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Survival of *Escherichia coli* Cells on Solid Copper Surfaces Is Increased by Glutathione

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Bacteria are rapidly killed on solid copper surfaces, so this material could be useful to limit the spread of multiple-drug-resistant bacteria in hospitals. In *Escherichia coli*, the DNA-protecting Dps protein and the NADH:ubiquinone oxidoreductase II Ndh were not involved in tolerance to copper ions or survival on solid copper surfaces. Decreased copper tolerance under anaerobic growth conditions in the presence of ascorbate and with melibiose as the carbon source indicated that sodium-dependent symport systems may provide an import route for Cu^{I} into the cytoplasm. Glutathione-free Δ*copA* Δ*gshA* double mutants of *E. coli* were more rapidly inactivated on solid copper surfaces than glutathione-containing wild-type cells. Therefore, while DNA protection by Dps was not required, glutathione was needed to protect the cytoplasm and the DNA against damage mediated by solid copper surfaces, which may explain the differences in the molecular mechanisms of killing between glutathione-containing Gram-negative and glutathione-free Gram-positive bacteria.

Copper surfaces, long known for their beneficial effect on public health, may be useful to limit the spread of antibiotic-resistant bacteria in hospitals, long-term care facilities, and public places (1, 2). In contrast to other surfaces such as stainless steel or plastic material, microorganisms are rapidly killed on the surfaces of copper and its alloys (3). This effect has been demonstrated for bacteria such as pathogenic *Escherichia coli* strains (4), *Pseudomonas aeruginosa* (5), methicillin-resistant *Staphylococcus aureus* (6), *Burkholderia cepacia* (7), and *Salmonella enterica* (8), as well as for viruses (9, 10) and yeasts (11). Copper ions need to be released from the copper surfaces to mediate the killing process (12, 13), and there has to be direct contact between the cell and the copper surface (14). Therefore, a variety of parameters influence the success of the inactivation process by interfering with copper release and the availability of the copper ions for the killing action: (i) temperature, (ii) humidity, (iii) ionization/corrosion, (iv) the dry/wet test protocol used, and (v) the content of organic material (6, 15, 16).

If copper surfaces are employed to limit the spread of bacteria in hospitals (17, 18), it is essential to understand the molecular mechanisms behind the killing process, to optimize the handling and cleaning of copper surfaces, and to avoid the evolution of copper-surface-resistant pathogenic bacteria (19). Since copper ions are the mediators of the inactivation process, the presence of efflux pumps that remove copper ions from the cells extends the survival time of bacteria and yeasts on these surfaces, although they are killed later on nevertheless (11, 12, 16, 20). All cells suffered from rapid damage of the membranes (11, 21) by membrane lipid peroxidation (22), oxidative modifications of their proteins (23), and degradation of their DNA (24–26).

On solid copper surfaces, *Firmicutes* such as *S. aureus* may be predominantly killed by DNA degradation, but *Proteobacteria* such as *E. coli* may be predominantly killed by membrane damage (27). This difference was explained by protection of the nucleic acid by the periplasm of the Gram-negative proteobacteria (27). If this is true, copper import into the cytoplasm should be a slow process in proteobacteria. The zinc importer ZupT is a low-rate transport system for Cu^{II} (28) and should supply only minor amounts of copper to the cytoplasm. In contrast, import of the more toxic Cu^{I} could be mediated unspecifically and rapidly by sodium or potassium transporters (29). The ionic radius of Cu^{I} is 0.96 Å and therefore similar to that of Na^{I}, 0.95 Å (30). Na^{I} ions bind to copper-binding proteins (31) despite the differences in their ligand preferences. Thus, Cu^{I} is more likely to use Na^{I} import pathways than those for K^{I}. Time course experiments have revealed that the *copA* gene for the Cu^{I}-exporting P-type ATPase of *E. coli* is expressed stronger and for a longer time under anaerobic than under aerobic conditions (32) because Cu^{II} is anaerobically reduced to Cu^{I} and subsequently, more CopA efflux pumps are needed to balance the rapid and unspecific Cu^{I} uptake (33). This explains also the necessity of the periplasmic Cu^{I} oxidase CueO and the transenvelope Cu^{I}/Ag^{I} efflux complex CusCBA/CusF to remove Cu^{I} from the periplasm by oxidation and by export to the outside, respectively (29, 34–38).

