Applied and Environmental Microbiology, Sept. 2011, p. 6049–6059 Vol. 77, No. 17
0099-2240/11/$12.00 doi:10.1128/AEM.00597-11

Mechanism of Copper Surface Toxicity in Vancomycin-Resistant Enterococci following Wet or Dry Surface Contact[▽]

S. L. Warnes* and C. W. Keevil

Environmental Healthcare Unit, School of Biological Sciences, Life Sciences Building, Highfield Campus, University of Southampton, Southampton SO17 1BJ, United Kingdom

Received 16 March 2011/Accepted 27 June 2011

Contaminated touch surfaces have been implicated in the spread of hospital-acquired infections, and the use of biocidal surfaces could help to reduce this cross-contamination. In a previous study we reported the death of aqueous inocula of pathogenic *Enterococcus faecalis* or *Enterococcus faecium* isolates, simulating fomite surface contamination, in 1 h on copper alloys, compared to survival for months on stainless steel. In our current study we observed an even faster kill of over a 6-log reduction of viable enterococci in less than 10 min on copper alloys with a "dry" inoculum equivalent to touch contamination. We investigated the effect of copper(I) and copper(II) chelation and the quenching of reactive oxygen species on cell viability assessed by culture and their effects on genomic DNA, membrane potential, and respiration *in situ* on metal surfaces. We propose that copper surface toxicity for enterococci involves the direct or indirect action of released copper ionic species and the generation of superoxide, resulting in arrested respiration and DNA breakdown as the first stages of cell death. The generation of hydroxyl radicals by the Fenton reaction does not appear to be the dominant instrument of DNA damage. The bacterial membrane potential is unaffected in the early stages of wet and dry surface contact, suggesting that the membrane is not compromised until after cell death. These results also highlight the importance of correct surface cleaning protocols to perpetuate copper ion release and prevent the chelation of ions by contaminants, which could reduce the efficacy of the surface.

Contaminated surfaces in a clinical environment can be a source of hospital-acquired infection (3, 6). This is true especially when pathogens, such as enterococci, are robust and can survive on surfaces for months (15, 33). This not only increases the possibility of the transfer of viable microorganisms perpetuating infection but also increases the potential for genetic transfer between microorganisms, including the spread of antibiotic resistance. Stainless steel is a ubiquitous surface primarily because of its resistance to corrosion and its ability to be cleaned. However, many microorganisms can survive for many months on this surface, and microscopic analysis of the surface reveals striations on even the most polished of surfaces where bacteria can persist (49). The use of biocidal surface materials in conjunction with improved disinfection and hygiene protocols could eliminate this phenomenon, rather than relying solely on surface cleaning agents or irradiation methods, which may not reach all contaminating microorganisms. As such, biocidal surfaces could be invaluable in reducing the incidence of nosocomial and potentially community-acquired infections.

The antimicrobial properties of copper have been known for centuries. Laboratory studies and early results from clinical trials (4, 5) suggested that the use of copper alloy biocidal surfaces could help to reduce the spread of bacterial (27, 34, 35, 49, 50, 52–55), viral (36), and fungal (51) pathogens. In addition, the efficacy of copper surfaces is retained at a range of temperatures and humidity levels, unlike other potential antibacterial surfaces, including silver (28), and although biofilm formation has been reported for copper water pipes, this does not occur under dry surface conditions, and exposure to copper affects the ability to form biofilms (2).

Some enterococcal species, including *Enterococcus faecalis* and *Enterococcus faecium*, are common intestinal commensals that are now responsible for a range of hospital- and community-acquired infections worldwide (32). This is due in part to a robust nature, which ensures prolonged survival in the environment (15, 32, 49), but also because of their propensity for genetic transfer, which has resulted in the spread of antibiotic resistance even to other bacterial species. Recent work in our laboratory has observed that, along with rapid death and the inhibition of respiration, the destruction of bacterial nucleic acid occurs in multiantibiotic-resistant pathogenic enterococci as well as control strains on copper and copper alloy surfaces but not stainless steel, which could halt that transfer of resistance genes from enterococci (49).

At present, the actual mechanism of copper toxicity on surfaces is unclear but requires elucidation if copper biocidal surfaces are to be employed on a large scale, not only in clinical settings but also possibly in industrial environments, for example, in areas of large-scale food preparation, and eventually in the home. It is essential that biocidal surfaces retain the ability to provide a "constant-killing" surface for long periods and under changing environmental conditions. In addition, efficacy has to be retained for both "wet" fomite and "dry-touch" surface contamination. Previous studies suggested that reactive oxygen species (ROS), radical by-products of aerobic respiration, are an important part of the killing mechanism upon exposure to soluble copper, which can directly inflict damage to nucleic acids, proteins, and lipids (including those in the cell

* Corresponding author. Mailing address: Environmental Healthcare Unit, School of Biological Sciences, Life Sciences Building 85, University of Southampton, Highfield, Southampton SO17 1BJ, United Kingdom. Phone: 44(0)23 80594301. Fax: 44(0)23 80595159. E-mail: s.l.warnes@soton.ac.uk.

▽ Published ahead of print on 8 July 2011.

membrane) (13, 14, 17, 23, 24, 46). However, although damage to DNA resulting from the Fenton reaction with hydrogen peroxide and reduced copper in eukaryotic cells is well documented, studies of yeasts and *Escherichia coli* suggest that DNA is not the primary target in these organisms, with copper ions accumulating in the periplasmic space and protecting the nucleic acid in the latter (7, 25, 37).

In this study we have investigated the role of copper ionic species and reactive oxygen species in the killing mechanism of copper and copper alloy surfaces, including the effect on survival, status of nucleic acid integrity, bacterial membrane potential, and metabolic processes in pathogenic enterococci. We also investigated whether the DNA destruction that we have observed for enterococci is part of the active copper killing process or occurs after cell death. These results provide valuable information to retain the efficacy of copper alloys as biocidal surfaces in the long term.

MATERIALS AND METHODS

Bacterial strains. Vancomycin-resistant control strains, *Enterococcus faecalis* ATCC 51299 (VanB phenotype) and *Enterococcus faecium* NCTC 12202 (VanA phenotype), were supplied by Oxoid, United Kingdom. Clinical isolates of vancomycin-resistant enterococci (two *E. faecalis* and four *E. faecium* strains) were supplied by Health Protection Agency (HPA) Laboratories, Southampton, United Kingdom, in 2009 (details are provided in reference 49 and summarized in the Fig. 2 legend).

Culture preparation. Bacteria were maintained on Glycerol Protect beads (Technical Service Consultants, United Kingdom) at −80°C. For each experiment, one bead was inoculated into 15 ml sterile vancomycin-resistant enterococcal broth (VREB; Oxoid, United Kingdom) and incubated aerobically at 37°C for 17 h ± 2 h.

Coupon preparation. Various metal surfaces were tested: UNS C11000 (100% copper), UNS C26000 (cartridge brass; 70% copper and 30% zinc), and stainless steel, which was used as a control surface throughout (UNS S30400; 74% iron, 18% chromium, and 8% nickel). Metal coupons (10 by 10 by 0.5 mm) were degreased in acetone, stored in ethanol, and flamed prior to use as previously described (49). All data presented are from new, degreased coupons; however, experiments were also repeated using aged and recycled copper, alloys, and stainless steel, with the same results (data not shown).

Inoculation of metal surfaces with enterococci in the presence or absence of ionic chelators or ROS quenchers. Aliquots of the bacterial suspension were washed in phosphate-buffered saline (PBS) to remove any traces of medium and immediately resuspended in either PBS alone (control) or PBS supplemented with a chelator or ROS quencher (supplied by Sigma UK). Cells were resuspended in either the same volume as that of the culture (approximately 5×10^8 CFU/ml) for the wet inoculum or a 1/20 original volume (approximately 10^{10} CFU/ml) for the dry inoculum. Supplements and final concentrations were as follows: 20 mM EDTA, 20 mM bathocuproine disulfonic acid (BCS), 20 mM D-mannitol, 500 U/ml superoxide dismutase (SOD), 20 mM 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron), 500 U/ml catalase, and 10% (wt/vol) sucrose (pH adjusted to 7.2 for all supplements). Approximately 10^7 CFU in either 1 or 20 μl of the bacterial cell suspension (corresponding to dry or wet fomite inoculum conditions, respectively) was spread evenly over the surface of each coupon and incubated at room temperature (21°C ± 2°C) for various time periods. Bacteria were removed from the coupons and assessed by culture on solid medium as described previously (49) (vancomycin-resistant enterococcal agar [VREA] containing 6 mg/liter vancomycin [Oxoid, United Kingdom] and Columbia blood agar [CBA] containing 5% [vol/vol] sheep blood [bioMérieux UK Limited]).

