Biocidal efficacy of copper alloys against pathogenic enterococci involves degradation of genomic and plasmid DNA

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Abstract

The increasing incidence of nosocomial infections caused by glycopeptide-resistant enterococci is a global concern. Enterococcal species are also difficult to eradicate with existing cleaning regimes; they can survive for long periods on surfaces thus contributing to cases of reinfection and spread of antibiotic resistant strains. We have investigated the potential use of copper alloys as bactericidal surfaces. Clinical isolates of vancomycin-resistant Enterococcus faecalis and Enterococcus faecium were inoculated onto copper alloy and stainless steel surfaces. Samples were assessed for the presence of viable cells by conventional culture, detection of actively respiring cells and assessment of cell membrane integrity. Both species survived for up to several weeks on stainless steel. However, no viable cells were detected on any alloys following exposure for 1 hour at an inoculum concentration of $\leq 10^4$ colony forming units per cm$^2$. Analysis of genomic and plasmid DNA from bacterial cells recovered from metal surfaces indicates substantial disintegration of the DNA following exposure to copper surfaces that is not evident in cells recovered from stainless steel. The DNA fragmentation is so extensive, and coupled with the rapid cell death which occurs on copper surfaces, that it suggests mutation is less likely to occur. It is therefore highly unlikely that genetic information can be transferred to receptive organisms re-contaminating the same area. A combination of effective cleaning regimes and contact surfaces containing copper could be useful to not only prevent spread of viable pathogenic enterococci but also to mitigate against the occurrence of potential
resistance to copper, biocides or antibiotics, and spread of genetic determinants of
resistance to other species.

Keywords: *Enterococcus faecalis, Enterococcus faecium*, infection, surfaces, hospital
acquired infection, vancomycin-resistance, DNA disintegration, bactericidal copper

**Introduction**

Enterococci are an important cause of nosocomial infections worldwide\(^3^4\) and in the
USA approximately one third of enterococcal infections in intensive care units are
caused by vancomycin-resistant enterococci (VRE)\(^3^5\).

There is now a significant problem of enterococci acquiring resistance to clinically
important antibiotics particularly aminoglycosides, glycopeptides including
vancomycin and quinolones \(^1^8, 2^3, 2^4, 4^5, 4^7, 5^0\). Recent reports have identified enterococci
resistant to the latest antimicrobial agents including linezolid, daptomycin, tigecycline
and quinupristin-dalfopristin. This coupled with the bacteriostatic properties of
linezolid and tigecycline is limiting the treatment options for severe infections with
Gram-positive bacteria.\(^1, 1^2, 2^9\).

Enterococci have a propensity for genetic transfer via transposons and plasmids which
has resulted in the dissemination of antibiotic resistance genes \(^2^8, 4^1, 5^4\). In addition, a
recent report has highlighted the gap between the development of new antibiotics and
an increasing number of infections caused by multi-antibiotic resistant organisms \(^1^6\).

Enterococci are intestinal commensals and can withstand high salt concentrations and
pH values found in the bowel and are known to be able to survive for long periods in
the environment. They can also survive on soft surfaces including hospital linens and
plastics \(^3^6\), upholstery, floor and wall coverings\(^2^7\) and can exhibit resistance to some
routinely used cleaning agents including sodium hypochlorite \(^1^0, 2^5\). Any enterococci
not removed by routine cleaning procedures can therefore persist in a viable state and pose a risk of further infection. Hayden et al. discovered that health care workers were almost as likely to contaminate their gloves or hands after touching the environment in a room occupied with VRE colonised patients than from the patient themselves.9 22 Contaminated surfaces are known to contribute to infection spread4 8 45 therefore the use of bactericidal surfaces alongside rigorous disinfection protocols could potentially reduce the incidence of horizontal disease transmission. The biocidal properties of copper have been known for centuries3. More recently, the potential use of copper alloys as microbicidal surfaces has been described. The rapid kill of Escherichia coli O15738 52, Listeria monocytogenes53, MRSA37, Clostridium difficile48 51, Mycobacterium tuberculosis32, Candida albicans and other pathogenic fungi49 and influenza A39 have been observed on copper compared to stainless steel surfaces which are prevalent throughout the healthcare environment. In the USA the Environment Protection Agency (EPA) approval has been granted for the use of alloys containing greater than 65% copper for use as bactericidal surfaces7, and has recently been extended down to greater than 60% copper. It is estimated that biofilm formation on surfaces is a contributing factor in 65% cases of nosocomial infections43. There is no evidence that pathogens can form biofilms on dry copper surfaces37 and the effectiveness of copper alloys as bactericidal agent is not affected by temperatures and humidity, unlike silver33. At present trials of copper surfaces used alongside conventional cleaning regimes in the clinical environment are under way. In the UK a pilot study is investigating the effectiveness of corrosion resistant copper alloys to reduce the microbial burden of ‘constant touch’ bare metal surfaces in a busy hospital ward (tap handles, ward door
push plates, grab rails, door handles, sink traps)\textsuperscript{5,6}. In addition plastic toilets seat were compared with those coated with a copper resin composite (approximately 70% copper) Preliminary results suggest a significant reduction in bacterial contamination on all copper alloy surfaces and investigations are now proceeding to a larger scale trial.\textsuperscript{5,6} Other trials are in progress worldwide including the US, Germany, Chile, Japan and South Africa\textsuperscript{7}.