In this study, we tested whether (i) Ndh, (ii) Dps, (iii) sodium-dependent uptake systems, or (iv) glutathione (GST) might be involved in survival of *E. coli* on solid copper surfaces and its tolerance to copper ions. Copper ions in liquid culture served as a model for copper ions released by solid copper surfaces during the inactivation process. The NADH:ubiquinone oxidoreductase II Ndh might reduce Cu^{II} to Cu^{I}, thus providing Cu^{I} for rapid import of Cu^{I}, maybe by Na^{I}-dependent symporters. Ndh is a copper-dependent (39) cupric reductase (40) expressed under aerobic conditions (41, 42). Dps protects the genomes of stationary-phase cells of *E. coli* by binding nonspecifically to the DNA and sequesters iron in the interior of the dodecameric protein. Although Dps does not store copper, Δ*dps* mutants of *E. coli* were

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
W3110	Wild type (K-12 derivative)	72
ECA769	Δ*copA*	44
ECA464	Δ*copA* Δ*cueO* Δ*cusCFBA*	This study
ECA657	Δ*ndh*	This study
ECA659	*usA* Δ*cueO* Δ*ndh*	This study
ECA658	Δ*cueO* Δ*cusCFBA* Δ*ndh*	This study
ECA765	Δ*dps*	This study
ECA766	Δ*copA* Δ*dps*	This study
ECA768	Δ*copA* Δ*cueO* Δ*cusCFBA* Δ*dps*	This study
ECA461	Δ*copA* Δ*ghsA*	44
ECA462	Δ*copA* Δ*ghsB*	44
ECA764	Δ*copA* Δ*ghsA* Δ*dps*	This study
ECA767	Δ*copA* Δ*ghsB* Δ*dps*	This study
Plasmids		
pASK-IBA3	Vector	IBA GmbH, Göttingen, Germany
pECD1256	pASK-IBA3::*gshA*	This study
pECD1257	pASK-IBA3::*gshB*	This study

more sensitive to copper under anaerobic conditions (43). If DNA is a target of copper surface-mediated cell killing in proteobacteria, Δ*dps* mutants should therefore show decreased survival on copper surfaces, especially in cells without copper efflux systems such as CopA. Glutathione is also involved in copper ion tolerance (44). In contrast to most proteobacteria, Gram-positive bacteria contain no glutathione (45–47). The presence or absence of this important cellular thiol compound might explain the fundamental difference in the ways Gram-negative and -positive bacteria are killed on solid copper surfaces.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strains (Table 1) were grown in Luria-Bertani (LB) medium or in Tris-buffered mineral salts medium (TMM) (48) containing 2 ml of glycerol and 3 g of Casamino Acids per liter. Anaerobic growth curves were recorded in Hungate tubes in TMM with 2 g/liter of glucose, lactose, or melibiose. Hungate tubes were used for growing and maintaining anaerobic bacteria and culture conditions (49). Solid media contained 20 g of agar/liter. Antibiotics (25 μg/ml of chloramphenicol, 50 μg/ml of kanamycin, and 125 μg/ml of ampicillin) and copper ions as chloride were added when appropriate.

Dose-response growth experiments. LB cultures of *E. coli* strains were diluted 1:400 into TMM, cultivated overnight, and diluted again 1:400 into fresh TMM. After 2 h of incubation at 37°C, they were diluted 1:400 into fresh TMM with increasing metal cation concentrations and cultivated for 16 h with shaking at 37°C. Optical density was measured at 600 nm using a SmartSpec3000 photometer (Bio-Rad, Munich, Germany). For anaerobic growth, the 2-h cultures were diluted 1:400 into fresh medium in Hungate tubes and cultivated without shaking at 37°C for 16 h. TMM with added resazurin was boiled for reduction, immediately used to fill Hungate tubes, and autoclaved. Optical density was measured using a Spectronic20+ photometer (Milton Roy, Ivyland, PA).

Time-dependent growth experiments. LB cultures of *E. coli* strains were diluted 1:400 into TMM, cultivated overnight, and diluted again 1:400 into fresh TMM. After 2 h of incubation at 37°C, they were diluted 1:400 into fresh TMM in 12-well plates (Greiner Bio-One, Frickenhausen, Germany) with 50 μM $CuCl_2$ (final concentration) and cultivated with shaking at 37°C. Optical density was measured every 30 min at 600 nm using a TECAN Infinite 200 PRO reader (TECAN, Männersdorf, Switzerland). Anaerobic growth cultures were measured every 2 h using a Spectronic20+ photometer (Milton Roy).

Gene deletions and other genetic techniques. Genes were deleted by insertion of resistance cassettes using the λ red recombinase system (50). Initial deletions were performed in *E. coli* strain BW25113, in which the target genes were exchanged for a chloramphenicol (*cat*) resistance cassette, and subsequently transferred by general transduction with phage P1 into *E. coli* strain W3110 or its derivatives. In the resulting mutant strains, the genes were disrupted by insertion of the *cat* resistance cassette through homologous recombination. Multiple deletions were constructed by FLP recombination target (FRT)-dependent elimination of the respective resistance cassette assisted by flippase from plasmid pCP20 (50) and subsequent general phage P1 transduction. For construction of plasmids pECD1256 and pECD1257, the genes *gshA* and *gshB* were amplified by PCR from start to stop codon and cloned with SacII and XhoI in the vector pASK-IBA3 (IBA GmbH, Göttingen, Germany), respectively. Due to the stop codon, no fusion with the C-terminal StrepTagII resulted. Both plasmids were checked by restriction analysis and DNA sequencing. Otherwise, standard molecular genetic techniques were used (51). PCR was performed with *Taq* or *Taq*/*Pwo* DNA polymerase (Roche, Mannheim, Germany). All primer sequences can be obtained upon request.

