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Killing of Bacteria by Copper Surfaces Involves Dissolved Copper[▽]

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Bacteria are rapidly killed on copper surfaces. However, the mechanism of this process remains unclear. Using *Enterococcus hirae*, the effect of inactivation of copper homeostatic genes and of medium compositions on survival and copper dissolution was tested. The results support a role for dissolved copper ions in killing.

The rapid killing of bacteria by solid copper surfaces is receiving rapidly growing attention. In laboratory experiments, it has been shown that many bacterial species, such as *Escherichia coli* O157, *Staphylococcus aureus*, *Salmonella enterica*, *Campylobacter jejuni*, *Clostridium difficile*, *and Mycobacterium tuberculosis*, are efficiently killed on copper or copper alloy surfaces (1, 3, 4, 6, 7, 12–14). In contrast, on stainless steel, living cells could be recovered even after 28 days. The antimicrobial activity of copper and copper alloys is now well established, and copper has recently been registered at the U.S. Environmental Protection Agency as the first solid antimicrobial material. A key focus is the use of copper in health care facilities, food processing plants, and other areas where clean or aseptic working procedures are required (2). In this connection, it has become important to understand the mechanism of bacterial killing, as it may bear on the possibility of the emergence of resistant organisms, cleaning procedures, and material and object engineering. We here used wild-type and mutant strains of *Enterococcus hirae* to investigate the influence of copper resistance genes on killing rates. We also evaluated copper dissolution by various media and its relation to killing efficiency. Our findings provide support for a prominent role for dissolved copper in the killing process.

An *E. hirae* copper export mutant is more sensitive to killing by copper. To test how mutations in copper homeostatic genes of *E. hirae* affect killing, cells were plated by the "wet plating" procedure described by Wilks et al. (13). Wild-type *E. hirae* ATCC 9790 or the Δ*copA*, Δ*copB*, Δ*copAB*, and Δ*copY* knockout mutants (8, 9) were grown semianaerobically (air-saturated medium in sealed tubes) to stationary phase in 5-ml cultures at 37°C in M17 medium (11). Culture aliquots of 20 μl (final pH = 5) were applied to coupons of 1 by 2 cm consisting of either 99.9% copper or stainless steel (74% Fe, 18% Cr, 8% Ni). Following incubation at 22°C in a water-saturated atmosphere, the coupons were washed with 5 ml of 140 mM NaCl and 10 mM NaP_i, pH 7, by vortexing for 30 s with 20 sterile, 2-mm glass beads. Serial dilutions were spread on N plates (1% peptone, 0.5% yeast extract, 1% glucose, 1% Na_2HPO_4, 1.5% agar), which were incubated overnight at 37°C. Survival was evaluated by determining the number of CFU.

The CopB copper ATPase appears to be the sole copper export mechanism of *E. hirae* (10). A Δ*copB* mutant was more sensitive to killing than the wild type (Fig. 1A). The Δ*copB* strain was completely killed in 75 min, while wild-type cells still showed substantial survival at this time. No significant killing of either strain was observed on stainless steel coupons (a stainless steel control was also performed in all subsequent experiments and always resulted in complete survival; this control is thus shown only here). The higher sensitivity of the Δ*copB* strain than of the wild type suggests a role for copper influx into the cell in the killing mechanism. The killing rate of several other mutants was in line with this concept. A deletion in *copA*, encoding the copper import ATPase, did not significantly affect survival (Fig. 1B). In contrast, a Δ*copA* Δ*copB* double mutant was fully killed in 75 min, like the *copB* single-deletion strain, albeit with different kinetics. Finally, a mutant deleted in the *copY* repressor gene and constitutively expressing CopA and CopB exhibited the same sensitivity as the wild type. This indicates that the wild-type copper homeostatic machinery already provides maximal protection against killing. Clearly, an operational copper export mechanism delays killing of *E. hirae* by copper, supporting a direct role for copper ions in the killing mechanism. Similar observations were reported for *Pseudomonas aeruginosa* mutants (3).

