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Antimicrobial Properties of Copper in Gram-Negative and Gram-Positive Bacteria

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***Abstract*—**For centuries humans have used the antimicrobial properties of copper to their advantage. Yet, after all these years the underlying mechanisms of copper mediated cell death in various microbes remain unclear. We had explored the hypothesis that copper mediated increased levels of lipid peroxidation in the membrane fatty acids is responsible for increased killing in *Escherichia coli*. In this study we show that in both gram positive (*Staphylococcus aureus*) and gram negative (*Pseudomonas aeruginosa*) bacteria there is a strong correlation between copper mediated cell death and increased levels of lipid peroxidation. Interestingly, the non-spore forming gram positive bacteria as well as gram negative bacteria show similar patterns of cell death, increased levels of lipid peroxidation, as well as genomic DNA degradation, however there is some difference in loss in membrane integrity upon exposure to copper alloy surface.

***Keywords*—**Antimicrobial, copper, gram positive, gram negative.

I. Introduction

COPPER is a trace element required for several essential biological processes that exhibit remarkable structural and functional conservation from bacteria to human. The fact that copper exists in two oxidation states, cuprous Cu(I) (reduced) and cupric Cu(II) (oxidized), contributes to its ability to serve as a catalytic co-factor in diverse biological systems. Studies of copper acquisition, distribution, and homeostasis have shown that, while at appropriate concentrations copper is an essential micronutrient, it can however be toxic at higher concentrations [1]-[3]. In excess, copper affects the biochemistry of macromolecules and reportedly leads to a rapid decline in membrane integrity [4], [5]. Thus, it is no surprise that species have evolved tightly regulated mechanisms for copper homeostasis.

Reference [6] opened a new realm of possibilities for copper and copper alloy surfaces, that of passive antimicrobial sanitizing agents. Studies from several laboratories clearly demonstrate efficient and rapid killing of bacteria, fungi, and viruses upon exposure to surfaces composed of copper or copper-containing alloys but not stainless steel [7]-[9]. These findings were confirmed by standardized testing in an approved "Good Laboratory Practices" facility and the US Environmental Protection Agency registered over 350 copper alloys as having antimicrobial activity against 6 different bacteria. In view of the potential importance of copper alloy surfaces in the battle against hospital-acquired infections, it is essential to understand the mechanism of contact-mediated killing by copper.

Studies of the mechanism of contact killing in *Escherichia coli* implicate the membrane as the target yet the specific component and underlying biochemistry remains unknown. Funds from previous PSC-CUNY and Copper Development Association grants to PI Gadura, enabled us to publish [7] where our data shows that membrane lipid peroxidation in involved in copper alloy mediated contact killing of *Escherichia coli.* Briefly, we explored the hypothesis that non-enzymatic peroxidation of membrane phospholipids is responsible for copper alloy surface killing. Lipid peroxidation was monitored with the TBARS assay. Survival, TBARS levels, and DNA degradation were followed in cells exposed to copper alloy surfaces containing 60 to 99.90% copper or in media containing $CuSO_4$. In all cases, TBARS levels increased with copper exposure levels. Cells exposed to the highest copper content alloys, 100% and 80% Cu, exhibited novel characteristics. TBARS increased immediately at a very rapid rate but peaked at about 30 minutes. This peak was associated with the period of most rapid killing, loss in membrane integrity, and DNA degradation. DNA degradation is not the primary cause of copper surface killing. Cells exposed to the Steel surface (control) for 60 minutes had fully intact genomic DNA but no viable cells.

In a fabR mutant strain (published results in [7]) with increased levels of unsaturated fatty acids, sensitivity to copper alloy surface mediated killing increased, TBARS levels peaked earlier, and genomic DNA degradation occurred sooner than in the isogenic parental strain. Taken together, our results suggested that the mechanism of copper alloy surface mediated killing of *E. coli* is triggered by non-enzymatic oxidative damage of membrane phospholipids that results in loss of membrane integrity and cell death.

Our published results indicated a strong correlation between copper surface exposure and increased lipid peroxidation levels leading to faster cell death. Given the differences between the cell wall and plasma membrane of gram positive and gram-negative bacteria we decided to study this further. We decided to take a closer look at bacterial strains that include more clinically relevant gram-positive bacteria, *Staphylococcus aureus*, frequently found in human respiratory tract and on skin. We also studied a gram-negative bacteria frequently found in man-made environment and is less susceptible to antibiotics, *Pseudomonas aeruginosa,* to help us understand how differences in cell membrane might be the underlying cause in rapid cell death on copper surfaces.

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Results from *Bacillus subtilis* (a gram positive, spore producing bacteria) have shown differences in the rate of cell death as well as lipid peroxidation rates compared to that of our previously published *E. coli* results *(Manuscript in preparation).* The PI serves as a research mentor to several QCC students from NSF-STEM, US DOE MSEIP programs. Three Queensborough students were engaged to do these experiments. Each student was responsible for one of the three strains and results were compared to our published results from *E. coli* [7].