RNA isolation and RT-PCR. *E. coli* wild-type and mutant cells were cultivated as described below for the survival assay. Total RNA was isolated and the reverse transcriptase (RT) reaction performed as previously described (52). To exclude experimental artifacts resulting from DNA contaminations, only RNA preparations that did not generate products in a PCR with chromosomal primers without a previous RT reaction were used. As an endogenous control, *rpoZ* was used. All cDNAs displayed the same expression level when amplified with primers for the gene *rpoZ*. A no-template control was performed under identical conditions as for the target genes. Two independent biological samples were used. Details for PCR protocols and primer sequences are available on request.

Glutathione determination. Overnight cultures were diluted 1:100 into fresh TMM without or with $CuCl_2$ and cultivated with shaking at 37°C until the optical density (600 nm) reached 2.25 ± 0.25 (late exponential phase of growth). Cells representing 5 mg (dry mass) were harvested by centrifugation (15 min at 4,500 × *g* and 4°C) and washed twice in medium. The pellet was suspended in 3 volumes of 5% sulfosalicylic acid (SSA) and disrupted by two freeze-thawing cycles. Cell debris was removed by centrifugation (15 min at 15,300 × *g* and 4°C). The supernatant was used to determine the protein concentration with the QuantiPro bicinchoninic acid (BCA) assay kit (Sigma-Aldrich, Taufkirchen, Germany) using bovine serum albumin as a standard and to measure the reduced glutathione (GSH) content with a glutathione assay kit (CS0260; Sigma-Aldrich) according to the manufacturer's instructions. The enzymatic determination of the total amount of glutathione (GSH and oxidized glutathione [GSSG]) after deproteinization with SSA was conducted photometrically using TNB [5,5′dithiobis(2-nitrobenzoic acid)].

Survival on metal surface assay. As copper alloy coupons, copper C11000 (99.9% Cu), cupronickel C75200 (maximum of 62% Cu, maximum of 18% Ni, and maximum of 21% Zn) and, as a control, stainless steel (AISI 304) were used and treated prior to each experiment (12). As previously described, overnight cultures of *E. coli* were concentrated 10-fold by centrifugation and cells were suspended in phosphate-buffered saline (PBS) (12). A 40-μl sample (approximately 1.5×10^9 cells) was applied to a sterile cotton swab and spread evenly once across a 2.5- by 2.5-cm metal coupon. All samples dried completely within 5 s after contact with the surfaces. Coupons were transferred in 10 ml of PBS with 20 glass beads (2 mm; Carl Roth, Karlsruhe, Germany) and vortexed vigorously for 1 min to remove the cells. Samples were diluted in PBS and plated on LB agar. Surviving bacteria were counted as CFU.

DNA degradation assay. For the DNA assay, a 20-fold-higher cell density (cell cultures were concentrated 200-fold in PBS) compared to the survival experiments were spread on the coupons as described above. After cell removal from the coupons into PBS, DNA was isolated with the

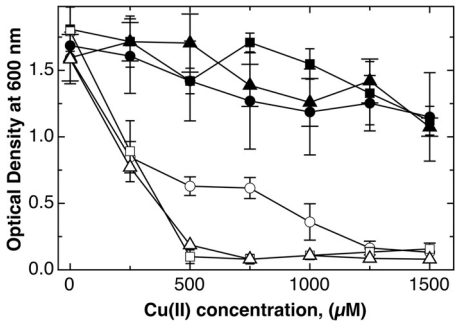

FIG 1 Aerobic growth of *E. coli* mutant strains in the presence of different carbon sources. Dose-response curves (16 h at 37°C with shaking in TMM containing 2 g/liter of the respective carbon source) were recorded for *E. coli* wild-type strain W3110 (closed symbols) and the Δ*copA* Δ*cueO* Δ*cusCFBA* mutant strain (open symbols) in the presence of glucose (circles), lactose (triangles), or melibiose (squares). The experiment was repeated at least three times; error bars show standard deviations.

GeneJet genomic DNA purification kit (Thermo Scientific/Fermentas, St. Leon-Rot, Germany), concentrated in a SpeedVac, separated by agarose gel electrophoresis on a 1% (wt/vol) gel, stained with ethidium bromide, and documented with a UV system (gel imager; INTAS, Göttingen, Germany).

RESULTS

Import of copper ions. To test the hypothesis that Ndh may reduce Cu^{II} released by copper surfaces to Cu^{I}, which is subsequently imported into the cytoplasm by sodium symporters, the *ndh* gene was deleted in the *E. coli* W3110 wild type, the Δ*cueO* Δ*cusCFBA* double mutant, and the Δ*cueO* single mutant. Tolerance to copper ions of the strains with and without *ndh* was tested under aerobic and anaerobic conditions, but no difference was found (data not shown). Thus, the Ndh cupric reductase (alone) did not increase copper sensitivity in *E. coli*.