We have investigated whether various copper-based alloys, compared with stainless steel, may kill pathogenic vancomycin-resistant and sensitive enterococcal isolates. In particular, we have investigated the effect of exposure to copper on enterococcal DNA. This is significant because to effectively prevent the spread of nosocomial enterococcal infections and antibiotic resistance not only do the cells have to be killed but the DNA must be compromised. If the DNA remains intact there may still be the possibility of resistance mutations occurring with the potential transmission of genetic material to other species.

Methods

Bacterial strains:

Vancomycin-resistant control strains, \textit{E. faecalis} ATCC 51299 (VanB phenotype) and \textit{E. faecium} NCTC 12202 (VanA phenotype) were supplied by Oxoid, UK. Clinical isolates of vancomycin-resistant \textit{E. faecalis} (n=2), \textit{E. faecium} (n=5) and \textit{E. gallinarum} (n=1) were obtained from patients (aged 11 d to 70 y) between June 2004 and June 2006 at Southampton General Hospital, Southampton, UK (Table 1). The enterococcal genomes exhibited 54-93% similarity (Dice similarity coefficient) by pulsed-field gel electrophoresis of \textit{Sma} I-digested DNA (data not shown).

Vancomycin-sensitive \textit{E. faecalis} NCTC 775 was supplied by a local water authority
Culture preparation:

Bacteria were maintained on Glycerol Protect beads (Fisher Scientific, United Kingdom) and also in 1 ml aliquots of Vancomycin-resistant Enterococci Broth (VREB) (Oxoid, UK) containing 15% (w/v) glycerol at -80°C. For each experiment, one bead or vial of enterococci was inoculated into 15 ml sterile Brain Heart Infusion Broth (BHIB) (Oxoid, UK) or VREB and incubated aerobically at 37°C for 18±2 hours.

Coupon preparation:

Various copper alloys were tested (Table 2). Before analysis, sheets (1-3 mm thick) of each metal alloy (supplied by Copper Development Association, New York, USA) were cut into coupons (10 mm by 10 mm). Coupons were degreased and cleaned by vortexing in approximately 10 ml acetone containing 20-30 glass beads (2 mm diameter) for 30 seconds and then immersed in absolute ethanol. Prior to use, coupons were flamed in a Bunsen burner and placed, using forceps, in sterile Petri dishes for microbial inoculation.

Inoculation:

For each exposure time, duplicate coupons were analysed using either culture methods or staining methods; 20 µL of bacterial culture was spread evenly over the surface of each coupon, dried in sterile air flow in a Class II microbiological safety cabinet and these were incubated on the bench at room temperature (21±2°C) for various time periods. The use of bacteriological medium as an inoculation matrix was used as a worse case scenario to mimic contamination of hospital surfaces with complex organic material.

Culture analysis:
Coupons were aseptically transferred to 5 ml phosphate buffered saline (PBS) containing 2 mm diameter glass beads and vortexed for 30 seconds. (Preliminary experiments using PBS with 20 mM Ethylenediaminetetraacetic acid (EDTA) to chelate free copper ions gave no significant difference in all viability methods tested). Serial dilutions were prepared and 10 or 100 µL from each dilution was spread over 45 or 90 mm agar plates in triplicate: Slanetz and Bartley Agar (Merck) and Columbia Blood Agar (CBA) (BioMerieux) were used for recovery of the vancomycin-sensitive strains and vancomycin-resistant enterococcal agar (VREA) containing 6 mg/L vancomycin (Oxoid, UK) and CBA for VRE strains (but with no meropenem added as only used pure cultures were investigated). Plates were allowed to dry before inverting and incubating aerobically at 37°C for 24 and 48 hours. Colonies on plates were counted by eye and the concentration per coupon was calculated and recorded as colony forming units (CFU) per coupon (1 cm²). Staining protocols to detect actively respiring bacterial cells and membrane integrity in situ on metal surfaces:

**SYTO9 and CTC (5-cyano-2, 3-ditolyl tetrazolium chloride)**

30 µL of CTC (Sigma-Aldrich, UK) at a final concentration of 5 mM was pipetted onto the surface of inoculated coupons for the final 2 hours of required contact time. Coupons were placed in a humid chamber and incubated in the dark at 37°C for 2 hours. To stain all cells present on coupons SYTO9 (7 µM; Invitrogen, UK) was pipetted onto the coupons and incubated at room temperature, in the dark, for the final 30 minutes.