Assessment of bacterial genomic DNA following exposure to metal surfaces by use of a DNA fragmentation assay. The DNA fragmentation assay protocol was originally described by Fernandez et al. (10) and has been modified in our laboratory (49). Briefly, bacterial cells either exposed to test metal surfaces or untreated were removed from surfaces, pretreated with lysozyme (as described previously in reference 49), and trapped in low-melting-point agarose on a glass slide. The bacterial membranes were permeabilized, dried, and stained with the sensitive DNA stain SYBR gold (Invitrogen, United Kingdom). Intact genomic DNA was visualized by using epifluorescence microscopy, as described previously (18, 49).

Assessment of bacterial DNA integrity by use of the nucleic acid stain SYTO 9 in enterococci immediately upon contact with copper, alloy, and stainless steel surfaces with the dry inoculum. Bacterial cells were exposed to metal surfaces for various time points (approximately 10^7 CFU in 1 μl was inoculated onto eight 1-cm^2 coupons for each time point) and removed from coupons as described above for culture experiments. Cells were pooled, pelleted, and stained with 5 μM SYTO 9 (Invitrogen, United Kingdom) for 10 min. Stained cells were washed and resuspended in 20 μl PBS; 5 μl was applied onto a microscope slide, covered with a coverslip, and observed by using epifluorescence microscopy as described previously (49).

Detection of respiring cells *in situ* on metal surfaces using the redox dye CTC. Protons generated by electron transport in respiring bacterial cells reduce the redox stain CTC (5-cyano-2,3-ditolyl tetrazolium chloride) to insoluble formazan, which can be observed by using epifluorescence microscopy. Bacteria applied onto coupons as described above for culturing were stained with 5 mM CTC as described previously (49). However, because a minimum of 90 min of incubation with CTC was required to be able to visualize precipitated formazan, all *in situ* analyses were performed at 2 h of contact with the copper surface using the wet inoculum method only. For the dry inoculum, bacteria were applied onto metal coupons, incubated for the required time points, and removed from the coupons as described above for culturing. Cells were pooled, pelleted, and stained in suspension with 5 mM CTC for 60 min, 5 μl was transferred onto a microscope slide, and a coverslip was applied.

Detection of changes in bacterial membrane potential *in situ* on metal surfaces by using rhodamine 123. An electrical potential difference ($\Delta\Psi$) is generated and maintained in bacterial cells by ion gradients, affected by metabolism, and is usually 10 to 100 mV, with the inside of the cell having a negative charge. Rhodamine 123 is a cell-permeant lipidophilic cationic dye that accumulates on the inner side of the bacterial membrane and can be visualized by using epifluorescence microscopy (18). Depolarization of the membrane occurs if the membrane ruptures, which can be visualized as a reduction of fluorescence. Bacterial cells (approximately 5×10^8 CFU) were prestained with 10 μM rhodamine 123 in PBS for 10 min at room temperature. In initial experiments the stain that was not internalized was not subsequently washed away (to prevent the stain leaching out as the equilibrium shifts), and results of this procedure were compared to those obtained by washing the cells poststaining to remove excess dye. There was no difference in results for either method for the duration of the dry inoculum method. Prestained cells were observed untreated or *in situ* on metal surfaces (dry inoculum). Images were recorded over a 20-min period (the dye may affect viability at longer incubation times). For wet inocula of 20 μl per 1 cm^2 with longer exposure times, staining was done postinoculation to avoid prolonged contact with the dye, which could affect cell viability (cells were removed from coupons as described above and stained in suspension). The proton ionophor carbonylcyanide 3-chlorophenylhydrazone (CCCP), which dissipates $\Delta\Psi$ and proton gradient (ΔpH), was used as a negative-staining control (10 μM), and uninoculated metal coupons were stained with the dye alone to determine any background fluorescence.

Statistical analysis. Statistical analyses were performed by using Sigma Stat, version 3.5, and graphical representations were performed by using Sigma Plot, version 11, as previously described (49).

RESULTS

Effect of chelators on the survival of enterococci on copper and stainless steel surfaces. Previous work in our laboratory showed that an inoculum of 10^7 enterococcal CFU in 20 μl spread per cm^2 copper alloys (>60% copper) died at between 1 and 2 h at 22°C (with a small proportion of *E. faecium* cells surviving longer than *E. faecalis*) and could take over 30 min to dry on the surface. However, viable cells were still detected after several months on stainless steel, with an initial 1-log reduction in the first 48 h of contact (49).

In the current study, when enterococci were applied onto copper surfaces in the presence of EDTA or BCS to chelate copper(II) and copper(I) ions, respectively, the survival time was extended (Fig. 1). For *E. faecium*, there was virtually no reduction in the number of viable cells if both chelators were present during 2 h of contact with the copper surface (Fig. 1B). For *E. faecalis* at the same time point, there was less than a

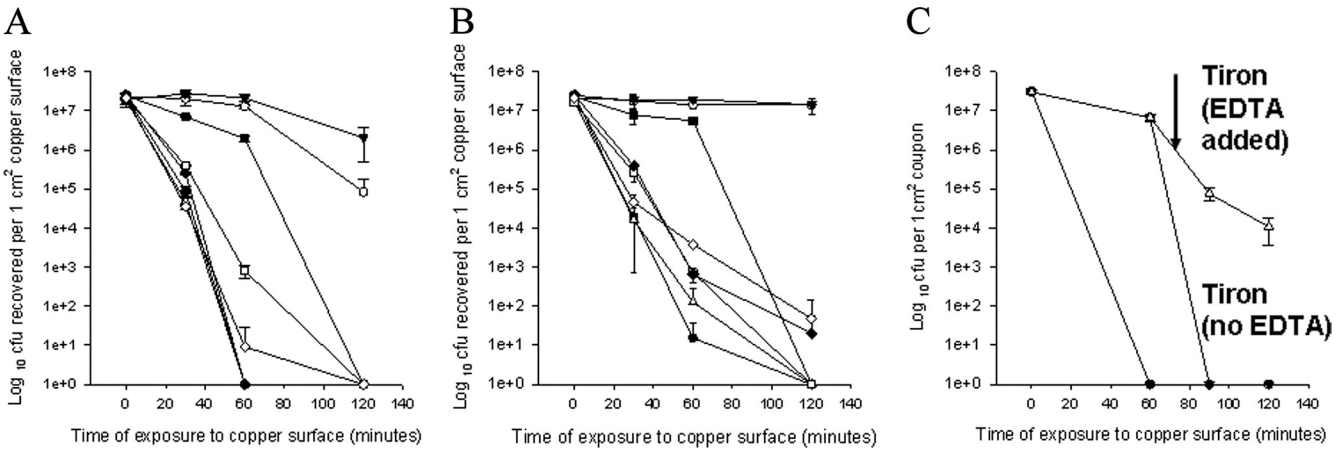

FIG. 1. Survival of vancomycin-resistant *E. faecalis* ATCC 51299 (A) and *E. faecium* NCTC 12202 (B) on copper surfaces with the wet fomite inoculum, with the addition of EDTA prolonging the protective effect of Tiron in *E. faecalis* ATCC 51299 bacteria exposed to copper surfaces (C). (A and B) Approximately 10^7 CFU in 20 μl was inoculated onto 1-cm^2 coupons in PBS (●) or PBS supplemented with EDTA (○), BCS (▼), D-mannitol (△), Tiron (■), SOD (□), catalase (◆), and sucrose (◇) at 22°C. (C) Approximately 10^7 CFU in 20 μl was inoculated onto 1-cm^2 coupons in PBS (● and ▼) or PBS supplemented with Tiron (○ and △). Cells were removed from coupons and assessed for culturability as described in the text. The addition of EDTA at 65 min of contact did not prolong survival if cells had been inoculated in PBS (▼) but did so in cells inoculated in Tiron (△).

1-log reduction in viable cells if copper(I) was removed with BCS but a 2-log reduction if copper(II) ions were chelated (Fig. 1A). A similar pattern was seen for clinical isolates of both species (Fig. 2), where a high degree of protection was still present even after 3 h of contact with the copper surfaces, but once again, more protection was observed with BCS than with EDTA for *E. faecalis*. Under anaerobic conditions, the chelation of both copper ionic species was still protective (not shown).