To test whether sodium-dependent transport systems might use Cu^{I} instead of Na^{I} in an unspecific transport process, growth of wild-type *E. coli* and its Δ*copA* Δ*cueO* Δ*cusCFBA* triple mutant in the presence of substrates for sodium-dependent import systems was studied. There was no effect on copper tolerance when each strain was cultivated in the presence of various amino acids (Gln, Ser, Pro, Ala, Ile, and Leu) under aerobic conditions, neither in the presence nor in the absence of additional glucose (data not shown).

Copper tolerance of the *E. coli* W3110 wild type under aerobic conditions with lactose or melibiose as a carbon source was not different from that in glucose; however, the Δ*copA* Δ*cueO* Δ*cusCFBA* triple mutant was more sensitive to copper on lactose or melibiose than on glucose (Fig. 1). Under anaerobic conditions and in the absence of ascorbate, wild-type cells cultivated in melibiose-containing medium were less tolerant to copper than those on lactose or glucose (Fig. 2A), while no difference was visible in the triple mutant. In the presence of ascorbate, which reduces Cu^{II} to Cu^{I}, wild-type and triple mutant cells were less tolerant to copper when grown in melibiose than in glucose; cells cultivated on lactose showed an intermediary level of copper tolerance (Fig. 2B and C). Since melibiose is imported by the sodium-dependent MelB system (53, 54) but lactose by the proton-dependent LacY system (55, 56), this may indicate that Cu^{I} may be indeed a substrate for this sodium-dependent uptake system in *E. coli*.

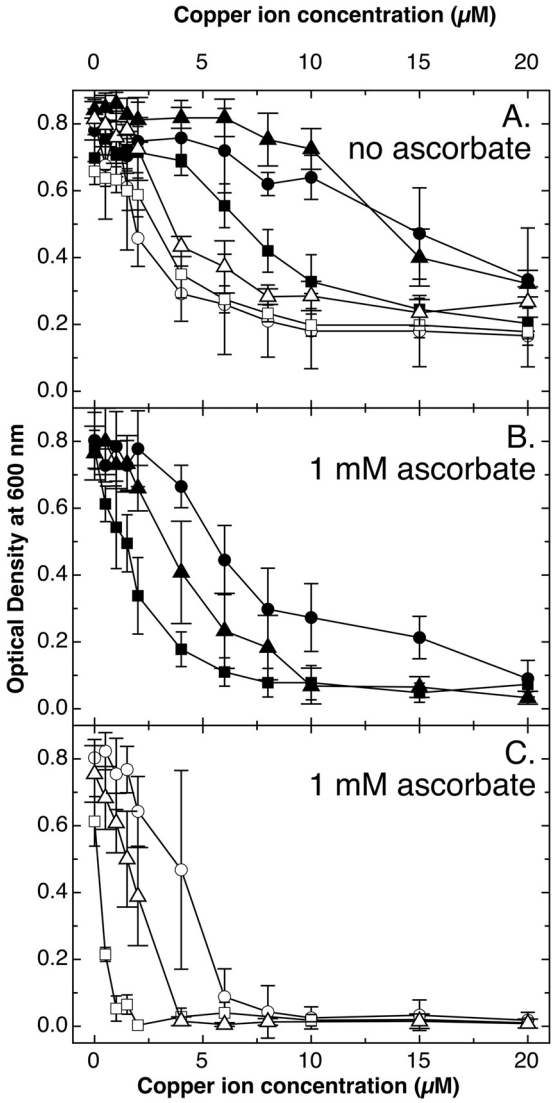

FIG 2 Anaerobic growth of *E. coli* mutant strains in the presence of different carbon sources. Dose-response curves (16 h at 37°C in TMM containing carbon source) were recorded for *E. coli* wild-type strain W3110 (closed symbols) and the Δ*copA* Δ*cueO* Δ*cusCFBA* mutant strain (open symbols) in the presence of glucose (circles), lactose (triangles), or melibiose (squares). The growth medium contained 1 mM ascorbate to reduce Cu^{II} to Cu^{I} (B and C) or not (A). The experiment was repeated at least three times; error bars show standard deviations.

Dps was not required for copper tolerance. To address the question of whether DNA damage is involved in killing of *E. coli* cells on copper surfaces, the *dps* gene was deleted in wild-type *E. coli* strain W3310 and various mutants carrying deletions in genes (*copA*, *cueO*, *cusCFBA*; *gshA* and *gshB* for synthesis of glutathione [GSH] via gamma-glutamyl-cysteine) that are involved in copper tolerance (32, 37, 38, 44, 57). RT-PCR controls were done in all experiments to demonstrate the absence or presence of the gene transcripts in the respective mutants (data not shown). The effect of the *dps* deletion in these strains was subsequently determined in aerobic and anaerobic liquid cultures in the presence of copper ions and on solid copper alloy surfaces.