II. Material and Methods

A. Strains and Growth Conditions

Unless otherwise noted, the work was carried out using *E. coli* strain 23724 (*F- supE44 lacY1 thr-1 leuB6 mcrA thi-1 rfbD1 fhuA21 lambda*), *S. aureus, P. aeruginosa* and *B. subtilis* were obtained from the American Type Culture Collection.

Strains were grown on Luria-Burtani (LB) broth and maintained and titered on LB agar plates. Cultures were grown with aeration at 37°C to early log phase, OD_{600} of 0.3.

B. Metal Coupon Cleaning Protocol

Metal coupons consisting of a 1 $inch^2$ sheet of a specified alloy composition were provided by the Copper Development Association, New York. The alloys used in this study are: C11000 (99.90% copper wt/wt, less than 0.04% oxygen, balance consisting of various trace elements depending on the ore body sources and the refining process)and S30400 (304 stainless steel – 18% chromium, 8% nickel, 74% iron, wt/wt).

The coupons were individually dipped into warm 3.5% NaOH (71°C) for 30 seconds to degrease the alloy surface. They were immediately rinsed in deionized water and allowed to dry. The coupons were then individually acid rinsed in 10% H_2SO_4 for 30 seconds, immediately rinsed in deionized water, and allowed to dry. Following drying, the coupons were individually flame sterilized by dipping in 95% ethanol, igniting the ethanol by passing through a flame, and allowing it to burn off. The sterilized coupons were stored in sterile Petri dishes.

C. TBARS Assay

The TBARS (Thiobarbituric Acid Reactive Substances) assay is described in detail at [8]. For this study, *all bacterial* strains were grown to early log phase (OD_{600} about 0.3) in LB media. Cells were harvested from 100 ml of culture medium, washed once with 0.85% NaCl, and resuspended in 0.85% NaCl to give a final volume of 200 or 500 µl, as indicated. For cells exposed to $CuSO_4$ in liquid cultures, 100 µl samples of concentrated cells were used for the TBARS assay. For exposure to metal coupons, 100 µl samples of concentrated cells were spread on the coupon surface in a sterile Petri dish and allowed to air dry. Drying was complete in about 15 minutes. Cells were collected from the coupon surface by repeated washing and scraping with the micropipette tip into 100 µl of sterile 0.85% NaCl, and harvested from the coupon wash by centrifugation. The entire sample was used for the TBARS assay that was performed as described by the manufacturer (ZeptoMetrix Corporation). To summarize, the harvested cells were resuspended in 100 µl of SDS by gentle swirling, 2.5 ml of TBA buffer reagent added, and the covered tubes were incubated in a water bath at 95°C for 60 minutes. After the tubes were allowed to cool to room temperature, they were put into an ice bath for 10 minutes, and 2 ml of the reaction mixture was transferred to microcentrifuge tubes for debris removal by centrifugation at 3000 rpm for 15 minutes. The absorbance at 532 nm of the supernatant was determined using a Shimadzu Biospec-mini spectrophotometer. The amount of malondialdehyde (MDA) was determined by comparison to an MDA standard curve and is reported as nmoles per 10^9 cells. Each experiment was repeated with three independent cultures.

D. Microscopic Cell Viability Assay

The LIVE/DEAD® BacLight Bacterial Viability Kit (Invitrogen) for microscopy and quantitative assays was used to visually monitor cell viability. The kit contains a mixture of two stains, SYTO®9 and propidium iodide, which differentially enter bacterial cells and monitor bacterial cell viability as a function of membrane integrity. SYTO®9, a green-fluorescent nucleic acid stain, is capable of passing the intact membrane of both viable and dead or dying bacterial cells. Propidium iodide, a red-fluorescent nucleic acid stain, only enters bacterial cells with intact but compromised membranes that are either dead or dying. When both dyes are present, the propidium iodide causes a reduction in the SYTO®9 fluorescence. Thus, cells with a compromised membrane will stain red, whereas cells with an intact membrane will stain green. Cells were stained as described in the manufacturer's protocol and observed using a Zeiss fluorescent Axioscope (GFP and Rhodamine filters) and an AxoCam ICm1 camera. Total magnification used was 1000X oil immersion.

III. Results

A. Both Gram Positive and Gram-Negative Bacteria Show Similar Cell Death Patterns on Copper Surface Exposure

We compared the copper mediated toxicity rates of cell death between *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Staphylococcus aureus*. Given the differences between the cell wall and plasma membrane structure we used the standard lab strain of *Pseudomonas aeruginosa* (Gram negative) and Staphylococcus *aureus* (Gram positive) in order to compare the rates of cell death to *E. coli results published already* [7] and *B. subtilis (not shown, manuscript in preparation)* obtained from ATCC. Briefly, all ATCC bacterial strains indicated were grown in LB media to mid-log phase (OD600 0.3), harvested by centrifugation from 100 ml of culture, and resuspended in 0.85% NaCl to a final volume of 500 µl. 100 µl of concentrated cells were spread over the surface of metal coupons of 304 stainless steel (S30400) and 99.90% copper (C11000). Following the indicated time of exposure, the cells were washed from the coupon surface with 100 µl of 0.85%

NaCl and samples taken to titer survival. