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ORIGINAL ARTICLE

Potential action of copper surfaces on meticillin-resistant *Staphylococcus aureus*

L. Weaver[1], J.O. Noyce[1], H.T. Michels[2] and C.W. Keevil[1]

1 Environmental Healthcare Unit, School of Biological Sciences, University of Southampton, Southampton, UK
2 Copper Development Association Inc, New York, NY, USA

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Correspondence
Louise Weaver, presently at Institute of Environmental Science and Research Ltd, Christchurch Science Centre, 27 Creyke Road, Ilam, Christchurch 8041, New Zealand.
E-mail: louise.weaver@esr.cri.govt.nz

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Abstract

Aims: Studies to date have shown rapid killing of bacterial cells when exposed to copper surfaces. The mechanistic action of copper on bacterial cells is so far unknown.

Methods and Results: To investigate potential mechanisms involved, meticillin-resistant *Staphylococcus aureus* (MRSA) cells (10^7 CFU) were inoculated onto coupons of copper or stainless steel and stained with either the viability fluorophore 5-cyano-2,3-ditolyl tetrazolium (CTC), to detect respiration, or BacLight™ (SYTO9/propidium iodide), to determine cell wall integrity. Coupons were then observed *in-situ* using epifluorescence microscopy. In addition, DNA from cells inoculated onto either copper or stainless steel surfaces was isolated and analysed by agarose gel electrophoresis. An effect on cellular respiration with CTC reduction was evident but no effect on cell membrane integrity (BacLight™) was observed. Results from the DNA isolation indicated a copper-induced detrimental effect on MRSA genomic material as no bands were observed after exposure to copper surface.

Conclusions: The results indicate that exposure to copper surfaces rapidly kills MRSA by compromising cellular respiration and damaging DNA, with little effect on cell membrane integrity.

Significance and Impact of the study: This research provides a mechanistic explanation in support of previous suggestions that although copper surfaces do not affect membrane integrity of cells, there is still a rapid antimicrobial effect.

Introduction

Meticillin-resistant *Staphylococcus aureus* (MRSA) is a serious health care pathogen that causes extensive morbidity and mortality (Karas *et al.* 2006). The most notable method of MRSA transmission between patients is in the hands of health care workers (Jarvis 1994). Consequently, hand hygiene either by washing or disinfection remains the single most effective strategy for preventing cross-contamination (Larson 1995). A recent study suggested the introduction of copper fittings, and work surfaces could potentially act as an additional barrier to prevent cross-contamination, because of its ability to rapidly kill MRSA (Noyce *et al.* 2006). A hospital trial using copper fittings and touch surfaces demonstrated median values of micro-organisms 90–100% lower on copper surfaces compared to conventional materials (Casey *et al.* 2010). Copper represents two ends of the viability spectrum for micro-organisms, being both an essential nutrient at one end but also highly toxic at the other (Faundez *et al.* 2004).

As a nutrient, copper is required for many biochemical and physiological functions, acting as a cofactor for several enzymes (Pena *et al.* 1999). The cellular uptake and intracellular distribution of this important nutrient is a precisely orchestrated process. Copper homeostasis is coordinated by several proteins to ensure that it is delivered to specific subcellular compartments and copper-requiring proteins without releasing free copper ions that

will cause damage to cellular components (Harris and Gitlin 1996; Gutteridge and Halliwell 2000). If, however, copper homeostasis is breached, copper has the ability to catalyse free-radical formation, leading to alteration of nucleic acids, lipids and proteins (Borkow and Gabbay 2004).