In cultures under aerobic conditions, deletion of *dps* led to a

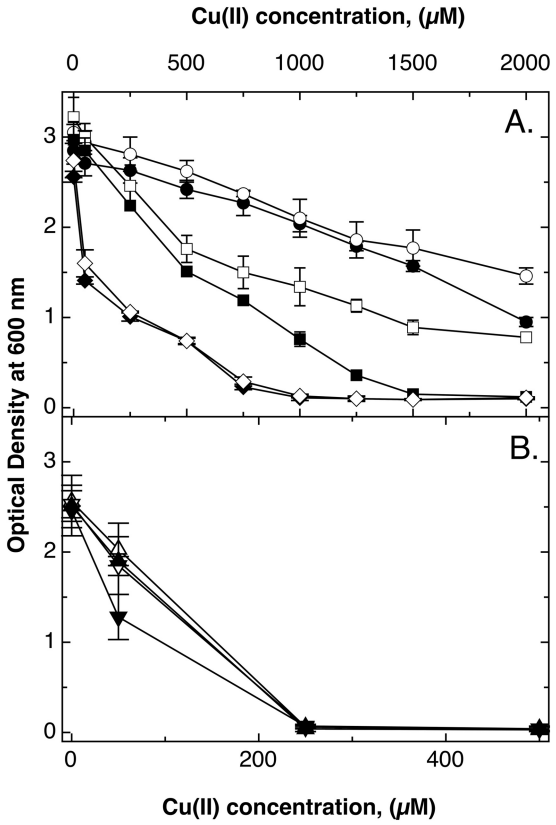

FIG 3 Aerobic growth of *E. coli* mutant strains with or without *dps*. Dose-response curves (16 h at 37°C in TMM) were recorded for *E. coli* wild-type strain W3110 (●) and Δ*dps* (○), Δ*copA* (■), Δ*copA* Δ*dps* (□), Δ*copA* Δ*cueO* Δ*cusCFBA* (◆), and Δ*copA* Δ*cueO* Δ*cusCFBA* Δ*dps* (◇) mutants (A). Panel B shows only the data points up to 500 μM $CuCl_2$ for the Δ*copA* Δ*ghsA* (▲), Δ*copA* Δ*ghsB* (▼), Δ*copA* Δ*ghsA* Δ*dps* (△), and Δ*copA* Δ*ghsB* Δ*dps* (▽) mutant strains. The experiment was repeated at least three times; error bars show standard deviations.

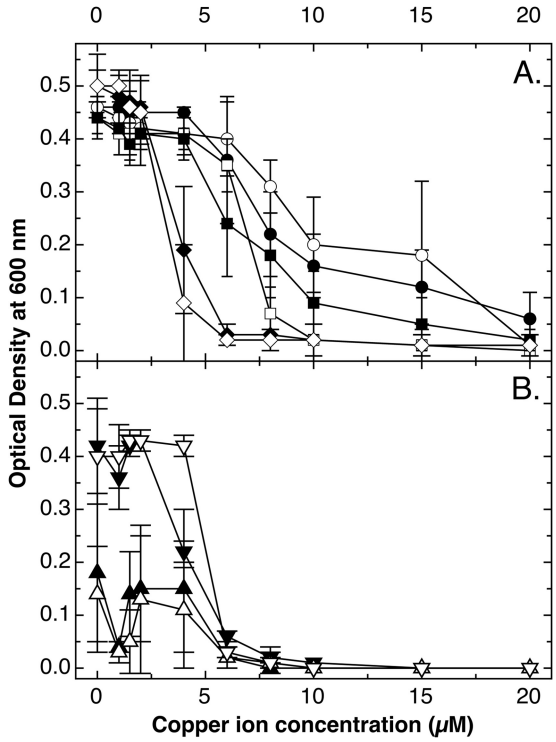

FIG 4 Anaerobic growth of *E. coli* mutant strains with or without *dps*. Dose-response curves (16 h at 37°C in TMM containing 2 g/liter of glucose and 1 mM ascorbate) were recorded for *E. coli* wild-type strain W3110 (●) and Δ*dps* (○), Δ*copA* (■), Δ*copA* Δ*dps* (□), Δ*copA* Δ*cueO* Δ*cusCFBA* (◆), and Δ*copA* Δ*cueO* Δ*cusCFBA* Δ*dps* (◇) mutants (A) and for the Δ*copA* Δ*ghsA* (▲), Δ*copA* Δ*ghsB* (▼), Δ*copA* Δ*ghsA* Δ*dps* (△), and Δ*copA* Δ*ghsB* Δ*dps* (▽) mutants (B). The experiment was repeated at least three times; error bars show standard deviations.

small effect (Fig. 3A), as published previously (43). To challenge the cells with increased cytoplasmic copper concentrations, the Δ*dps* effect was also tested in a Δ*copA* mutant strain. Deletion of *copA* significantly decreases copper tolerance in *E. coli* (33, 44), but additional deletion of the *dps* gene diminished this effect (Fig. 3A) by an unknown mechanism.

The gene *dps* was deleted in the Δ*copA* Δ*cueO* Δ*cusCFBA* triple mutant. There was no decrease of tolerance in the resulting Δ*dps* Δ*copA* Δ*cueO* Δ*cusCFBA* quadruple mutant compared to that of the triple mutant strain (Fig. 3A). Finally, *dps* was removed from Δ*copA* Δ*gshA* and Δ*copA* Δ*gshB* double mutants that were not able to produce glutathione (Δ*gshA*) or produced equal amounts of γ-glutamyl-cysteine (γEC) instead (Δ*gshB*) (44). Again, there was no effect under aerobic growth conditions (Fig. 3B).