Killing rates depend on the medium composition. When cells were washed and resuspended in 100 mM Tris-Cl, pH 7, before being applied to coupons, killing was much more rapid, but the qualitative differences between strains persisted (Fig. 2A). Complete killing of the wild type occurred in 12 min, and that of the Δ*copB* and Δ*copAB* mutants occurred in 10 min. Tris is known to complex copper and has been shown to be able to extract copper from enzymes (5). The more efficient killing in Tris may thus be due to accelerated copper dissolution from the coupons and/or more-facile membrane penetration of neutral $Tris_2$-Cu complexes. Indeed, preliminary experiments indicated that the growth of *E. hirae* or *Lactococcus lactis* was more strongly inhibited by 1 to 3 mM copper if Tris buffer was added to the growth medium (not shown).

We also assessed killing in the absence of copper-complexing chemicals. Figure 2B shows the killing rates of *E. hirae* wild-type and mutant cells in water. Interestingly, killing in water was considerably slower than in M17 medium or Tris buffer, and there was no difference between wild-type and mutant strains. Complete killing in all cases took 6 h. This supports the notion that the more rapid killing in Tris buffer is a specific property of this buffer substance. Since phosphate

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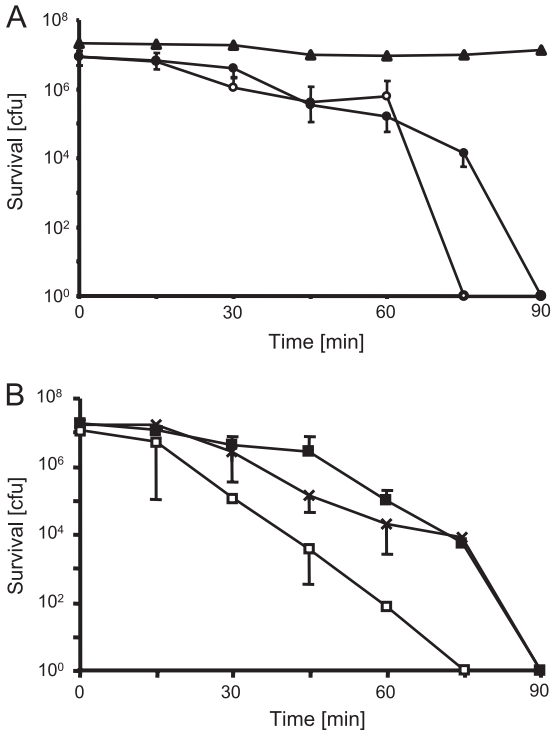

FIG. 1. Survival of *E. hirae* wild-type and mutant cells in growth media on copper and stainless steel surfaces. Cells in growth media were applied to copper or stainless steel coupons, incubated at room temperature for the times indicated, and washed off with phosphate-buffered saline. Survival was determined by plating serial dilutions on N plates. (A) Survival of wild-type *E. hirae* on stainless steel coupons (▲) or copper coupons (●) and survival of a Δ*copB* mutant on copper coupons (○). (B) Survival of *E. hirae* Δ*copA* (■), Δ*copAB* (□), and Δ*copY* (×) mutants on copper coupons. All measurements were conducted in triplicate, and the error bars indicate standard deviations.

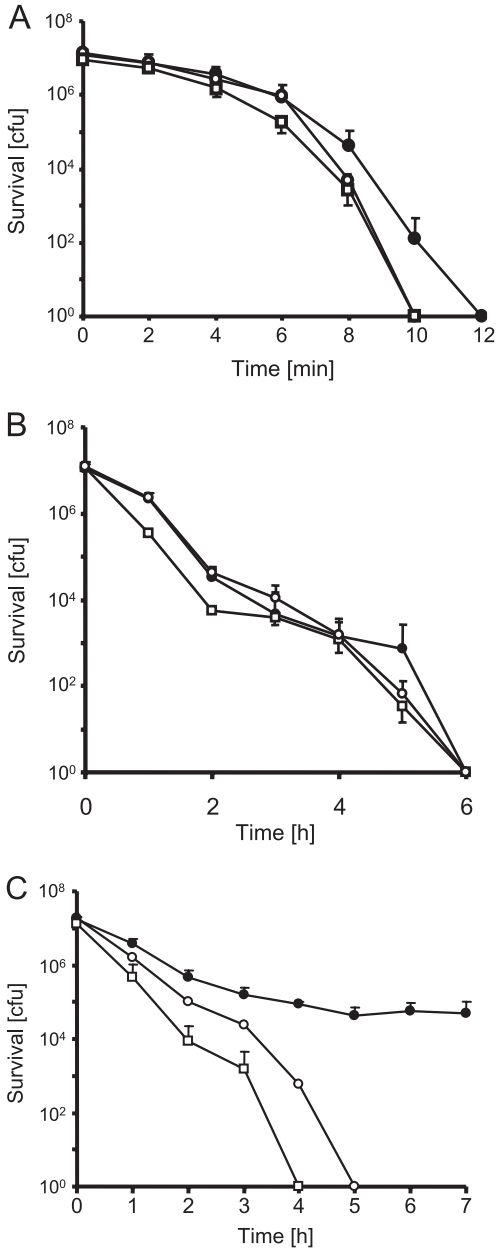

FIG. 2. Survival of *E. hirae* wild-type and mutant cells on copper surfaces in different media. Cells were washed and applied to copper coupons in 0.1 M Tris-Cl, pH 7 (A), water (B), or 100 mM NaP_i, pH 7 (C). Survival was assessed as described in the legend for Fig. 1. ●, wild type; ○, Δ*copB* mutant; □, Δ*copAB* mutant. All measurements were conducted in triplicate, and the error bars indicate standard deviations.

can also complex copper and is a common growth medium component, killing in phosphate buffer was assessed. Figure 2C shows that the killing rates of wild-type and mutant cells in 100 mM NaP_i, pH 7, were strikingly different from those in the other media tested; Δ*copAB* and Δ*copB* cells were completely killed in NaP_i after 4 and 5 h, respectively, while killing of the wild type ceased after 5 h, leaving a residual fraction of 0.4% long-term survivors. Conceivably, phosphate can participate in copper detoxification, and this could require adaptation, which only a fraction of cells can undergo before being killed.

Largely different amounts of copper are dissolved by different media. Copper release from coupons was determined by withdrawing 15-μl samples from the incubations, diluting them 100-fold with 1% HNO_3, and measuring the copper content by inductively coupled plasma atomic emission spectroscopy (Fig. 3). The different copper concentrations at time zero are due to copper contamination of the chemicals used. Tris-Cl, pH 7, accumulated 42 mM copper after 90 min, spent M17 medium 14 mM, and water and phosphate buffer only 55 and 57 μM, respectively. Overall, the dissolved copper concentrations correlated with the rates of killing. Spent M17 medium and Tris-Cl, which accumulated 14 and 42 mM copper, respectively, effected complete killing of the wild type in 90 and 12 min, respectively. In water, which dissolved only 55 μM copper, complete killing of the wild type took 6 h. Phosphate buffer, which dissolved 57 μM copper, killed mutants at rates similar to those observed in water but allowed a small fraction of wild-type cells to survive even after 7 h.

Taken together, our data suggest that aqueous, ionic copper released from solid copper surfaces is an important factor in the killing of *E. hirae*. This conclusion rests on the observations that (i) mutants unable to extrude copper were killed more rapidly under most conditions and (ii) media which released

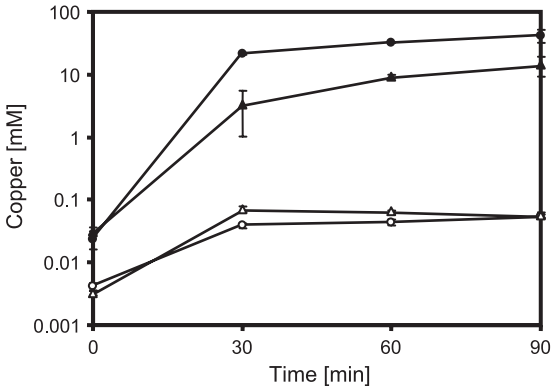

FIG. 3. Copper release from coupons into the aqueous phase. Aliquots of 20 μl of 0.1 M Tris-Cl buffer, pH 7 (●), spent M17 medium (▲), NaP_i, pH 7 (△), and water (○) were applied to copper coupons and incubated at room temperature for the times indicated, and the copper content of the aqueous phase was determined. All measurements were conducted in triplicate, and the error bars indicate standard deviations.

more copper from the solid copper surface also killed cells more rapidly. The residual survival of wild-type *E. hirae* in phosphate buffer is of interest in the context of copper use as antibacterial material and deserves further investigation.

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