A 20-μl wet inoculum can take over 30 min to dry, and it has been suggested that copper toxicity may differ depending on the aqueous nature of the contaminating inoculum. The same number of cells was applied onto copper surfaces in a reduced inoculum of 1 μl (dry), which dried in seconds. Death was much faster (Fig. 3A and B), and no viable cells were detected for *E. faecalis* in PBS after 10 of min of contact with copper surfaces at room temperature; once more, *E. faecium* was more resilient, with several thousand cells remaining viable at this

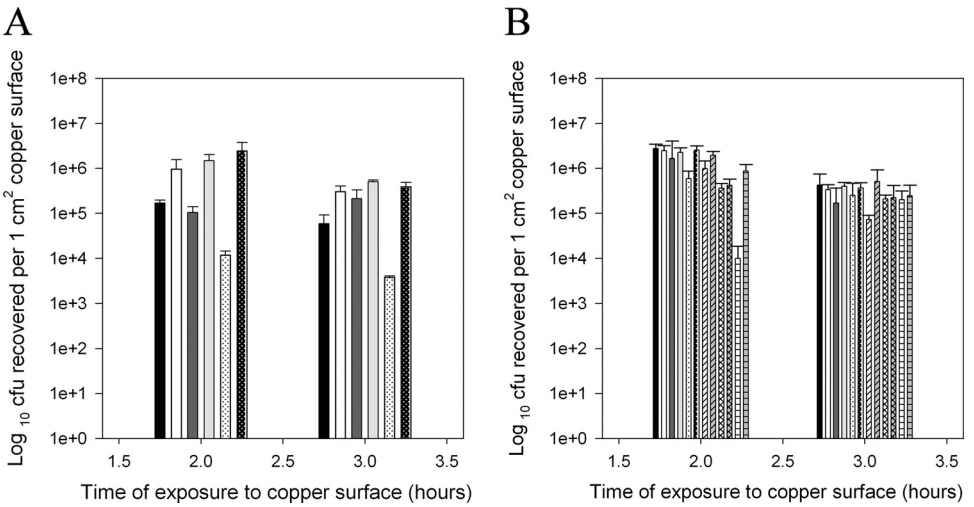

FIG. 2. Survival of enterococcal clinical isolates on copper surfaces in the presence of chelators of Cu(I) (BCS) and Cu(II) ions (EDTA) with the wet fomite inoculum. Approximately 10^7 CFU of *E. faecalis* (A) or *E. faecium* (B), resuspended in 20 μl of 20 mM EDTA or 20 mM BCS, was inoculated onto 1-cm^2 copper coupons. Cells were removed and assessed for culturability after 2 and 3 h of contact with the surface at 22°C. Strains used were as follows: ATCC 51299 in EDTA (black bars) or BCS (white bars), wound swab isolate 1 in EDTA (dark gray bars) or BCS (light gray bars), and fecal isolate 2 in EDTA (white spotted bars) or BCS (black spotted bars) (A) and NCTC 12202 in EDTA (black bars) or BCS (white bars), ascitic fluid isolate 1 in EDTA (dark gray bars) or BCS (light gray bars), swab isolate 2 in EDTA (white spotted bars) or BCS (black spotted bars), blood culture isolate 3 in EDTA (white diagonal bars) or BCS (gray diagonal bars), gastric aspirate isolate 4 in EDTA (white crossed bars) or BCS (gray crossed bars), and isolate from central venous catheter tip 5 in EDTA (white horizontal striped bars) or BCS (gray horizontal striped bars) (B). (For ease of observation, time zero results are not shown for each graph.) No viable cells were present in PBS alone at these time points.

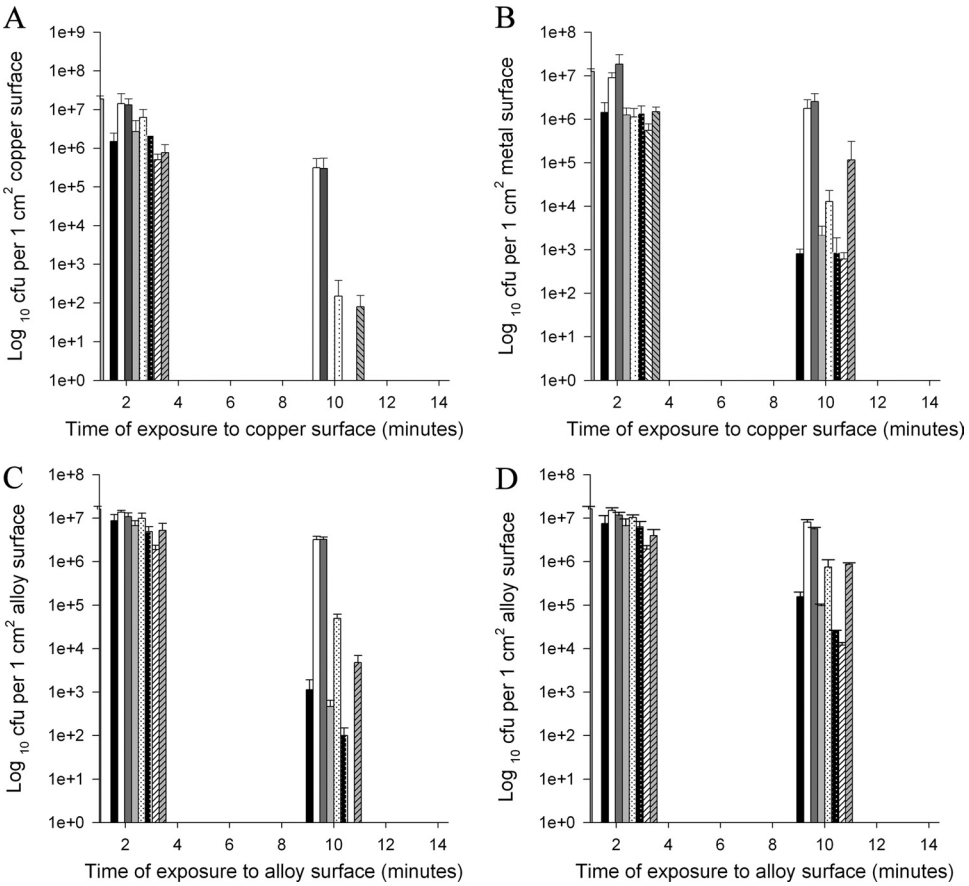

FIG. 3. Survival of vancomycin-resistant *E. faecalis* ATCC 51299 (A and C) and *E. faecium* NCTC 12202 (B and D) on copper (A and B) and copper alloy C26000 (C and D) surfaces with the dry inoculum. Approximately 10^7 CFU in 1 μl was inoculated onto 1-cm^2 coupons in PBS (black bars) or PBS supplemented with EDTA (white bars), BCS (dark gray bars), D-mannitol (light gray bars), Tiron (white spotted bars), SOD (black spotted bars), catalase (white diagonal striped bars), and sucrose (gray diagonal striped bars) at 22°C (time zero results not shown).

time point. The addition of chelators also gave a high degree of protection against copper with the dry inoculum (Fig. 3). A similar result was observed with cartridge brass (70% copper and 30% zinc) (Fig. 3C and D). For both enterococcal species viable cells were still present at 10 min (approximately a 4-log reduction for *E. faecalis* and a 2-log reduction for *E. faecium*). Significant protection was afforded by EDTA and BCS.

There was no significant difference ($P < 0.05$) between cells inoculated in PBS and those inoculated in PBS supplemented with chelators on stainless steel surfaces for either inoculum volume (results not shown). At 3 h of contact, there was less than a 0.5-log reduction in viable cells, as detected by culturing.

Effect of ROS quenchers on survival of enterococci on copper and stainless steel surfaces. To determine if Fenton chemistry is involved in copper surface toxicity, quenchers of reactants and end products were used. Catalase was used to detoxify hydrogen peroxide, the driving force of the Fenton reaction; superoxide was quenched with SOD and Tiron; and D-mannitol was used to quench hydroxyl free radicals. Sucrose was used to determine the role of osmotic stress in survival on surfaces.