The same mutant strains with or without *dps* were also cultivated under anaerobic conditions in the presence of ascorbate. The *dps* gene was not important for copper tolerance in *E. coli* W3110 wild-type cells and increased copper tolerance only a little in the Δ*copA* mutant (Fig. 4A). There was also no effect when *dps* was deleted in the Δ*copA* Δ*cueO* Δ*cusCFBA* triple mutant (Fig. 4A).

In contrast to findings under aerobic conditions in LB medium (44), the Δ*copA* Δ*gshB* mutant was more tolerant to copper ions than the Δ*copA* Δ*gshA* mutant. This indicated that γEC, which slightly increased copper toxicity under aerobic conditions, was important for copper tolerance under anaerobic conditions. The Δ*copA* Δ*gshA* mutant strain, however, grew poorly even in the absence of copper (Fig. 4B). There was, however, again no effect of Δ*dps* on copper tolerance of either mutant strain with a defect in the glutathione biosynthesis pathway. This indicated that Dps did not contribute to copper tolerance in *E. coli*.

Survival on surfaces of solid copper alloys. Survival of the various Δ*dps* and Δ*gsh* mutants on the surfaces of solid copper alloys was determined. For 99.9% "pure" copper (alloy C11000), all strains could not be cultivated after incubation of just a few seconds on this surface (data not shown). To get a better differentiation, the experiments were repeated on cupronickel (alloy C75200; 62% Cu, 18% Ni, and 21% Zn). The number of culturable cells on cupronickel coupons was decreased by a factor of about 10 within a minute in the cases of wild-type strain W3110, the Δ*copA* single mutant, the Δ*copA* Δ*cueO* Δ*cusCFBA* triple mutant, and the Δ*copA* Δ*gshB* double mutant, all with or without *dps* (see Fig. S1 in the supplemental material for the triple mutant; all other data are not shown).

In contrast, no survivors were found among the Δ*copA* Δ*gshA* double mutants with and without *dps* after 30 s (see Fig. S1B in the supplemental material), while no difference between these mutants was observed in the case of stainless steel (see Fig. S1A). When DNA was isolated from wild-type and Δ*copA* Δ*gshA* mutant

TABLE 2 Total glutathione contents in *E. coli* strains in the absence or presence of 50 μM $CuCl_2$

Bacterial strain	GSH content (mg/g of released protein) with Cu^{II} in growth medium at the indicated concn (μM)[a]	
	0	50
Wild-type W3110	97 ± 3	146 ± 34
Δ*copA* (pASK-IBA3) mutant	119 ± 19	124 ± 28
Δ*copA* Δ*gshA* (pASK-IBA3) mutant	0 ± 1	0 ± 0
Δ*copA* Δ*gshB* (pASK-IBA3) mutant	3 ± 2	0 ± 0
Δ*copA* Δ*gshA* (pASK-IBA3::*gshA*) mutant	132 ± 10	195 ± 32
Δ*copA* Δ*gshB* (pASK-IBA3::*gshB*) mutant	115 ± 14	163 ± 27

[a] Shown are contents of total glutathione (GSH and GSSG); values are means ± standard deviations.

strains, DNA remained intact for 3 h for wild-type cells incubated on stainless steel and for 60 min for mutant cells on steel and wild-type cells on cupronickel, while DNA from mutants on cupronickel showed beginning degradation after only 30 min (see Fig. S2). This clearly indicated that glutathione (or at least presence of γEC) but not the Dps protein may be essential for survival of *E. coli* on solid copper surfaces and for stability of its nucleic acids.

Glutathione is required for survival of *E. coli* on solid copper surfaces. The contribution of GSH was subsequently investigated in more detail with Δ*copA* Δ*gshA* and Δ*copA* Δ*gshB* double mutants that were also complemented in *trans* with the *gshA* or the *gshB* gene cloned into vector pASK-IBA3. To increase the sensitivity of the glutathione determination, the cells were disrupted not by ultrasonication as published previously (44) but by a freeze-thaw cycle. This led to a 15.3-fold-lower release of proteins from the cells (data not shown), enhancing the efficiency of the subsequent deproteinization step, and consequently to a higher quotient of glutathione to released protein. The glutathione content of wild-type *E. coli* strain W3110 was 97 ± 3 mg of GSH per g of released protein (Table 2), 7-fold higher than the published value of 13.7 ± 1.6 mg of GSH per g of total protein (44). The value did not change when *copA* was deleted but increased 50% when 50 μM Cu^{II} was present in the growth medium. GSH was not detected in Δ*gshA* and Δ*gshB* mutant cells even in this assay with increased sensitivity. Expression of the respective gene in *trans* restored the GSH content of the cells (Table 2) and restored copper tolerance under aerobic (see Fig. S3) and anaerobic (see Fig. S4) conditions.