The precise mechanism of copper toxicity is postulated to occur through one or more of several possible mechanisms including: the displacement of essential metals from their native binding sites, interference with oxidative phosphorylation and osmotic balance, and alterations in the conformational structure of nucleic acids, membranes and proteins (Faundez *et al.* 2004). Numerous studies have confirmed the antimicrobial properties of copper and copper alloys, including brass and bronze against other pathogenic bacteria, including *Escherichia coli* O157, *Salmonella enterica*, MRSA, *Campylobacter jejuni* and *Clostridium difficile* (Bartosch *et al.* 2003; Faundez *et al.* 2004; Wilks *et al.* 2005; Noyce *et al.* 2006; Casey *et al.* 2008; Mehtar *et al.* 2008; Weaver *et al.* 2008). The United States Environmental Protection Agency (USEPA) has granted copper surfaces antimicrobial claim based on stringent laboratory testing including repeated soiling of surfaces (Michels and Anderson 2008). Recent studies have reported MRSA was not killed on copper surfaces after repeated cleaning (Airey and Verran 2007). The research findings were based on the uptake of propidium iodide (BacLight^{TM}) within the cell because of the loss of cell membrane integrity. There have been questions raised on reliance on one method of viability assessment, and previous research has indicated the action of copper on cells is not involved in the loss of cell membrane integrity.

To elucidate a potential method of the action of copper ions on or in cell's survival of MRSA on copper alloys and stainless steel using viability fluorophores designed to detect cellular respiration, 5-cyano-2,3-ditolyl tetrazolium (CTC) (Boulos *et al.* 1999), cell wall integrity BacLight^{TM} (Noyce *et al.* 2006), culture analysis and DNA degradation were tested.

Materials and methods

Preparation of MRSA

MRSA (NCTC 10442) was supplied by TCS Biosciences, (Code MM37; Buckingham, UK) and maintained on glycerol protect beads (Fisher Scientific, Loughborough, UK) at −80°C. For experiments, 15 ml of tryptone soy broth was aseptically inoculated with a bead and incubated at 37°C for 16 h. After this incubation period, the culture contained $\sim 5 \times 10^8$ CFU ml^{-1}. Unless otherwise stated, media were obtained from Oxoid (Basingstoke, Hampshire, UK).

Preparation of copper and stainless steel coupons

Sample sheets (0·5-mm thickness) of copper (unified numbering system, C11000) and stainless steel (grade 304000) were prepared as previously described (Noyce *et al.* 2006). Briefly, each sheet was sectioned into coupons (1 × 1 cm) and, prior to testing, degreased and cleaned by vortexing for 30 s in 10 ml of acetone with 2- mm-diameter glass beads (Merck, Nottingham, UK).

Inoculation of coupons

Coupons of stainless steel and copper were inoculated with 20 μl of the 16-h culture and air-dried rapidly, in a flow of air (Class II cabinet), as described previously (Noyce *et al.* 2006).

Epifluorescence microscopy analysis

To elucidate the condition of cells inoculated onto copper and stainless steel samples, images were taken using epifluorescence microscopy. For the epifluorescence analysis, MRSA cells on inoculated coupons were stained with either CTC (Polysciences Inc., Warrington, PA, USA) or BacLight^{TM} (SYTO9 and propidium iodide; Molecular Probes, Invitrogen, Paisley, UK). Coupons were sterilized and inoculated with 20 μl of MRSA culture. After 1 and 1·5-h exposure at room temperature, coupons were transferred to Petri dishes, and 50 μl of either 5 mmol l^{-1} CTC or a mixture of 10 μmol l^{-1} SYTO9 and 60 μmol l^{-1} propidium iodide were added to the coupon surface. For CTC, coupons were incubated for 2 h at 37°C in the dark. For coupons stained with BacLight^{TM}, incubation occurred in the dark at room temperature for 30 min. Postincubation, the coupons were thoroughly examined using an epifluorescence microscope (Nikon Eclipse Model ME600; Best Scientific, Swindon, UK) equipped with a 40× and 100× (long phase) objective with epifluorescence filters appropriate for CTC, SYTO9 and propidium iodide. For each coupon tested, representative images were taken using a digital camera (Model CoolSnap CF; Roper Industries, Bury St Edmunds, UK) connected to a PC with digital image analysis software (Image-Pro Plus, ver. 4.5.1.22; Media Cybernetics, Bethesda, MD, USA).

Culture analysis

To determine the number of viable organisms recovered from the coupons, 100 μl of the culture was pipetted and serially diluted to 10^{-4} in sterile phosphate-buffered saline (PBS). Nutrient agar plates were then inoculated with 50 μl of each dilution, which was spread evenly over the surface of the agar with a sterile, L-shaped spreader.