For the wet inoculum on copper, significant protection for both enterococcal species was observed initially with Tiron, with the protection peaking at 80 min; however, unlike the results for chelators, all cells were dead at 120 min. This represents a doubling of the kill time for *E. faecalis* compared to that of the PBS control on copper (Fig. 1A), and although the kill time was the same for *E. faecium* at 60 min of contact with copper, there were approximately 6 logs more viable cells with Tiron present (Fig. 1B). If cells were inoculated in the presence of Tiron, and EDTA was added after 65 min of contact with copper surfaces, survival could be prolonged (Fig. 1C). There was no difference in survival rates between cells inoculated in PBS alone with and those without the addition of EDTA at 65 min; i.e., death occurred by 60 min of contact on copper. Similarly, there was no difference if cells were inoculated in PBS or Tiron with or without the addition of EDTA on stainless steel surfaces, i.e., less than a 1-log reduction in cell viability following 120 min of contact (results not shown). These combined results suggest that superoxide generation occurs initially but that it is the toxicity of copper(II) that continues in the long term. There was no protection afforded with catalase or mannitol for *E. faecalis*, but some protection was observed at 60 min for SOD and sucrose. This was also seen with *E. faecium*, and marginal protection with catalase and D-mannitol was seen (Fig. 1B). The same results were observed for both species with the dry inoculum, with signifi-

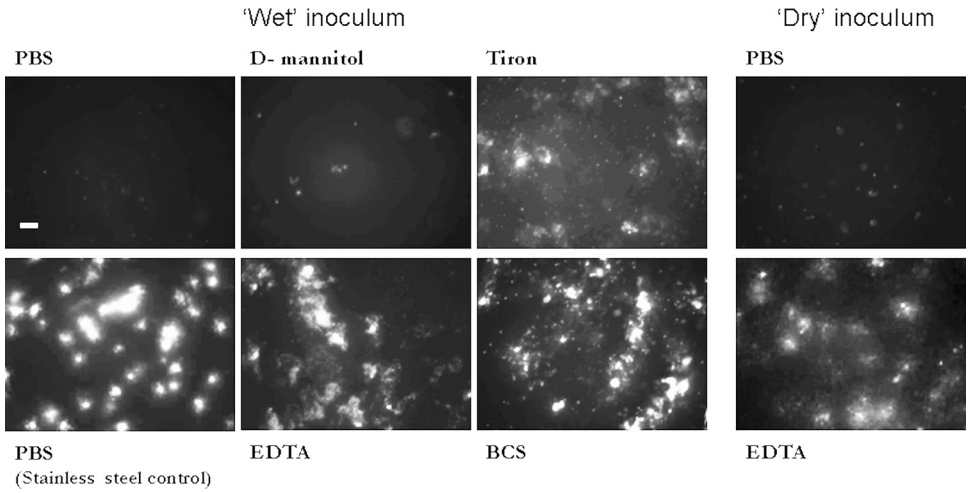

FIG. 4. Protection of bacterial genomic DNA by chelators and ROS quenchers determined by use of a DNA fragmentation assay with the wet and dry inocula. Approximately 10^7 bacterial cells (in 20 μl [wet] or 1 μl [dry]) were inoculated onto copper coupons in PBS (control) or PBS supplemented with chelators or ROS quenchers and incubated at 22°C for 2 h (wet) or 10 min (dry). The cells were removed from the coupons, and genomic DNA was analyzed with a DNA fragmentation assay. For cells that had been inoculated onto coupons in PBS or PBS supplemented with mannitol or SOD, very little DNA was visible, presumably because the fragments were too small to be visualized and had diffused away from the body of the cell. However, in the presence of EDTA, BCS, and Tiron, intact DNA was visible, suggesting that they exerted a protective effect on the nucleic acid. On stainless steel all supplements demonstrated intact genomic DNA (not shown for the dry inoculum). The bar represents 10 μm.

cant protection by Tiron and sucrose on copper and cartridge brass at 10 min (Fig. 3).

There was no significant difference between cells inoculated in PBS alone (control) and those inoculated in PBS supplemented with ROS quenchers on stainless steel surfaces for either inoculum volume. At 3 h of contact time there was less than a 0.5-log reduction in viable cells, as detected by culture (not shown).

Status of DNA of enterococci exposed to copper and copper alloy surfaces compared to stainless steel in the presence of chelators or ROS quenchers. We previously observed a disintegration of the DNA of bacteria exposed to copper surfaces but not stainless steel using a DNA fragmentation assay (49). This assay allows assessments of the status of genomic DNA in whole bacterial cells: an intact genome can be visualized by using the sensitive DNA stain SYBR gold as loops of nucleic acid protruding through the permeabilized membrane. Damaged or disintegrated DNA can be seen in stages of fragments that have diffused away from the cell until they are too small to be detected.

Cells inoculated onto copper in PBS (wet inoculum) had very little detectable DNA following 30 min of contact with copper surfaces (Fig. 4). However, if EDTA or BCS was present, the DNA was visualized, suggesting that the removal of copper(I) and copper(II) ions either directly or indirectly protects the bacterial DNA. A similar pattern was seen in the presence of Tiron, as well as weak protection with mannitol, but not with the remaining ROS quenchers.

A similar response was observed for dry inocula of *E. faecalis* inoculated onto stainless steel and copper surfaces in the presence of EDTA (Fig. 4). On stainless steel in the presence of PBS or PBS supplemented with EDTA, the DNA was brightly staining and characteristic of an intact genome. Interestingly, the DNA on stainless steel was less diffuse with EDTA present, which may reflect protection from metal ions leaching from the surface, which can occur on this surface (not shown). No DNA was detectable in cells inoculated in PBS alone exposed to copper surfaces (no viable cells in culture were recorded at this exposure time), but if EDTA was present, DNA was protected from damage and was clearly visible but more diffuse than that on stainless steel, possibly reflecting DNA of dead cells (a 1-log reduction in viability was observed with EDTA present).

Is the DNA damage observed on copper and copper alloy surfaces part of the killing mechanism or post-cell-death breakdown? SYTO 9 is a cell-permeant, low-toxicity cyanine nucleic acid stain that increases fluorescence when bound to double-stranded DNA upon excitation at 485 nm. The disruption of intact DNA can be visualized as a reduction in fluorescence. Enterococci exposed to copper surfaces demonstrate reduced staining and cell clumping immediately upon contact (Fig. 5A). Even cells removed immediately upon contact (time zero) were already affected. On copper alloy (Fig. 5B), the effect was the same but slightly delayed, with significant fluorescence reduction evident at 1 to 2 min of contact. On stainless steel the DNA retained bright staining for all time points tested (Fig. 5C). These results suggest that DNA degradation is an active part of the copper killing mechanism in enterococci, which begins immediately upon contact and appears to be an early stage of cell death (the results shown are for *E. faecalis* ATCC 51299, and culture results indicate high viability after 2 min of contact on copper [Fig. 3] and that viable cells were still detectable at 10 min on alloy [Fig. 3]). Similar results were obtained for *E. faecium* (not shown). The same results were also obtained if cells were stained *in situ* and also by using the alternative DNA stain 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), which associates in AT clusters in the minor groove of double-stranded DNA (not shown).