**BacLight™ (SYTO9 and propidium iodide)**

A staining solution containing 7 µM SYTO9 and 40 µM propidium iodide (L7012; Invitrogen, UK) was prepared in filter sterilised deionised water (2 µL per ml of each
stain); 50 µL of this solution was pipetted onto the coupons and incubated at room
temperature for the final 30 minutes of required contact time in the dark.

After staining with SYTO9/CTC or BacLight™, coupons were tipped to remove stain
and 1 drop of sterile deionised water gently pipetted onto the coupon from a disposable
pastette and tipped off to remove remaining excess stain.

Episcopic differential interference contrast microscopy combined with epifluorescence
(EDIC/EF microscopy) was used to scan the coupons directly (Nikon Eclipse Model
ME600; Best Scientific, Swindon, United Kingdom) 26. A minimum of 10 fields of
view were photographed using a digital camera (Model Coolsnap CF; Roper Industries,
United Kingdom) connected to a computer with digital image analysis software
(Image-Pro Plus, version 4.5.1.22; Media Cybernetics, United Kingdom). Total cells
(SYTO9 stained), respiring cells (CTC stained) and membrane damaged/intact cells
(propidium iodide/SYTO9 stained) were enumerated.

Genomic DNA assay

The protocol is described in detail elsewhere 17 and allows analysis of whole bacterial
genomes. Briefly, approximately 10^7 bacterial cells, untreated, heat/alcohol killed or
exposed to copper or stainless steel surfaces were trapped in low melting point agarose
on a slide previously coated with standard agarose (the bacteria had been pre treated
with lysozyme (4 mg/mL (approximately 30,000U)) for 15 minutes at 37°C.) The cell
membrane was permeabilised with a lysing solution (2% SDS, 0.05M EDTA, 0.1M
DTT, pH 11.5) for 5 minutes at 37°C before drying in ethanol baths and leaving
overnight in a 65°C oven. Dried slides were heated in a microwave oven (4 minutes,
750W) before staining with the sensitive nucleic acid stain SYBR Gold (Invitrogen,
UK), which detects single and double stranded DNA, for 5 minutes at room
temperature in the dark. Epifluorescence microscopy was used to analyse the DNA fragments produced.

**Purification of bacterial DNA and separation of fragments by gel electrophoresis**

Enterococcal DNA (50 kb fragments) were purified using the Qiagen DNeasy Blood and tissue kit (following pre treatment with lysozyme) and preparations separated on a 1, 2 or 3% (w/v) agarose gel containing DNA stain SYBRsafe (Invitrogen) exposed to a current of 300 mA for 90 minutes. Plasmid DNA was extracted using the Qiaprep Spin Miniprep kit (Qiagen) and preparations separated on 0.9% agarose gels. Gels were observed in a UV light box and photographed using GeneScan software.

**Statistical analysis:**

Data are expressed as mean ± standard errors of the mean (SEM). Differences between duplicate samples were assessed using the Mann-Whitney rank t-test. Group comparisons were analysed using the Mann-Whitney U test where statistical significance was expressed as p < 0.05. Statistical analyses were performed using Sigma Stat version 3.5 and graphical representations were performed using Sigma Plot version 10.

**Results**

**Culture analysis**

The survival times of all strains of enterococcal species tested was significantly less on copper and copper alloys containing 60-95% copper than on stainless steel for all inoculum concentrations studied (Figures 1-4).

On stainless steel using the highest inoculum of $10^6$ cfu/cm² of metal surface there was only a 1-log reduction in viable cells after 2 and 6 days for vancomycin-resistant *E.*
faecium and E. faecalis, respectively, and viable cells were still present at 60 days for both species (Figure 3).

In contrast at the same inoculum concentration no viable cells were detected on pure copper at a contact time of 1 hour and on alloys after 1-3 hours for both species tested (Figures 1 and 2).

Although all alloys are very effective at killing control and clinical strains of E. faecium, compared to stainless steel, survival appears to be related to the percentage copper in the alloys (Figure 1). The most rapid kill occurs on pure copper and alloys containing > 90% copper (C51000 and C706000) with increased survival times on the remaining alloys containing 60-70% copper (C28000, C75200 and C26000) (Figure 1). Alloys containing greater than 95% copper (C51000, C70600) are as effective as pure copper for isolates #2 and #3 resulting in cell death at 1 hour but small numbers of cells remained viable in isolates #1 and #4 (86% similarity) up to 2 hours after contact. Viable cells of all isolates were detected following 2 hours contact with alloy C28000 which has the lowest copper content tested here (60%). There are however exceptions: viable cells of clinical isolate #3 were detected following 2 hours contact on alloy C26000 (Cartridge brass, 70% copper) but C75200 (Nickel silver) which contains 5% less copper was a more effective bactericidal surface.