Plates were incubated at 37°C for 18 h. The number of colonies (CFU) was counted, and the number of viable CFU per coupon was calculated. Triplicate plates were completed for each dilution, and the mean was calculated. Experiments were repeated in triplicate. Control coupons for stainless steel and copper were removed immediately after inoculation at time zero to determine the initial number of viable bacteria.

Genomic DNA analysis

Sterile coupons (×5) of either copper or stainless steel were inoculated with 100 μl of MRSA culture ($\sim 10^8$ CFU). After 1-h exposure at 22°C, coupons were transferred to a sterile disposable tube containing 10-ml sterile PBS (containing 20 mmol l^{-1} EDTA to complex free copper) with 2-mm glass beads and vortexed for 30 s. When all five coupons had been cleaned, the cells were pelleted by centrifugation (6000 ***g***, 3 min at 22°C).

DNA from the pelleted cells was isolated and purified using the GenElute™ Bacterial Genomic DNA Miniprep Kit (Sigma, Dorset, UK), following the instructions for Gram-positive bacteria. A series of three centrifugation and washing steps was followed prior to DNA isolation with 50 μl of supplied elution solution. The purified DNA was stored at 4°C until required. The size and quality of the DNA was determined by agarose gel electrophoresis.

Samples (7 μl), Lambda/Hind III DNA ladder (Sigma) and 100- kb ladder (Invitrogen) were pipetted into the wells of a 1% agarose gel (containing 0·5 μg ml^{-1} ethidium bromide) using 3 μl of loading buffer (Sigma). Samples were run in TBE (40 mmol l^{-1} Tris, 20 mmol l^{-1} boric acid, 1 mmol l^{-1} EDTA, pH 8·0) using a Fisher Scientific Power 300 horizontal gel electrophoresis unit at 100 V for 60 min. Gels were then analysed under ultraviolet illumination and images recorded.

Results

Epifluorescence microscopy and digital image analysis

Epifluorescence images taken of coupons stained with CTC showed no actively respiring cells on the pure copper coupons after 1 h at room temperature (Fig. 1). In contrast, images of cells exposed to stainless steel after 6-h incubation at 22°C clearly show the presence of respiring cells as shown by the numerous points of red emission within the image, because of the intracellular reduction in CTC to the water-insoluble fluorescent product 3-cyan-1,5-di-tolyl-formazan. In addition, images of MRSA cells on pure copper and stainless steel stained with SYTO9 and propidium iodide (Fig. 2) showed that

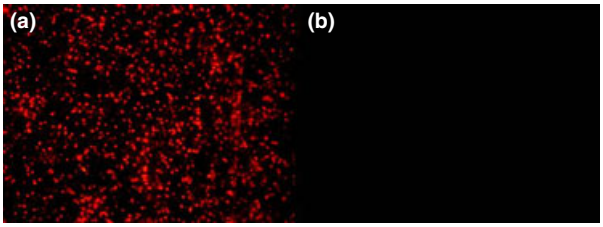

Figure 1 Epifluorescent images stainless steel (a) and copper (b) inoculated with meticillin-resistant *Staphylococcus aureus* and stained with 5-cyano-2,3-ditolyl tetrazolium. Red points represent actively respiring cells. Magnification ×400.

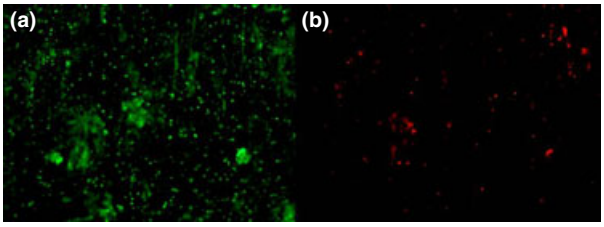

Figure 2 Epifluorescent images of copper inoculated with meticillin-resistant *Staphylococcus aureus* and stained with Baclight™, Syto 9 (a) and propidium iodide (b). Green points (a) represent cells with integral membranes and red points (b) represent cells with disrupted membranes. Magnification ×400.

the vast majority of cells were green fluorescent, indicative of having intact membranes, with only a small minority displaying compromised cell walls as demonstrated by the uptake of propidium iodide and subsequent red fluorescent emission.