To determine if the DNA of dead cells also degrades on

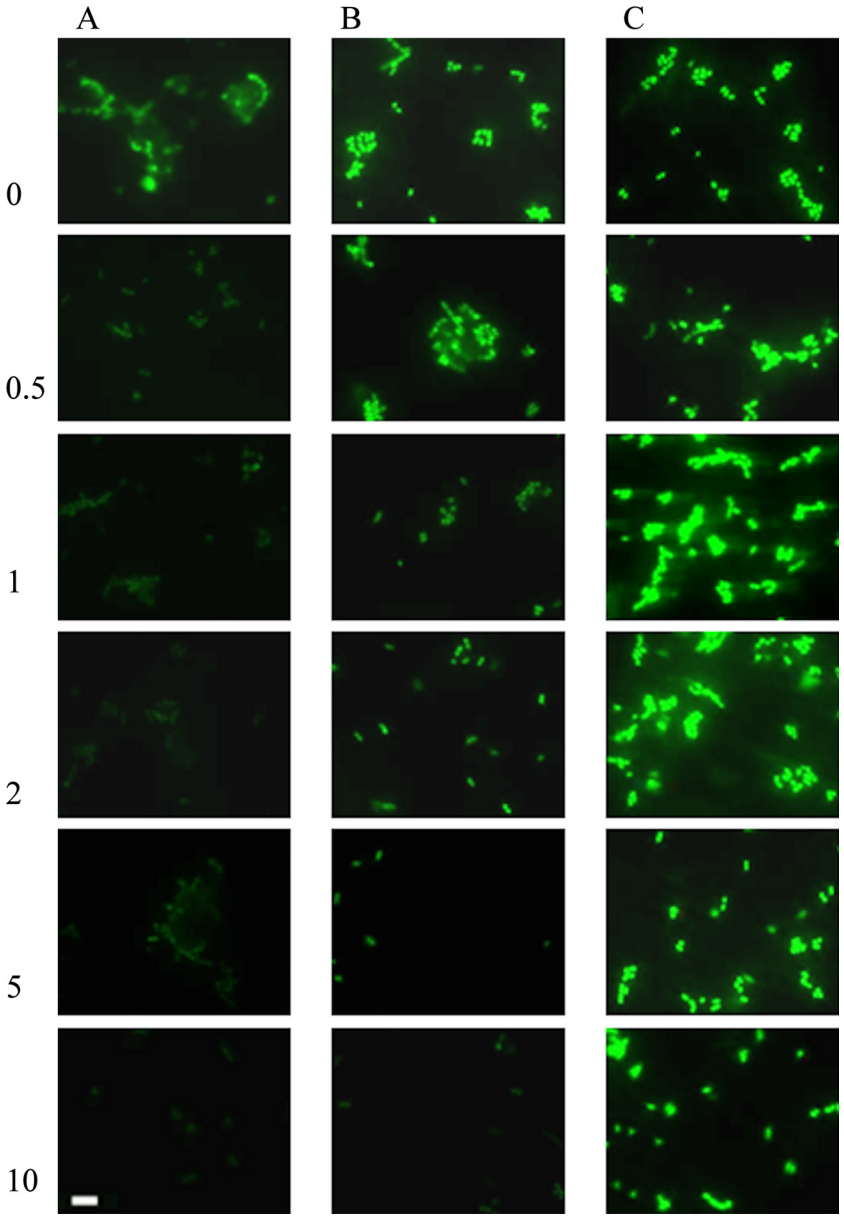

FIG. 5. Rapid breakdown of bacterial DNA on copper (A) and alloy (C26000) (B) surfaces compared to stainless steel (C) occurs as part of the killing mechanism in enterococci. Approximately 10^7 CFU in 1 μl was inoculated onto 1-cm^2 coupons, and cells were removed at the time points indicated (minutes of contact) with glass beads and PBS-EDTA (20 mM) as described in the text. Cells were pooled and stained with SYTO 9 (5 μM) to detect double-stranded DNA and observed by using epifluorescence microscopy. Clumping and disintegration of DNA (reduced staining) were seen at 0 to 30 s of contact with copper surfaces and at 30 s to 1 min of contact with alloy but not on stainless steel. Results for *E. faecalis* ATCC 51299 are shown. Bar, 10 μM.

copper, enterococci were heat killed (>95°C to inactivate nucleases) and exposed to metal surfaces. The DNA of dead cells exposed to copper did degrade but did so more slowly than the DNA in live cells (approximately 60 min for the dry inoculum). This degradation could be prevented by the addition of EDTA (DNA was still detectable at 2 h of contact) but not BCS, Tiron, catalase, or mannitol, suggesting that copper(II) may have a direct effect on the nucleic acid. Similar results were obtained for ethanol- and formalin-killed cells.

Effect of chelators or ROS quenchers on the respiration of enterococci exposed to copper and stainless steel surfaces. Actively respiring cells accumulate reduced tetrazolium salts as fluorescent red formazan, which can be visualized microscopically. Previously reported data showed that enterococci exposed to stainless steel and stained *in situ* with CTC on stainless steel surfaces continued to respire for several days, but on copper, respiration ceased in less than 2 h with a wet inoculum (49).

In the present study, after 2 h of contact with copper surfaces in the presence of EDTA, BCS (not shown), or Tiron, respiring cells continued to produce fluorescent formazan, suggesting that respiratory pathways had been protected from copper toxicity (Fig. 6A).

Positive staining by the nucleic acid stain SYTO 9 in the

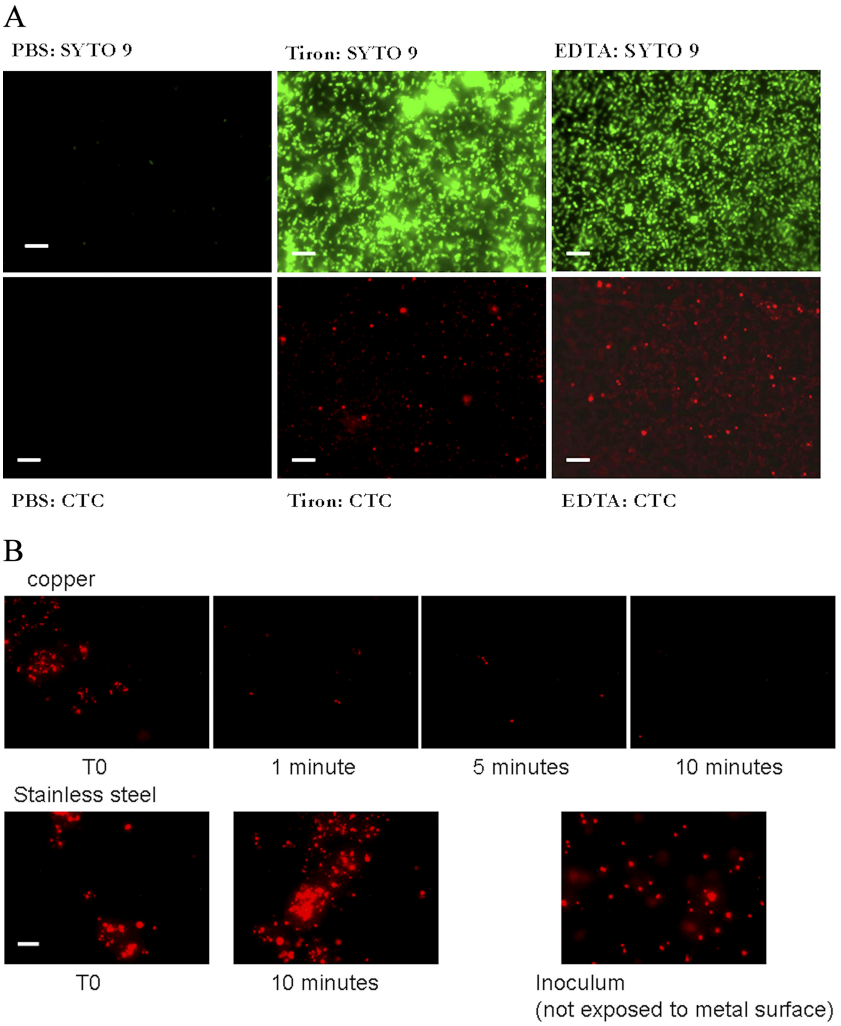

FIG. 6. Protection of bacterial respiratory pathways by chelators and ROS quenchers on copper surfaces with the wet (A) or dry (B) inoculum. Approximately 10^7 bacterial cells (in 20 μl) were inoculated onto copper coupons in PBS or PBS supplemented with chelators or ROS quenchers and incubated at 22°C for 2 h. Cells were stained *in situ* with the redox stain CTC to detect actively respiring cells (fluorescing red) and a nonspecific bacterial stain, SYTO 9, for total cell numbers (green fluorescence DNA stain). In the presence of EDTA, BCS, or Tiron, respiring cells are visible, and the bright staining with SYTO 9 suggests that the DNA is intact. However, no respiring cells were visible in PBS or PBS supplemented with mannitol, catalase, or SOD (not shown). Respiring cells were detected on stainless steel for all supplements tested (not shown). For the dry inoculum (1 μl), cells were removed from coupons as described in the text, and at the times indicated, for culture; pooled; and stained with CTC for 1 h before transferring them onto a microscope slide. Cells were actively respiring at 10 min on stainless steel, but very few respiring cells were visible following 1 min of contact with copper surfaces. Bar, 10 μM.

presence of EDTA and Tiron but not PBS also suggests that the DNA is protected if copper ions and superoxide are suppressed.

Bacterial respiration was also affected in the dry inoculum on copper surfaces (Fig. 6B). Respiration was significantly diminished after 1 min of contact. No effect on bacterial respiration on stainless steel was observed for the duration of the experiment.

Changes in bacterial membrane potential *in situ* on copper and copper alloy surfaces. We have previously tried to investigate bacterial membrane integrity in enterococci using *Bac*Light (Invitrogen) viability stains. However, this was unsuccessful, because this method requires fluorescent SYTO 9 and propidium iodide stains to bind to intracellular DNA as a measure of cell permeability, but the DNA degrades rapidly on copper (49). Other conventional methods to investigate $\Delta\Psi$ in bacteria are usually performed on cells in suspension using cationic or anionic lipophilic dyes, measuring fluorescence changes using a fluorometer or flow cytometry. To investigate bacteria on surfaces, we devised a staining protocol to observe cells *in situ* on copper, alloy, and stainless steel surfaces: rhodamine 123 is internalized and accumulates in the bacterial membrane and rapidly equilibrates to a level of fluorescence determined by the $\Delta\Psi$. Any changes in the $\Delta\Psi$ can be observed with depolarization resulting in reduced fluorescence as the membrane becomes leaky and eventually ruptures. Enterococcal species did not demonstrate a depolarization of the membrane on copper surfaces until 9 to 10 min of contact in the dry inoculum. This finding suggests that the membrane of enterococci is compromised only after the cells are dead (Fig. 7). No