The results are similar for control and clinical strains of E. faecalis (Figure 2) with a rapid kill achieved with all copper alloys compared to stainless steel. However, unlike E. faecium, no viable cells were detected on alloy C28000 at 2 hours contact. It is interesting to note that alloy C75200 is once again a more effective bactericidal surface than alloy C26000 for the E. gallinarum isolate tested (Figure 2:4). This species, although not as prevalent as E. faecium and E. faecalis was reported to be isolated in 2.6% enterococcal bacteremia infections in the UK in 2007.23
rapid kill was observed with the vancomycin-sensitive \textit{E. faecalis} (Figure 2:5) where all cells were killed on pure copper and copper alloys at 30 minutes and 80 minutes respectively (C28000 not tested) but it is difficult to assess the significance unless further sensitive strains are tested and the results may not be clinically relevant.

Survival time is also dependant on inoculum concentration. If the inoculum was increased to $5 \times 10^7$ cfu per cm$^2$ low numbers of viable bacterial cells could be recovered after 2.5 hours contact with alloys C26000 and C70600 but not C75200 after 2.5 hours contact for \textit{E. faecalis} (data not shown). Reducing the inoculum concentration of the \textit{E. faecium} control strain to $10^5$ cfu / cm$^2$ and below minimised the discrepancy between pure copper and the copper alloys as all alloy surfaces were even more effective at killing enterococcal cells (Figure 4). All cells were killed in 60 minutes except for alloy C70600 where a few cell remained viable from an inoculum of $10^5$ cfu / cm$^2$. However contact for 20 minutes on pure copper killed all cells at an inoculum concentration of 1000 cells /cm$^2$.

Analysis of respiring cells (CTC) and membrane integrity (SYTO9/PI) following contact with copper and stainless steel

To determine if non-cultivable cells were indeed dead, and to investigate the potential mechanism of copper’s antibacterial action, copper and steel coupons were inoculated with \textit{E. faecalis} vancomycin-resistant control strain (ATCC51299) and directly stained with the redox dye, CTC, at various time points. Actively respiring cells accumulate the insoluble red fluorescent product, CTC-formazan, which can be visualised \textit{in situ} using epifluorescence microscopy and a long working distance objective lens. Figure 5A demonstrates CTC staining of the cells after 4 hours contact at room temperature.
with copper and steel. Actively respiring bacterial cells can be seen on stainless steel coupons (Figure 5A: 1b CTC) but not on copper (Figure 5A: 1a CTC). The membrane permeable DNA stain SYTO9 was used to determine the total bacterial count but the staining intensity on copper (Figure 5A: 1a SYTO 9) appeared greatly diminished relative to the stainless steel (Figure 5A: 1b SYTO 9). Similar results were obtained for the control strain of *E. faecium* (not shown) Actively respiring cells were also present on alloys C26000 (Figure 5B) and C70600 as well as stainless steel when a higher inoculum of $5 \times 10^7$ cfu/cm$^2$ was used for 2 hours contact time but not on copper, C51000 and C75200 (data not shown, alloy C28000 not tested). At this higher concentration the cells are piled on top of each other and are not necessarily in direct contact with the alloy surface (Figure 5B). This appears not to be a problem for copper and alloys C75200 (65% Cu) and C51000 (95% Cu) but may be important if there is a reduced copper release rate of a particular alloy.

Enumeration of CTC-positive cells on stainless steel gave consistently higher counts than results obtained from culture but the difference was not statistically significant. *BacLight* comprises two nucleic acid stains, SYTO9 and the higher affinity propidium iodide (PI). SYTO9 can permeate the membrane and will stain all cells regardless of viability. If the cell membrane is damaged PI can enter the cell and displace the lower affinity SYTO9. As the cells die the ratio of cells staining green (membranes intact) changes to predominantly red (dead cells with compromised membranes). *E. faecalis* vancomycin-resistant control strain was inoculated onto all metal samples. At the earliest time point of 30 minutes, green staining *in situ*, revealed a large number of viable cells and few red staining with damaged membranes for all samples. After 4 hours contact with stainless steel the number of bright SYTO9 stained cells was reduced while red PI staining had increased, suggesting cells die slowly on the stainless steel.
steel and cytoplasmic membranes are compromised (Figure 5A: 2b). It was not possible to enumerate viable cells \textit{in situ} beyond 30 minutes on copper and copper alloys because preparations were negative for both stains. (Figure 5A: 2a). If the cells were removed from the coupons prior to staining there was still no uptake of PI suggesting membranes were not damaged. A similar finding was demonstrated for MRSA (Weaver et al. (paper submitted)) where contact with copper resulted in respiratory failure but not damage to the bacterial cell membrane.

However at a higher inoculum of $5 \times 10^7$ cfu/cm$^2$ viable cells were detectable not only on stainless steel after 2 hours contact but again also on alloys C26000 and C70600, with significantly higher counts compared to culture for C706000 ($P<0.001$).