Time-dependent growth experiments under aerobic conditions indicated that at high copper concentrations (500 μM $CuCl_2$), in *trans* complementation with *gshA* and *ghsB* increased the tolerance level of the Δ*gshA* and Δ*gshB* mutants, respectively, again but not to the level of the parent strain (Fig. 5B), while tolerance was back to the level of the Δ*copA* parent strain at 50 μM $CuCl_2$ (data not shown). Survival rates of the strains tested were not different on stainless steel (Fig. 6A), but the Δ*ghsA* and Δ*ghsB* mutant strains were again rapidly killed on cupronickel alloy (Fig. 6B). Since the Δ*gshA* mutant was more sensitive than the Δ*ghsB* mutant, the gamma-glutamyl-cysteine present in the Δ*ghsB* strain (44) might offer a small degree of protection. Expression of the *gshA* and the *ghsB* gene in *trans* again restored the tolerance level of the parent strain. Thus, glutathione increased survival of *E. coli* on metallic copper surfaces.

DISCUSSION

Bacteria, bacterial biofilms, yeasts, and viruses are inactivated on moist or dry copper surfaces, and this may be useful to control the spread of pathogenic entities in hospitals (5, 7, 9, 17, 58). On the other hand, some bacterial strains are able to survive on solid copper surfaces, such as on coins (19). Evolution of copper surface-resistant pathogenic bacteria, however, has to be prevented if indeed copper surfaces are to be used in hospitals and elsewhere. It is therefore essential to understand the molecular mechanisms underlying the killing process.

Copper ions are released from solid copper surfaces (13) and are bound by the cells (21), which leads to killing. Differences in the cellular morphology or physiology between Gram-negative *Proteobacteria* and Gram-positive *Firmicutes* and *Actinobacteria* may decide how these copper ions kill the respective bacterial cells (27). In Gram-negative bacteria, copper ions need to be imported into the periplasm first. OmpC and ComC (YcfR) play a role here (57, 59). Although a strain deficient in the gene for the NADH: ubiquinone oxidoreductase II (Ndh) suffered a faster inactivation than its parent strain in the presence of copper and tert-butyl hydroperoxide (60), Ndh was not involved in copper tolerance in the absence of this substance (data not shown). So, Ndh seems not to be required for reduction of Cu^{II} to Cu^{I}. This process can be mediated by respiratory chain components (61).

A comparison of aerobic and anaerobic growth of *E. coli* (Fig. 3 and 4) shows the higher toxicity of copper ions under anaerobic conditions in the presence of ascorbate. This may indicate that Cu^{I} is more toxic than Cu^{II}; however, no uptake system was known for

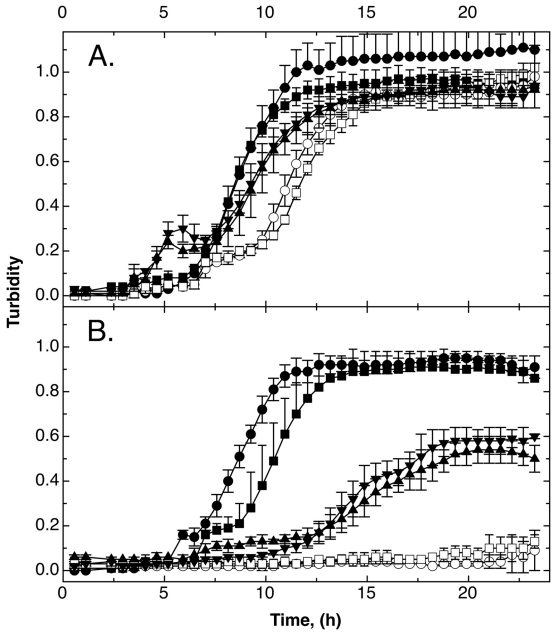

FIG 5 Expression of *gshA* or *gshB* in *trans* in a Δ*ghsA* or Δ*ghsB* mutant strain, respectively, restores copper tolerance under aerobic conditions but not completely. Time-dependent growth curves (37°C in TMM containing 2 g/liter of glucose) in the absence (A) or presence (B) of 500 μM $CuCl_2$ were recorded as turbidity at 600 nm in a TECAN reader for *E. coli* wild-type strain W3110 (●) and Δ*copA* (■), Δ*copA* Δ*ghsA* (○), and Δ*copA* Δ*ghsB* (□) mutants, all containing the vector plasmid pASK3, and Δ*copA* Δ*ghsA*(pASK-IBA3::*gshA*) (▲) and Δ*copA* Δ*ghsB*(pASK-IBA3::*ghsB*) (▼) mutants. The experiment was repeated at least three times; error bars show standard deviations.