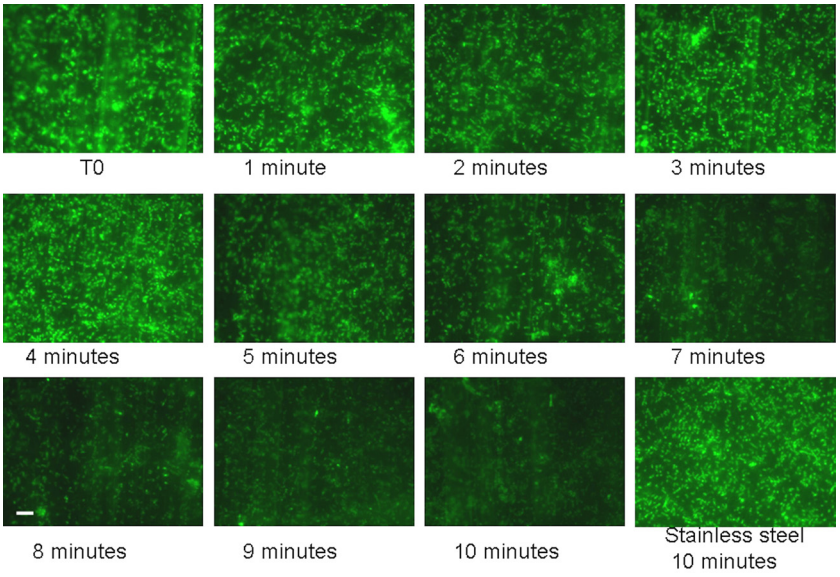

FIG. 7. Detection of changes in membrane potential changes *in situ* in *E. faecalis* ATCC 51299 cells exposed to copper (for the times indicated) and stainless steel (only the 10-min time point is illustrated) surfaces. Approximately 10^7 bacterial cells (in 1 μl) that had been stained with rhodamine 123 were inoculated onto metal coupons for 10 min. Images were recorded every minute and were observed by using epifluorescence microscopy. Bright staining indicates that membranes are not compromised until after 8 min of contact; i.e., many cells were already dead, as indicated by the culture results. The ionophore CCCP was used as a negative control to depolarize the membrane and eliminate rhodamine 123 staining (not shown). Bar, 10 μM.

effect on the bacterial membrane on stainless steel was observed (the image taken at 10 min of contact only is included in Fig. 7). Experiments to determine the role of copper ions and ROS in the membrane damage of Gram-negative organisms are currently being undertaken. No staining was observed in the presence of negative-control CCCP-treated cells (not shown), because it increases proton permeability and dissipates $\Delta\Psi$ and the proton gradient (ΔpH) (together making up the proton motive force), resulting in a compromised cell membrane. Minimal background staining was observed on metal surfaces alone. The same results were obtained for the wet inoculum, where the intensity of rhodamine 123 diminished only after prolonged contact (not shown).

DISCUSSION

In this study we have demonstrated a rapid killing, particularly for "dry-touch" contamination, of important pathogenic enterococci, where death occurs in minutes on copper and, perhaps more importantly, copper alloys, which could be used practically as touch surfaces in health care settings and elsewhere. An understanding of the mechanism of copper surface toxicity requires the identification of the agents responsible and the targets affected. The prolonged survival that we observed in the presence of chelators suggests that both ionic species, copper(I) and copper(II), are important, directly or indirectly, in the killing mechanism for wet and dry surface contamination under aerobic and anaerobic conditions. This was surprising, because it was expected that for the wet inoculum with longer contact times, the rate of copper ion release would have been greater. Molteni et al. (29) previously quantified copper ion released from surfaces and observed that release was proportional to the killing rate and very dependent on the liquid matrix constitution. Perhaps, the most significant ion release is immediately upon contact. In *E. faecium*, both copper oxidative states were equally important, but in *E. faecalis*, copper(I) ions appeared to be more significant than copper(II) for the type strains and clinical isolates tested here. The chelation of copper ions also resulted in the protection of enterococcal genomic DNA and respiration on copper surfaces under wet and dry inoculum conditions.

Copper is an essential element in biological systems but highly toxic at elevated concentrations, which may be due to the generation of toxic radicals that damage cellular components such as superoxide (13, 20, 24, 41, 43). In enterococci, superoxide dismutase (SOD) dismutes superoxide to hydrogen peroxide under the acidic conditions of the intestine. Our results suggest that Tiron, a membrane-permeable quencher of superoxide, significantly protected enterococci from the toxicity of copper surfaces under wet and dry conditions. However, if SOD was present, protective effects were minimal for the dry inoculum but more evident under wet conditions, particularly for *E. faecalis*. However, these experiments were conducted at a neutral pH, and therefore, the dismutation rate may have been reduced. The protective effect of Tiron was initially considerable but rapidly declined after 60 min in the wet inoculum. It is unclear if this is due to a release of ROS, which occurs as part of a common lethal pathway in bacteria exposed to antimicrobials with completely different modes of action (21, 48). However, the addition of EDTA at the point where Tiron protection had begun to decline protected cells, suggesting that toxicity may involve a short-term generation of superoxide but prolonged copper(II) toxicity. Tiron also protected enterococci from DNA damage and respiratory failure on copper surfaces.

In vivo, enterococci exist primarily under the anoxic condi-

tions of the gut. However, *E. faecalis* is an unusual intestinal commensal, because under certain nutrient-limiting conditions, an incomplete respiratory chain results in fermentative metabolism that releases extracellular superoxide, which enhances virulence and has been linked to chromosomal instability (CIN), a possible precursor to colorectal cancer (CRC) and inflammatory bowel disease (16, 47). However, it may be that if small quantities of superoxide are being produced in our system, then the copper(II) released from the copper surface is reduced to copper(I).

This would explain the increased effect of the copper(I) chelator observed for *E. faecalis* compared to that observed for *E. faecium*. It is interesting that Baker et al. (2) previously observed extracellular copper(I) on the surface of *Staphylococcus aureus*, which is implicated in the toxicity of soluble copper. It is unclear if Tiron is quenching intracellular and extracellular superoxide, although the only partial protection afforded by SOD suggests that the majority of superoxide generation is intracellular, since the enzyme would be unlikely to penetrate the cell. The generation of superoxide, which is a virulence factor *in vivo*, may be a suicidal act when cells are exposed to copper and copper alloy surfaces.

Hydrogen peroxide is a 2-electron reductant of oxygen and therefore not a true radical but has the ability to diffuse through cell membranes. It has a long half-life in the presence of superoxide and can damage biomolecules directly by the oxidation of sulfur atoms in cysteine residues (16, 47); in the presence of the transition metals iron and copper, it is responsible for the generation of highly toxic hydroxyl free radicals (the Fenton reaction). Catalase was used to decompose hydrogen peroxide to water and oxygen but did not have a protective effect on survival for either species in the dry inoculum and only a slight effect at 60 min of contact for the *E. faecium* wet inoculum (although superoxide generated in *E. faecalis* is known to inactivate catalase but not SOD, and the production of hydrogen peroxide *in vivo* is a virulence factor for *E. faecium* [31]). No protective effect on bacterial DNA or respiration was seen.

The hydroxyl radical is a highly reactive oxidant with a half-life in aqueous solution of less than 1 nanosecond. This moiety has the ability to damage biomolecules directly, for example, by inducing strand breaks and base modifications in DNA at diffusion-limited rates (17). In this study, the quenching of hydroxyl radical generation with D-mannitol did not significantly prolong survival on copper surfaces or protect DNA or bacterial respiration even at a range of concentrations (data not shown). Tkeshelashvili et al. (44) observed previously that D-mannitol did not completely abolish lethal damage by soluble copper ions on purified *E. coli* DNA and suggested that other ROS may be involved in DNA damage, including copper peroxides. Savoye et al. (40) previously investigated the binding of soluble copper ions to purified irradiated DNA and found that conformational changes restricted the access of mannitol to the hydroxyl ions generated. It is uncertain if hydroxyl radical formation is occurring in enterococci, and we have not detected it either, because the site of generation is shielded from the quencher or too short-lived and escaped detection in our system. The enterococcal DNA destruction observed *in vitro* (cells exposed to copper surfaces and removed for analysis) and *in situ* on copper and copper alloy surfaces does appear to be part of the killing process, because significant breakdown begins to occur immediately upon contact but does not appear to be the result of hydroxyl radical toxicity. The DNA breakdown of dead cells exposed to copper surfaces that could be protected with EDTA suggests direct copper(II) involvement. In eukaryotic DNA, metal ions are known to bind at separate sites on DNA and can unwind the helix, affect DNA-associated proteins, and induce lesions (24). Copper bound to peptides is also known to result in damage to the DNA (42). Perhaps, the constant influx of ions into the bacteria on copper surfaces produces intracellular copper complexes with unknown proteins that induce damage to DNA and possibly free-ion-induced lesions. However, in viable cells the DNA breakdown is much faster than that in dead cells, suggesting that there is still a role for unknown radicals possibly generated by superoxide and cellular metabolism. Other radicals, including peroxynitrite, which are generated from superoxide and nitric oxide and are known to produce further radicals that cause DNA strand breakage and base damage, may possibly have a role but have not been addressed here. Moore et al. (30) observed previously that *in vivo*, *E. faecalis* produces thiyl radicals from the oxidation of cysteine residues, which, like superoxide, are virulence factors that damage epithelial cell DNA and affect the fluidity of the membrane. Tiron did not protect dead cell DNA on copper, allaying fears that Tiron may also be chelating ions as well as quenching superoxide, as reported previously by Ghosh et al. (12). A recent report (9) suggested that DNA damage is not occurring in *E. coli* but that the mutagenicity and comet assays used detect limited damage and not the extensive overall effect on the entire genome that we have observed for enterococci. Those authors also determined that the superior DNA repair mechanisms of the polyextremophile *Deinococcus radiodurans* did not protect the organism from death on copper surfaces, but it is unclear if the DNA was already extensively degraded.