Therefore enterococci at inoculum concentration of $\leq 10^6$ cfu/cm$^2$ on copper and copper alloy surfaces for at least 2 hours showed no detectable viable cells by culture and also demonstrated inhibition of respiration but membrane integrity did not appear to be compromised.

\textbf{Analysis of bacterial DNA following exposure to copper and stainless steel surfaces}

\textbf{DNA size separation by agarose gel electrophoresis}

The reduced SYTO9 staining data suggested that exposure to copper could be affecting bacterial DNA. This was investigated further using DNA purified from bacteria exposed to copper surfaces. Genomic DNA was purified using the Qiagen DNeasy Blood and Tissue kit which cuts the bacterial genome into approximately 50 kb fragments before separation by agarose gel electrophoresis. The results of DNA purified from the \textit{E. faecium} vancomycin resistant control strain are shown in Figure 6A and clinical isolates (Figure 6B): \textit{E. faecium} #5 (lanes B7 and B8) and \textit{E. faecalis} #2 (Lanes B9 and 10). DNA purified from cells that have not been exposed to metal
surfaces (live cells, Lane A2) and dead cells (not shown) exhibited fragments that are too large to migrate through the gel (>greater than 10Kb) remaining at the site of loading. The same pattern is seen with DNA purified from cells exposed to stainless steel for 2 hours (Lanes A3, B7 and B9). However the DNA from all strains tested when exposed to copper for 2 hours exhibit a pronounced ‘smearing’ effect characteristic of nucleic acid degradation (Lanes A4, B8 and B10). This suggests the DNA is being broken down but not at specific points because there is no accumulation of particular sized fragments. If cells are exposed to copper for longer, 4 hours, the denaturation has continued and no DNA can be detected at all (Lane A5). This may be because the fragments are too small to be visualised and may have run straight through the gel. Increasing the percentage agarose concentration did not allow further visualisation of the small DNA fragments generated on contact with copper surfaces suggesting extensive denaturation of the DNA has occurred (Figures 6A and 6B show 2% agarose gels). No viable cells were present at either time point on copper surfaces when assessed by all viability methods described previously. This suggests that disintegration of the DNA into very small fragments is continuing after the death of the bacteria. Similar results were obtained for plasmid DNA extracted from the E. faecium control strain exposed to copper and stainless steel (Figure 6C). Lanes 11 and 12 demonstrate plasmid DNA present in untreated cells and those exposed to stainless steel for 2 hours. No plasmid DNA bands are detected in those cells exposed to copper surfaces (lane 13). It must be noted that this is a crude, undigested plasmid preparation and therefore includes supercoiled and linear fractions of the same plasmids. However, the results suggest that both genomic and plasmid DNA are denatured upon exposure to copper surfaces.
Genomic DNA fragmentation assay

To determine if DNA disintegration into very small fragments was due to copper alone and not a preparation artefact, the DNA fragmentation assay was used to gently visualise the entire genome in situ of individual cells (Figure 7). Analysis of the untreated bacterial cells prior to inoculation of coupons revealed a pattern of DNA loops protruding through and close to the lysed cell membrane in live and heat killed cells (Figure 7: 3 and 7:4 respectively), characteristic of an intact genome. Following 2 hours contact with stainless steel the pattern of DNA staining was similar but there were more discreet fragments that have diffused away from the bacterial cell (Figure 7: 2).

The pattern of DNA staining after 2 hours contact with copper surfaces was very different (Figure 7:1). The absence of visible DNA strands suggests extensive damage to the nucleic acid has resulted in fragments that are too small to be visualised and have dispersed throughout the slide.

A similar result was observed for clinical isolates of E. faecalis and E. faecium exposed to copper surfaces for 1 hour at room temperature (Figure 8). Intact DNA was visible following contact with stainless steel (2, 4) but not visible on cells isolated from copper surfaces (1, 3) Preliminary results suggest that DNA damage may begin much earlier (data not shown)

Discussion

Contamination of hospital surfaces with pathogenic micro-organisms contributes to reinfection and spread of disease. Enterococci are not only hardy and able to survive on many types of ‘touch’ surfaces for several weeks but the ability of these organisms to easily transfer antibiotic resistance means it is essential that any environmental contamination with viable cells into the environment from infected individuals is
effectively destroyed. A combination of bactericidal surfaces providing a constant ‘killing surface’ and regular effective disinfection could greatly reduce the spread of disease. Stainless steel is a commonly used hospital surface for many reasons including resistance to corrosion and ability to withstand regular disinfection. However our research has shown that vancomycin-resistant isolates of the two main pathogenic enterococcal species are able to survive for several months on stainless steel surfaces which could potentially contribute to reinfection of personnel, especially vulnerable patients.

In our studies on alloy surfaces containing at least 65% copper, the enterococci are rapidly killed over a few hours contact for a high contamination concentration of $10^6$ cfu/cm$^2$ compared to survival for several months on stainless steel. Pure copper was the most effective surface at killing bacterial cells.