Culture analysis

After exposure to copper for 1 h, no cells were able to be cultured on nutrient agar plates. This supported the observation that copper had interrupted the respiratory pathway, leading to death in the cell. In contrast, no significant difference was seen in the number of CFU per coupon before or after 6-h incubation on stainless steel (data not shown).

DNA analysis

To further confirm that cells were killed on copper (and not in a viable but not culturable state), analysis of the cellular genomic DNA recovered was visualized in electrophoresis gels. Isolated genomic DNA should migrate as a single, high-molecular weight band, typically 50 kb in size when isolated using the Miniprep kit. Clear bands can be seen in lanes 7 and 14 representing DNA recovered from MRSA cells in culture (Fig. 3). Bands are seen in lanes 5,

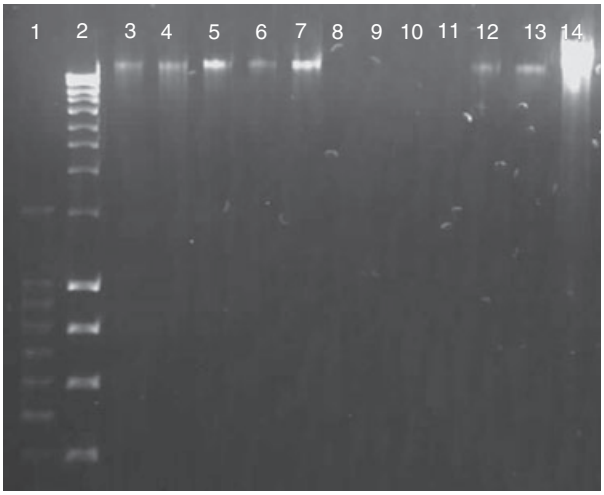

Figure 3 Gel electrophoresis analysis of DNA isolated from meticillin-resistant *Staphylococcus aureus* (MRSA) on copper and stainless steel coupons. Lanes: (1) Lambda/HIND III DNA ladder, (2) 100- bp ladder, (3) MRSA after 1-h exposure on copper, (4) MRSA after 1-h exposure on copper, (5) MRSA after 1-h exposure on stainless steel, (6) MRSA after 1-h exposure on stainless steel, (7) MRSA culture, (8) negative control, (9) blank well, (10) MRSA after 1.5-h exposure on copper, (11) MRSA after 1.5-h exposure on copper, (12) MRSA after 1.5-h exposure on stainless steel, (13) MRSA after 1.5-h exposure on stainless steel, (14) MRSA culture.

6, 12 and 13, corresponding to DNA recovered from MRSA after exposure to stainless steel for 1 h (lanes 5 and 6) and 1·5 h (lanes 12 and 13). In contrast, no bands are present in lanes 10 and 11 that represent cells inoculated onto and recovered from copper after 1·5-h exposure. Only faint bands are present after 1-h exposure to copper (lanes 3 and 4) indicating possible damage to DNA. The DNA ladders (lanes 1 and 2) indicate that the genomic DNA has been degraded or broken into small molecular weight fragments that cannot be detected in this molecular weight range.

Discussion

Copper ions are essential for bacteria, but they can cause a number of toxic cellular effects if levels of free ions are not controlled. Exposure to copper surfaces has been shown to be detrimental to MRSA viability with a log 7 kill in 45 min at room temperature (Noyce *et al.* 2006; Mehtar *et al.* 2008). The rapid inactivation of microorganisms exposed to copper has to date been attributed to either one or a combination of the following mechanisms; loss of structural integrity of the cell membrane, alteration to the conformational structure of proteins or degradation of the cellular DNA and or RNA (Stohs and Bagchi 1995; Faundez *et al.* 2004).