Our results suggest that for Gram-positive enterococci, the Fenton reaction-generated hydroxyl radicals may not be as important on copper surfaces, in contrast to recent reports for Gram-negative organisms (7). Fenton chemistry has been observed *in vitro* with purified DNA, but concern about its relevance *in vivo* has been expressed (39, 41). Macomber et al. (25) found previously that soluble copper decreased the rate of hydrogen peroxide-induced DNA damage in *E. coli* and suggested that oxidative stress was not the only mechanism responsible for copper killing. The survival of enterococci on copper surfaces was prolonged in the presence of 10% sucrose, which was also observed previously for *E. coli* by Espirito Santo et al. (7). This may be due to protection from osmotic stress, the reduction of water activity (1), or antioxidant-scavenging properties that have been attributed to some sugars (38). Membrane damage in *E. coli* on copper has been reported (9); however, our *in situ* staining with the lipophilic cationic dye rhodamine 123 indicates that extensive membrane depolarization does not occur upon the prolonged contact of enterococci on copper and alloy surfaces when DNA destruction and cell death have already occurred. Consequently, membrane damage cannot be assumed to be a universal mechanism of copper toxicity.

To summarize, in the two main pathogenic species of enterococci, copper surface toxicity is implemented by copper(I)

and copper(II) ions and superoxide under both fomite and dry-touch conditions. Killing is 80 to 90% faster under dry conditions, and rapid DNA degradation is followed by a reduction in bacterial respiration. Finally, the membrane is slowly depolarized. The same killing mechanism also exists with cartridge brass, a commonly used copper alloy, but takes slightly longer, presumably due to the reduced copper content.

The bacterial DNA is denatured, primarily by copper(II) but also by superoxide, whose toxicity is thought to arise indirectly from the generation of hydroxyl radicals (Fenton and Haber-Weiss reactions) (19, 26). We have not found any evidence to support hydroxyl radical generation in enterococci, with superoxide being the principal ROS generated: superoxide was reported previously to have some direct effects on enzymes and small molecules (11). The DNA of dead cells also denatures on copper surfaces but much more slowly, suggesting that metabolic activity is required for the initial superoxide-mediated killing, while direct copper effects take longer.

Respiration is also affected by copper ions and superoxide on copper surfaces. *E. faecalis* possesses cytochrome *bd* in the membrane (56), and copper(II) ions are known to bind and inhibit certain cytochromes by altering the conformation and electron transfer of associated reductases (22). This may explain why respiration is affected so quickly in the dry inoculum. Moreover, a respiratory block may result in the buildup of toxic intermediates. Although the ATPase activity in *Enterococcus hirae* cell membranes was reported previously to be affected by soluble copper (46), our results suggest that little damage to the enterococcal membrane occurs on copper surfaces until after cells are dying. It is unlikely that the thick cell wall of Gram-positive organisms helps to maintain the integrity of the cell, shielding the membrane from direct contact with copper, since copper ion uptake is rapid and contributes quickly to DNA destruction and respiratory inhibition, but bacterial morphology does appear to affect the mechanism of copper toxicity.

Our results also highlight that if any contaminating substance can inhibit the access of copper ions to pathogens contained within it, this can affect the efficiency of the copper surface as a killing surface. Molteni et al. (29) observed previously that ion release in Tris HCl buffer was 100 times greater than that in water or phosphate buffer. These experiments were performed by using phosphate buffer, and a rapid killing of enterococci was achieved unless chelating substances were present. This was observed by previous studies of contaminated copper coins, where blood, pus, and natural soiling delayed the copper killing mechanism, presumably due to a chelation effect (8, 45).

Regardless of differences in mechanisms, copper alloy surfaces may prove invaluable for the reduction of the spread of viable organisms in health care facilities and food preparation areas, but because copper ion release is the limiting factor in surface efficacy, constant surface care to ensure that soiling or cleaning agents do not interfere with copper(I) and copper(II) ion release is essential.

ACKNOWLEDGMENTS

This research was supported by the Copper Development Association, New York, and the International Copper Association, New York.

REFERENCES

1. **Akiyama, H., O. Yamasaki, H. Kanzaki, J. Tada, and J. Arata.** 1998. Effects of sucrose and silver on *Staphylococcus aureus* biofilms. J. Antimicrob. Chemother. **42:**629–634.
2. **Baker, J., et al.** 2010. Copper stress induces a global stress response in *Staphylococcus aureus* and represses sae and agr expression and biofilm formation. Appl. Environ. Microbiol. **76:**150–160.
3. **Boyce, J. M.** 2007. Environmental contamination makes an important contribution to hospital infection. J. Hosp. Infect. **65**(Suppl. 2)**:**50–54.
4. **Casey, A. L., et al.** 2010. The role of copper in the reduction of contamination of the hospital environment. J. Hosp. Infect. **74:**72–77.
5. **Copper Development Association.** 2010. Reducing the risk of healthcare-acquired infections: the role of copper touch surfaces (publication 196). Copper Development Association, New York, NY. http://www.copperinfo.co.uk/antimicrobial/downloads/pub-196-reducing-risk-hcais-report.pdf.
6. **Drees, M., et al.** 2008. Prior environmental contamination increases the risk of acquisition of vancomycin-resistant enterococci. Clin. Infect. Dis. **46:**678–685.
7. **Espirito Santo, C. E., N. Taudte, D. H. Nies, and G. Grass.** 2007. Contribution of copper ion resistance to survival of *Escherichia coli* on metallic copper surfaces. Appl. Environ. Microbiol. **74:**977–986.
8. **Espirito Santo, C. E., P. V. Morais, and G. Grass.** 2010. Isolation and characterization of bacteria resistant to metallic copper surfaces. Appl. Environ. Microbiol. **76:**1341–1348.
9. **Espirito Santo, C. E., et al.** 2011. Bacterial killing by dry metallic surfaces. Appl. Environ. Microbiol. **77:**794–802.
10. **Fernandez, J. L., et al.** 2008. DNA fragmentation in microorganisms assessed in situ. Appl. Environ. Microbiol. **74:**5925–5933.
11. **Fridovich, I.** 1986. Biological effects of the superoxide radical. Arch. Biochem. Biophys. **247:**1–11.
12. **Ghosh, M., H. D. Wang, and J. R. McNeill.** 2002. Tiron exerts effects unrelated to its role as a scavenger of superoxide anion: effects on calcium binding and vascular responses. Can. J. Physiol. Pharmacol. **80:**755–760.
13. **Halliwell, B., and J. M. C. Gutteridge.** 1989. Free radicals in biology and medicine, 2nd ed. Clarendon Press, Oxford, United Kingdom.
14. **Harrison, J. J., et al.** 2009. Chromosomal antioxidant genes have metal ion-specific roles as determinants of bacterial metal tolerance. Environ. Microbiol. **11:**2491–2509.
15. **Hayden, M. K., D. W. Blom, E. A. Lyle, C. G. Moore, and R. A. Weinstein.** 2008. Risk of hand or glove contamination after contact with patients colonized with vancomycin-resistant enterococcus or the colonized patients' environment. Infect. Control Hosp. Epidemiol. **29:**149–154.
16. **Huycke, M. M., V. Abrams, and D. R. Moore.** 2002. *Enterococcus faecalis* produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. Carcinogenesis **23:**529–536.
17. **Imlay, J. A.** 2003. Pathways of oxidative damage. Annu. Rev. Microbiol. **57:**395–418.
18. **Keevil, C. W.** 2003. Rapid detection of biofilms and adherent pathogens using scanning confocal laser microscopy and episcopic differential interference contrast microscopy. Water Sci. Technol. **47:**105–116.
19. **Keyer, K., and J. A. Imlay.** 1996. Superoxide accelerates DNA damage by elevating free-iron levels. Proc. Natl. Acad. Sci. U. S. A. **93:**13635–13640.
20. **Keyhani, E., F. Abdi-Oskouei, F. Attar, and J. Keyhani.** 2006. DNA strand breaks by metal-induced oxygen radicals in purified *Salmonella typhimurium* DNA. Ann. N. Y. Acad. Sci. **1091:**52–64.
21. **Kohanski, M., D. J. Dwyer, B. Hayete, C. A. Lawrence, and J. J. Collins.** 2007. A common method of cellular death induced by bactericidal antibiotics. Cell **130:**797–810.
22. **Letelier, M. E., et al.** 2009. Mechanisms underlying the inhibition of the cytochrome P450 system by copper ions. J. Appl. Toxicol. **29:**695–702.
23. **Li, Y., and M. A. Trush.** 1993. DNA damage resulting from the oxidation of hydroquinone by copper: role for a Cu(II)/Cu(I) redox cycle and reactive oxygen generation. Carcinogenesis **14:**1303–1311.
24. **Lloyd, D. R., and D. H. Phillips.** 1999. Oxidative DNA damage mediated by copper(II), iron(II) and nickel(II) Fenton reactions: evidence for site-specific mechanisms in the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intrastrand cross-links. Mutat. Res. **424:**23–36.
25. **Macomber, L., C. Rensing, and J. A. Imlay.** 2006. Intracellular copper does not catalyze the formation of oxidative DNA damage in *Escherichia coli*. J. Bacteriol. **189:**1616–1626.
26. **Macomber, L., and J. A. Imlay.** 2009. The iron-sulfur clusters of dehydratases are primary targets of copper toxicity. Proc. Natl. Acad. Sci. U. S. A. **106:**8344–8349.
27. **Mehtar, S., I. Wiid, and S. D. Todorov.** 2008. The antimicrobial activity of copper and copper alloys against nosocomial pathogens and *Mycobacterium tuberculosis* isolated from healthcare studies in the Western Cape: an in vitro study. J. Hosp. Infect. **68:**45–51.
28. **Michels, H. T., J. O. Noyce, and C. W. Keevil.** 2009. Effects of temperature and humidity on the efficacy of methicillin-resistant *Staphylococcus aureus* challenged antimicrobial materials containing silver and copper. Lett. Appl. Microbiol. **49:**191–195.