In general alloys containing greater than 90% copper were as effective as pure copper with all isolates of *E. faecalis* and two of *E. faecium* achieving complete cell death by 1 hour (with a few cells remaining viable for another hour in the remaining *E. faecium* isolates but still with a 3-4 log reduction in number). For both species no viable cells were detected after 2 hours contact with alloys containing 60-70% copper. Occasionally alloy C75200 (nickel silver) outperformed alloys with a higher copper content e.g. C26000 (cartridge brass). The other metal constituents and physical properties of each alloy, particularly the rate of release of copper, may have a role in bactericidal activity reported.

However at a bacterial contamination level of $10^5$ cfu/cm$^2$ and below all copper alloys tested were virtually indistinguishable from pure copper and were very effective at killing pathogenic enterococcal cells within 1 hour, and in 20 minutes at less than $10^3$ cfu per square cm. This reinforces the potential for use of these alloys in the clinical
environment because a recent study in the USA enumerated vancomycin-resistant enterococcal contamination on surfaces to be at most a few hundred cells /100 cm² at 3 large hospitals. (M. Schmidt, personal communication 42)

Our experiments were done under ‘worst case scenario’ conditions: inoculation of surfaces with aqueous samples in a nutritious and isotonic medium. It has been reported that the presence of biological fluids or meat juices can delay the copper killing mechanism of \textit{Staphylococcus aureus} and \textit{E. coli} O157 respectively 46, 38.

Recent work in our laboratories has also determined the importance of chelating substances on bacterial survival on copper surfaces (paper in preparation). Experiments with a rapidly drying swabbed inoculum in PBS or water suggest killing times are even more rapid (data not shown) but these conditions may be not entirely relevant to actual contamination with organic specimens in a clinical environment.

Assessment of the number of viable cells recovered from metal surfaces by respiratory staining was not significantly different from that obtained by culture, providing more evidence that enterococci survive for long periods on stainless steel but also suggesting the absence of a viable-but-nonculturable (VNC) state on copper surfaces under these conditions.

Assessment of the viability of cells recovered from stainless steel using \textit{BacLight} SYTO9/PI staining also demonstrated no significant difference to results obtained by culture but suggested some damage to the cell membrane does occur on this surface. However, staining with \textit{BacLight} does not always produce distinct ‘live’ and ‘dead’ populations, particularly because membrane intact cells are not necessarily alive 2. The \textit{BacLight} staining method was difficult to interpret for enterococci exposed to copper alloys because of the diminished and frequently absent staining with SYTO9 and PI, respectively, except at time of inoculation. This suggested that the bacterial DNA has
been affected to the extent that intercalating DNA stains cannot now bind. There does
not appear to be any uptake of PI by enterococci exposed to copper alloys suggesting
damage to cell membrane is not occurring, but these results may be misleading if the
DNA is too damaged to bind PI.

Targets of copper toxicity are thought to include nucleic acid, structural and
functional proteins, lipids, inhibition of metabolic processes such as respiration and
osmotic stress resulting in cell lysis. In mammalian cells soluble copper (II) ions are
known to bind to DNA bases resulting in unwinding of the double helix, and in
aerobic conditions the Fenton reaction with bound and free ions and hydrogen
peroxide results in the production of reactive oxygen species (ROS) that cause double
and single strand breaks and intrastrand cross linking. Macomber et al. reported
that exposure of E. coli mutants lacking in copper export systems to copper solutions
resulted in copper overloaded cells and no detectable oxidative damage to the DNA
using a PCR gene specific assay. The reasons suggested were compartmentalisation of
hydroxyl radicals generated in the periplasm of the cell. The effect of hydroxyl radical
damage is short reaching and therefore damage to the DNA could not occur if
spatially separated. They also described the existence of ligands, perhaps glutathione,
complexing with copper ions and suggested that copper toxicity may primarily be due
to damage of metalloenzymes by the ROS: dihydrogen peroxide, hydroxyl radicals
and superoxides.

