased 30-fold, *cueO* expression increased 9-fold, and *cusCFBA* expression increased 1,500-fold, in agreement with results obtained in the laboratory of O'Halloran (47). However, when cells were challenged with 60 μM H_2O_2, these copper resistance genes were not induced at all. The same exposure did cause an eightfold increase in HPI (KatG) activity, demonstrating that the OxyR regulon was successfully activated. These results suggest that there has been little evolutionary pressure to limit intracellular copper levels during periods of H_2O_2 stress, further supporting the idea that copper, unlike iron, is not an effective catalyst of cytoplasmic hydroxyl radical formation in *E. coli*.

DISCUSSION

Cells of all types struggle to grow in media that contain high levels of copper. The sensitivity is especially acute in bacterial and mammalian cells that lack the pumps that maintain tolerable levels of intracellular copper. The mechanism of injury has not been resolved yet. However, a recurring theme in discussions of copper toxicity has been the expectation that copper participates in Fenton-like reactions, generating hydroxyl radicals that might then damage DNA, lipids, and proteins. Indeed, exposure of mammalian cells to high doses of copper resulted in some indications of accelerated DNA damage (18, 55, 67). However, these experiments did not unam-

biguously imply the mechanism by which copper was acting, since in addition to providing a metal catalyst for hydroxyl radical formation, copper treatment may also have increased the rate of H_2O_2 generation (3, 19, 37, 41), which could have accelerated iron-mediated oxidative DNA damage.

In the present study saturating doses of H_2O_2 were added exogenously, thereby rendering moot the effect of copper on intracellular H_2O_2 formation. What we observed was that copper not only failed to contribute to oxidative DNA damage but also suppressed the damage that would otherwise have been catalyzed by intracellular iron. While the data clearly show that copper toxicity does not involve oxidative DNA damage, the results pose several mechanistic questions.

Why doesn't copper cause significant oxidative DNA damage? *E. coli* has been an effective system for testing the involvement of specific metals in oxidative DNA damage. The predominant role of iron in catalyzing hydroxyl radical formation has been demonstrated through studies of the kinetics of damage, through genetic manipulations that overload the cells with iron, and through the protective action of iron chelators. Knowing that a mixture of copper and H_2O_2 efficiently oxidizes DNA in vitro, we suspected that the reason that copper has little effect on DNA damage in vivo is that efflux systems efficiently exclude copper from the cell interior. However, having seen that copper does not damage DNA even in efflux mutants, we now need to consider other issues that might blunt its genotoxicity.

One possibility is that even in the copper export mutants the copper overloading occurs primarily in the periplasm rather than in the cytosol. However, *copA* mutants, which lack only the exporter that pumps copper out of the cytosol, grow poorly in the presence of elevated copper levels, indicating that there must be some overloading of copper in the cell interior.

Perhaps it is more likely that the excess copper is chelated by ligands that either suppress the release of hydroxyl radicals or ensure that they are formed in the bulk solution away from DNA. Copper(II) is rapidly coordinated and reduced by sulfhydryl ligands in solution, including glutathione and cysteine (21). While the resultant copper(I) complex can be easily oxidized by H_2O_2 (27), the oxidative species that is formed, whether it is a transient copper(III) equivalent or a hydroxyl radical, may immediately accept an electron from the coordinating sulfhydryl. The result would be oxidation of the ligand and a failure to release a hydroxyl radical. Addition of dipyridyl, a nonsulfurous chelator, greatly amplified the release of hydroxyl radicals by copper inside overloaded cells, strongly suggesting that in the absence of dipyridyl the copper was coordinated by ligands that suppressed the release of a free hydroxyl radical.

Copper complexation can also protect DNA by a second mechanism. Hydroxyl radicals are so reactive that they are believed to diffuse an insignificant distance in vivo before they collide with a biomolecule and react. Iron-mediated hydroxyl radical formation, therefore, probably threatens DNA because loose ferrous iron binds directly to the phosphodiester backbone, so that hydroxyl radicals are generated on the surface of the target. Copper also tends to bind DNA in vitro, but stronger competing ligands may complex copper away from the DNA in vivo. Hence, both the addition of cysteine and the addition of histidine suppressed DNA damage by copper-H_2O_2 in our in vitro system.

Glutathione is often considered the most likely ligand for loose copper in vivo. We tested whether elimination of glutathione through a biosynthetic mutation enabled copper to drive DNA damage in copper export mutants. The resultant strain (*gshA copA cueO cusCFBA*) was somewhat more sensitive to growth inhibition by copper, but there was no indication of accelerated DNA damage when H_2O_2 was added to *gshA recA copA cueO cusCFBA* mutant cells (data not shown).