29. **Molteni, C., H. K. Abicht, and M. Solioz.** 2010. Killing of bacteria by copper surfaces involves dissolved copper. Appl. Environ. Microbiol. **76:**4099–4101.
30. **Moore, D. R., Y. Kotake, and M. M. Huycke.** 2004. The effects of iron and phytic acid on production of extracellular radicals by *Enterococcus faecalis*. Exp. Biol. Med. (Maywood) **229:**1186–1195.
31. **Moy, T. I., E. Mylonakis, S. B. Calderwood, and F. M. Ausbel.** 2004. Cytotoxicity of hydrogen peroxide produced by Enterococcus faecium. Infect. Immune. **72:**4512–4520.
32. **Murray, B. E.** 1998. Diversity of multi-drug resistant enterococci. Emerg. Infect. Dis. **4:**37–47.
33. **Neeley, A., and M. P. Maley.** 1999. Survival of enterococci and staphylococci on hospital fabrics and plastic. J. Clin. Microbiol. **38:**724–726.
34. **Noyce, J. O., H. Michels, and C. W. Keevil.** 2006. Potential use of copper surfaces to reduce survival of epidemic methicillin-resistant Staphylococcus aureus in the healthcare environment. J. Hosp. Infect. **63:**289–297.
35. **Noyce, J. O., H. Michels, and C. W. Keevil.** 2006. Use of copper cast alloys to control Escherichia coli O157 cross-contamination during food processing. Appl. Environ. Microbiol. **72:**4239–4244.
36. **Noyce, J. O., H. Michels, and C. W. Keevil.** 2007. Inactivation of influenza A virus on copper versus stainless steel surfaces. Appl. Environ. Microbiol. **73:**2748–2750.
37. **Quaranta, D., et al.** 2011. Mechanisms of contact-mediated killing of yeast cells on dry metallic copper surfaces. Appl. Environ. Microbiol. **77:**416–426.
38. **Sagone, A. L., J. Greenwald, E. H. Kraut, J. Bianchine, and D. Singh.** 1983. Glucose: a role as a free radical scavenger in biological systems. J. Lab. Clin. Med. **10:**97–104.
39. **Saran, M., C. Michel, K. Stettmaier, and W. Bors.** 2000. Arguments against the significance of the Fenton reaction contributing to signal pathways under in vivo conditions. Free Rad. Res. **33:**567–569.
40. **Savoye, C., R. Sabattier, M. Charlier, and M. Spotheim-Maurizot.** 1996. Sequence-modulated radiosensitization of DNA by copper ions. Int. J. Radiat. Biol. **70:**189–198.
41. **Solioz, M., H. K. Abicht, and M. Mermod.** 2010. Response of Gram-positive bacteria to copper stress. J. Biol. Inorg. Chem. **15:**3–14.
42. **Szczepanik, W., et al.** 2010. DNA oxidative cleavage induced by the novel peptide derivatives of 3-(quinoxalin-6-yl)alanine in combination with Cu(II) or Fe(II) ions. Bioinorg. Chem. Appl. **2009:**906836.
43. **Thieme, D., and G. Grass.** 2010. The Dps protein of Escherichia coli is involved in copper homeostasis. Microbiol. Res. **165:**108–115.
44. **Tkeshelashvili, L. K., T. McBride, K. Spence, and L. A. Loeb.** 1990. Mutation spectrum of copper-induced DNA damage. J. Biol. Chem. **266:**6401–6406.
45. **Tolba, O., et al.** 2007. Survival of epidemic strains of nosocomial- and community-acquired methicillin-resistant Staphylococcus aureus on coins. Am. J. Infect. Control **35:**342–346.
46. **Vardanyan, Z., and A. Trchounian.** 2010. The effects of copper(II) ions on *Enterococcus hirae* cell growth and the proton-translocating FoF1 ATPase activity. Cell Biochem. Biophys. **57:**19–26.
47. **Wang, X., and M. M. Huycke.** 2007. Extracellular superoxide production by *Enterococcus faecalis* promotes chromosomal instability in mammalian cells. Gastroenterology **132:**551–561.
48. **Wang, X., and X. Zhao.** 2009. Contribution of oxidative damage to antimicrobial lethality. Antimicrob. Agents Chemother. **53:**1395–1402.
49. **Warnes, S. L., S. M. Green, H. T. Michels, and C. W. Keevil.** 2010. Biocidal efficacy of copper alloys against pathogenic enterococci involves degradation of genomic and plasmid DNA. Appl. Environ. Microbiol. **76:**5390–5401.
50. **Weaver, L., H. T. Michels, and C. W. Keevil.** 2008. Survival of *Clostridium difficile* on copper and steel. J. Hosp. Infect. **68:**145–151.
51. **Weaver, L., H. T. Michels, and C. W. Keevil.** 2009. Potential for preventing spread of fungi in air conditioning systems constructed using copper instead of aluminum. Lett. Appl. Microbiol. **50:**18–23.
52. **Weaver, L., H. T. Michels, and C. W. Keevil.** 2010. Potential action of copper surfaces on meticillin-resistant *Staphylococcus aureus*. J. Appl. Microbiol. **109:**2200–2205.
53. **Wheeldon, L. J., et al.** 2008. Antimicrobial efficacy of copper surfaces against spores and vegetative cells of *Clostridium difficile*: the germination theory. J. Antimicrob. Chemother. **62:**522–525.
54. **Wilks, S. A., H. Michels, and C. W. Keevil.** 2005. The survival of *Escherichia coli* O157 on a range of metal surfaces. Int. J. Food Microbiol. **105:**445–454.
55. **Wilks, S. A., H. T. Michels, and C. W. Keevil.** 2006. Survival of *Listeria monocytogenes* Scott A on metal surfaces: implications for cross-contamination. Int. J. Food Microbiol. **111:**93–98.
56. **Winstedt, L., L. Frankenberg, L. Hederstedt, and C. von Wachenfeldt.** 2000. *Enterococcus faecalis* V583 contains a cytochrome *bd*-type respiratory oxidase. J. Bacteriol. **182:**3863–3866.