Exposure to relatively low soluble copper concentrations described by Macomber et
al. is very different to continual contact with copper and copper alloy surfaces. In
our system we have reported extensive disintegration of the genomic and plasmid
DNA of Gram-positive enterococci exposed to copper and copper alloy surfaces
because the DNA from cells exposed to copper appears to be (i) denatured over time
in agarose gel electrophoresis, (ii) does not bind intercalating stains SYTO9 and PI, and (iii) genomic DNA cannot be detected in the DNA fragmentation assay. These effects were not observed on stainless steel. The DNA fragmentation assay is a useful tool because it allows observation of the entire genome of individual cells without a purification step that could result in damage to DNA from stressed bacterial cells and lead to spurious results. This technology has been successfully used for the analysis of eukaryotic nucleic acid, for example in the analysis of sperm DNA and efficacy of specific anticancer agents on patient DNA. Fernandez et al. successfully adapted the method to investigate damage to bacterial DNA following treatment with quinolone antibiotics. We have also shown how exposure to copper resulted in the inhibition of respiration with minimal damage to the integrity of the bacterial cell membrane. We suggest that the absence of an outer membrane in Gram-positive cells and lack of a periplasmic space may facilitate copper (I)/(II) and generated ROS to access the DNA directly and rapidly inflict damage. The ligands described by Macomber et al. responsible for removing copper (II) in copper overloaded cells may still be present but the effect is insignificant when bacteria are constantly in contact with the copper surface and binding sites saturated with copper ions. Espirito Santo et al. determined that ROS are generated when *E. coli* is exposed to copper surfaces. They identified hydroxyl radicals generated in aerobic conditions, presumably by Haber-Weiss and Fenton reaction of reduced copper ions (supplied by redox cycling of copper (I) and (II)). Preliminary experiments in our laboratory with *E. coli* O157 exposed to copper surfaces using the DNA fragmentation assay have indicated that genomic DNA is also destroyed in this species but more slowly (manuscript in preparation). The DNA...
stability reported by Macomber et al. is probably due to exposure to soluble Cu (II) rather than the Cu (I)/Cu (II) redox cycling proposed in our studies. This DNA degradation effect on E. coli and other species will be investigated in future studies. Concerns have been expressed about the possibility of the development of copper resistance if alloy surfaces are constantly in use. Mutations in bacterial copper homeostasis mechanisms does affect survival times on copper alloys but because survival times on stainless steel and other commonly in-use surfaces are so much greater the significance may not be relevant in a real life situation. Further studies are required to elucidate the mechanism of copper killing and investigate this possibility. There has been much concern recently that the frequency of antimicrobial resistance in bacteria has increased in concert with increasing usage of antimicrobial compounds. A recent European Commission report has summarised the scientific evidence from bacteriological, biochemical and genetic data indicating that the use of active molecules in biocidal products may contribute to the increased occurrence of antibiotic resistant bacteria. The selective stress exerted by biocides may favour bacteria expressing resistance mechanisms and their dissemination. Some biocides have the capacity to maintain the presence of mobile genetic elements that carry genes involved in cross-resistance between biocides and antibiotics. In enterococci up to 25% of the genome has been found to contain mobile elements. The dissemination of these mobile elements, their genetic organisation and the formation of biofilms, provide conditions that could create a potential risk of development of cross-resistance between antibiotics and biocides. The case for the use of copper in antimicrobial products was considered but there was no evidence that this might lead to antibiotic resistance in the way that the widespread use of Triclosan has been associated with the emergence of
triclosan and mupirocin resistance in MRSA, although evidence for this is limited \textsuperscript{14, 15}. Plasmid-localised copper resistance \textit{tcrB} gene has been identified in \textit{E. faecium} and \textit{E. faecalis} thought to originate from pigs fed with copper sulphate supplemented food \textsuperscript{19}. The Tn\textit{1546} element and \textit{erm} genes conferring glycopeptide and macrolide resistance are located on the same plasmid but there is no significant evidence that use of copper in animal feeds co-selected for antibiotic resistance \textsuperscript{20} except under experimental conditions in piglets fed a high concentration of copper sulphate \textsuperscript{21}. However continued use of copper sulphate was not able to maintain high levels of antimicrobial resistance \textsuperscript{21}. The current study indicates that DNA is rapidly destroyed in enterococci exposed to copper surfaces, meaning that there is little chance of high level copper or antibiotic resistance developing. Consequently, this disintegration of the bacterial nucleic acid supports the use of copper alloys as contact surfaces in clinical environments to actively kill bacterial cells without the occurrence of DNA mutation and transfer of genetic material carrying antibiotic resistant genes.

Acknowledgements
References


and the Infectious Diseases society of America (IDSA) 46th Annual Meeting 2008.


22. **Hayden, M. K., Blom, D. W., Lyle, E. A., Moore, C. G. and R. A. Weinstein.** 2008 Risk of hand or glove contamination after contact with patients colonised with vancomycin resistant enterococcus or the colonised patients environment. Infect Control Hosp Epidemiol **29**: 149-154.


**Figure Legends**

**FIGURE 1** Survival of vancomycin-resistant *E. faecium* NCTC 12202 (1), clinical isolates #1(2), #2 (3), #3(4), #4(5) and #5 (6) on stainless steel, pure copper and copper alloys (S30400 (●), C28000 (○), C75200 (▼), C26000 (Δ), C70600 (■), C51000 (□) and copper C11000 (♦)) at 22ºC.

**FIGURE 2** Survival of vancomycin-resistant *E. faecalis* ATCC 51299 (1), clinical isolates *E. faecalis* #1(2), #2 (3), *E. gallinarum* (4) and vancomycin-sensitive *E. faecalis* NCTC775 (5) on stainless steel, pure copper and copper alloys (S30400 (●), C28000 (○), C75200 (▼), C26000 (Δ), C70600 (■), C51000 (□) and copper C11000 (♦)) at 22ºC.