The initial site of copper toxicity for MRSA is suggested to be the cell membrane with copper acting as a catalyst in the formation of reactive oxygen species which in turn catalyses peroxidation of membrane lipids (Ohsumi *et al.* 1988). In addition, research in yeast cells suggests that copper ions caused a selective change in the permeability barrier of the membrane, which induced a significant leakage of ions and low molecular weight substances from the cell (Marshall and Wilmoth 1981). However, our findings suggest no direct effect on the structure of the cell membrane of the Gram-positive MRSA with epifluorescence images showing only small amounts of uptake of the fluorophore propidium iodide, which because of its molecular weight can only cross disrupted membranes to intercalate into the DNA double helix. Studies of the yellow pigment in *Staph. aureus* have unravelled an elaborate biosynthetic pathway that produces a series of carotenoids (Krinsky 1993). Similar carotenoids produced in dietary fruits and vegetables are well recognized as potent antioxidants by virtue of their free-radical scavenging properties and exceptional ability to quench singlet oxygen (Airey and Verran 2007). In light of this, studies have confirmed decreased resistance in mutants with carotenoid manufacturing pathways removed compared to their wild-type counterparts (Kim *et al.* 2000). Thus, if copper-induced disruption of the cell membrane is believed to occur through the generation of reactive oxygen species, then MRSA would appear to have defensive strategies in place to counteract this, and our findings would corroborate this. Research has indicated that MRSA was not killed by copper because of findings that, using the SYTO9/propidium iodide BacLight^{TM} cell membrane integrity assay system, viable cells were visualized on the surface of copper after a repeated soil and clean cycle (Airey and Verran 2007). Our research has found that copper has little effect on the membrane integrity but renders the cells inactive by other mechanisms possibly disruption to the respiratory pathway and/or damage to genomic DNA. Hence, in the previous experiments, the cells may have appeared viable using Baclight^{TM} as they observed no damage to cell membrane integrity but may simply have shown no damage to the cell membrane.

Proteins might also be targeted by copper ions, resulting in altered structure and reduced biological activity (Mildažien *et al.* 2001). Consequently, the numerous enzymes and protein complexes involved in respiration could be possible targets; this could fit with our results clearly showing no actively respiring MRSA cells when *in situ* on copper surfaces. Another study with *E. coli* O157 on cast copper alloys has shown similar results with no respiring cells found on a 95% copper alloy after 75 min at room temperature (Trumpower 1990). An investigation

into the deleterious actions of metal ions on oxidative phosphorylation in rat liver mitochondria identified the target of respiratory toxicity by copper as cytochrome bc1 (E. Berry, Personal communication). This highly important electron transfer complex is found in the plasma membranes of phylogenetically diverse photosynthetic and respiring bacteria, in addition to the inner mitochondrial membrane of all eukaryotic cells (Rifkind *et al.* 1976). However, it is yet to be determined if *Staph. aureus* possesses the bc1 complex, although closely related Gram-positive *Bacillus* species do (Sagripanti *et al.* 1991). Regardless, an association between copper toxicity and respiration inactivation is apparent.

Evidence to date has demonstrated that copper ions have the ability to disorder DNA by binding to and cross-linking between and within strands. Studies have additionally shown that at least two binding sites exist for copper within the DNA double helix (Sagripanti *et al.* 1991). The results from this study would support a copper-activated deleterious effect on genomic DNA. However, although the degradation of the genomic DNA has been confirmed in MRSA, it is unclear at this stage if this is an effect of the cell dying from respiratory failure or whether it is in fact because of some type of copper–DNA interaction. It may well be a combination of the two, and further experimentation is required. The ability of copper and copper alloys to kill a range of micro-organisms on contact is undeniable, although to date it was unclear why MRSA specifically dies. From the simple application of epifluorescence microscopy with viability fluorophores, coupled with cellular DNA isolation, this initial study can conclude that MRSA inactivation occurs because of an effect on respiration and a loss of DNA integrity, with no effect on cell membrane integrity. This work supports previous studies and studies at a UK hospital (Casey *et al.* 2008, 2010), suggesting that the increased application of copper alloys in the critical care environment as antimicrobial surfaces, should provide an important component in the hygiene armoury of preventative measures used to minimize the risk of hospital acquired infection.

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