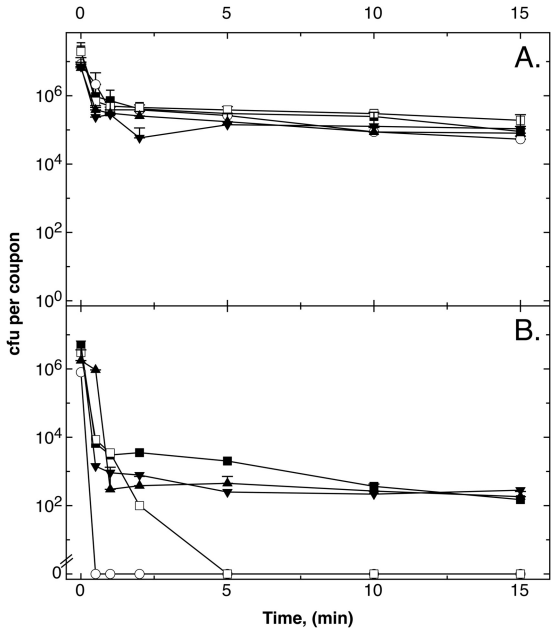

FIG 6 Expression of *gshA* or *gshB* in *trans* in a Δ*ghsA* or Δ*ghsB* mutant strain, respectively, confers protection from the toxic effects associated with metallic copper surfaces. Cells of *E. coli* W3110 Δ*copA* (■), Δ*copA* Δ*ghsA* (○), and Δ*copA* Δ*ghsB* (□) mutant strains, all containing the vector plasmid pASK-IBA3, and Δ*copA* Δ*ghsA*(pASK-IBA3::*gshA*) (▲) and Δ*copA* Δ*ghsB*(pASK-IBA3::*ghsB*) (▼) mutant strains were streaked on stainless steel (A) or cupronickel (B). After the indicated periods under ambient conditions (23°C), cells were removed from metal surfaces, diluted, and plated on LB agar. Surviving cells were counted as CFU after 16 h at 37°C. Values are means of results from three repetitions of the experiment; error bars show standard deviations.

copper ions in *E. coli* except some Cu^{II} transport by the ZupT protein as indicated by competition experiments (28). On the other hand, upregulation of *copA* expression from background expression to about 350 copies per cell took only 2 min after addition of Cu^{II} to the cells (32), and this regulatory event is performed by the MerR-type regulator CueR, which is usually constantly bound to its operator on the DNA (62). Thus, a Cu^{I} import pathway should exist.

Due to the similarities in charge and ionic diameter, Cu^{I} may be transported instead of Na^{I} by sodium-dependent transport systems such as MelB (53, 54) (Fig. 2). In agreement with this, *E. coli* cells suspended in 0.8% NaCl showed prolonged survival on copper surfaces (16) because the high concentration of Na^{I} may competitively block Cu^{I} uptake by these transport systems. Na^{I} binds to copper-binding proteins (31) and vice versa; copper ions also inhibit Na^{I} transport across membranes (63). So, copper ions could be able to enter the cytoplasm of *E. coli* by mimicking Na^{I} or by ZupT as an import route for Cu^{II} (28). As a practical consequence, sodium ions should not be present in solutions used to clean copper surfaces in hospitals.

In the cytoplasm, Cu^{II} and GSH yield rapidly $Cu^{I}GSH_2$, which reacts with O_2 to generate a superoxide radical (64) and $Cu^{II}GSSG$, which can be reduced back to $Cu^{I}GSH_2$ by GSH (65). Nevertheless, the presence of GSH increases copper ion resistance in the absence of the copper-exporting P_{IB1}-type ATPase CopA (44). Copper does not damage DNA by oxidation *in vivo* in *E. coli* (66) but destabilizes iron-sulfur clusters in *Bacillus subtilis* (67) and in *E. coli*, especially in dehydratases (68), and the copper efflux system CusCBA is needed to protect iron-sulfur clusters (69). This toxic action of copper ions is similar to that of Cd^{2+}, which is also a toxic "soft" transition metal cation (70).

Cells of *E. coli* and various yeasts suffered extensive membrane damage within minutes after exposure to copper surfaces, but the DNA was not immediately affected (11, 21). Copper leads first to oxidative damage of phospholipids and subsequent loss of membrane integrity and cell death before the DNA is fragmented (22). On the other hand, DNA as a primary target was observed in pathogenic enterococci (24, 26) and methicillin-resistant *Staphylococcus aureus* cells (25). DNA damage came before an effect on the cytoplasmic membrane in these examples. Indeed, while in Gram-positive bacteria the DNA seems to be the primary target of copper released from solid copper surfaces, Gram-negative bacteria and eukaryotes suffered primarily from damage of the cytoplasmic membrane (27), and damage of the plasma membrane of yeasts by cupric ions has already been described some time ago (71). In addition to differences in cellular morphology, *Proteobacteria* and *Eukaryota* contain large intracellular concentrations of glutathione, but *Firmicutes* such as enterococci or *S. aureus* contain much smaller concentrations and/or other thiol compounds (45, 46). Moreover, a Δ*copA* Δ*gshA* mutant of *E. coli* was more rapidly inactivated on cupronickel than its parent strain, and its DNA was also less stable. Taken together, the findings show that glutathione but not Dps was required to protect cytoplasm and DNA of *E. coli* against damage by copper ions released from solid copper surfaces. This effect may explain some of the differences in the modes of killing of Gram-negative and -positive bacteria on this material.

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