**FIGURE 3** Survival of vancomycin-resistant *E. faecalis* (ATCC 51299) (●) and *E. faecium* (NCTC 12202) (○) on stainless steel at 22ºC.

**FIGURE 4** Effect of inoculum concentration on survival of vancomycin-resistant *E. faecium* (NCTC 12202) on stainless steel, pure copper and copper alloys (S30400 (●), C75200 (○), C26000 (▼), C70600 (Δ), C51000 (■), or C11000 (□)) at 22ºC. Inoculum concentration 10⁵ cfu / cm² (1); 10⁴ cfu / cm² (2) and 10³ cfu / cm² (3).

**FIGURE 5A** Assessment of viability of *E. faecalis* (ATCC 51299) on copper (a) and stainless steel (b) surfaces using the redox dye CTC (positive for respiring cells) and SYTO 9 (total cell count regardless of viability)(1) or BacLight (2) to detect bacterial membrane integrity.
FIGURE 5B The effect of inoculum concentration and cell stacking on the susceptibility of alloy C26000 to inhibit respiration. The alloy was inoculated with *E. faecium* NCTC 12202 VRE strain at inoculum concentrations of $5 \times 10^7$ cfu/cm$^2$ (1, 3, 5) and $5 \times 10^6$ cfu/cm$^2$ (2, 4, 6) for 2 hours. Images 1 and 2 were captured using EDIC microscopy. The ringed areas in image 1 demonstrate areas where at the higher inoculum bacterial cells may not be in direct contact with metal surface as they are stacked on top of each other. At the lower inocula, (image 2), the cells are spread in small clumps and are all exposed to the alloy directly (this spread of individual cells is also clearly seen in epifluorescence image 4). Epifluorescence images 3 and 4 represent SYTO 9 total cell staining. CTC positive staining of respiring cells are present at higher inoculum (5) but not at the lower (6) Bar represents 10 microns.
FIGURE 6  Agarose gel electrophoresis of purified enterococcal DNA

A: purified genomic DNA of *E. faecium* NCTC 12202. Lane 2: cells not exposed to metal surfaces; Lane 3: cells exposed to stainless steel for 2 hours; Lane 4: cells exposed to copper for 2 hours; Lane 5: cells exposed to copper for 4 hours.

B: purified genomic DNA of clinical isolates *E. faecium* #5 (lanes 7 and 8) and *E. faecalis* #2 (lanes 9 and 10) exposed to stainless steel (lanes 7 and 9) or copper (lanes 8 and 10) for 2 hours at 22°C.

C: purified plasmid DNA of *E. faecium* NCTC 12202 (Lane 11) or exposed to stainless steel (Lane 12) or copper (Lane 13) for 2 hours at 22°C.

Control lanes are Bioline Hyperladder I (Lane 1) and Hyperladder II (Lane 6).

Genomic DNA was purified using Qiagen DNeasy Blood and Tissue kit (agarose 2 %) and Qiaprep Spin Miniprep Kit for plasmid DNA (agarose 0.9%).

FIGURE 7  Analysis of genomic DNA of *E. faecalis* ATCC 51299 in situ with DNA fragmentation assay following 2 hours exposure to copper (1) and steel (2). Images 3 and 4 are bacterial cells that have not been exposed to metal surfaces and are live and dead (heat killed) cells, respectively, which were then used in the DNA fragmentation assay. Loops of DNA are visible on all samples except 1 suggesting exposure to copper has resulted in disintegration of the bacterial DNA into fragments too small to be visualised even by sensitive nucleic acid stain SYBR Gold used for this assay.

FIGURE 8  Analysis of genomic DNA of clinical isolates of *E. faecalis* #2 (1, 2) and *E. faecium* #5 (3, 4) in situ with DNA fragmentation assay following a 1 hour exposure to copper (1, 3) and steel (2, 4). No visible DNA loops on cells that have
been exposed to copper whereas DNA fragments are visible emanating from cells isolated from stainless steel surfaces.

### TABLE 1. Characteristics of Enterococcus clinical isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Source</th>
<th>Sample Type</th>
<th>Antimicrobial Resistance&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>E. faecalis 1</td>
<td>Surgical ward</td>
<td>Wound swab</td>
<td>VAN, ERY, CHL, TET</td>
</tr>
<tr>
<td>E. faecalis 2</td>
<td>Surgical ward</td>
<td>Faeces</td>
<td>VAN, ERY, CHL, TET</td>
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<td>Ascitic fluid</td>
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<td>E. gallinarum</td>
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</table>

<sup>a</sup>Antimicrobial abbreviations: VAN, vancomycin; PEN, penicillin; ERY, erythromycin; CHL, chloramphenicol, TET, tetracycline; AMP, ampicillin.
TABLE 2. Composition of alloys tested

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\(^a\) Unified Numbering System