How does copper block oxidative DNA damage? While chelation of copper might explain its inability to contribute to DNA damage, we are still searching for the reason that it prevents iron-mediated damage. Hydroxyl radical generation appeared to be unabated. Therefore, copper treatment must in some way cause hydroxyl radical generation to take place away from DNA, either because copper directly displaces iron from the DNA or because copper overloading triggers a response in which iron is sequestered.

Recent studies have suggested that there are high-affinity metal binding sites on DNA, with the binding constant determined by the local nucleotide sequence (52), and that these sites are sites where there is preferential DNA damage. If this is true, then it is plausible that an excess of a competing metal might preclude DNA oxidation by iron. An obvious problem with this explanation for the present data is that copper should merely supplant iron as a site-specific generator of hydroxyl radicals. Furthermore, in our in vitro experiments, addition of copper did not suppress DNA oxidation by iron (data not shown).

Therefore, we considered the possibility that cells respond to excess copper by accumulating either a metabolite or a storage protein that sequesters metal ions, including iron. While mammalian cells effect such a response through the synthesis of metallothionine, no analogous system is known to exist in bacteria. We tested whether protein synthesis was required for copper-dependent hydrogen peroxide resistance, but our results were ambiguous (data not shown), because the inhibition of protein synthesis by chloramphenicol itself conferred resistance to hydrogen peroxide.

As a consequence of copper overload, we observed increased activity of hydroperoxidase I (KatG), hydroperoxidase II (KatE), and superoxide dismutase (Fig. 7 and data not shown). These results implied that copper stress induced the OxyR, RpoS, and SoxRS regulons. Therefore, we tested whether elimination of *oxyR*, *rpoS*, or *soxS* affected the copper-dependent H_2O_2 resistance of *copA cueO cusCFBA recA* cells. In each case, the elimination of the stress response regulator had no effect on either copper-dependent growth inhibition or H_2O_2 resistance (data not shown). In analogous experiments, we also tested whether the OxyR-regulated and RpoS-regulated iron storage protein encoded by *dps* played a role. It did not. At present, the ability of copper to block iron-mediated DNA damage remains a mystery.

How does copper poison cells? This investigation was motivated by the hypothesis that the toxicity of copper might result from its ability to catalyze a Fenton-like reaction. Evidently this is not the case, at least in *E. coli*. Several alternative mechanisms of copper toxicity have been suggested, and while we did not test these alternative mechanisms directly, some

observations made in the course of our experiments have some bearing on them. Studies with dihydrofluorescein have suggested that copper overloading increases the formation of an undefined oxidant in mammalian cells (3, 36), raising the possibility that copper exerts some effects through the action of reactive oxygen species other than the hydroxyl radical. In fact, we noted that both catalase and superoxide dismutase were strongly induced in copper-treated *E. coli*, consistent with the presence of elevated levels of hydrogen peroxide and superoxide, respectively (Fig. 7 and data not shown). These oxygen species can directly damage specific metalloenzymes (16, 31) and thereby debilitate the pathways to which they belong. Experiments are under way to test whether this occurs in copper-overloaded *E. coli*. Reactive oxygen species formation cannot be the sole mechanism of copper toxicity, however, as copper remains highly toxic to anaerobic cells, in which these species cannot form (5, 47). In fact, similar hypersensitivity to copper has been reported for anaerobic yeast (61).

The fact that two of the three known chromosomal copper resistance systems in *E. coli*, CueO and CusCFBA, work to clear copper(I) from the periplasm suggests that this compartment may be an important site of copper toxicity (17, 23, 24, 33). Indeed, our copper EPR studies suggest that in copper-overloaded cells the great majority of the EPR-detectable copper was located in the periplasm. Other workers have noted that the promoter of the copper stress response regulator, *cueR*, contains a putative binding site for CpxR, a regulator that responds to periplasmic stress signals (12, 48). Furthermore, *dsbC* mutants, which lack periplasmic disulfide isomerase, are especially sensitive to growth inhibition by copper (28).

Thus, while the mechanism of copper toxicity is fundamentally unsolved, intriguing observations point in new directions. It will be particularly interesting to determine the mechanism by which excess copper suppresses oxidative DNA damage. Oxidative resistance also appears to be conferred when bacteria are overloaded with other metals, including manganese (10) and zinc (20).

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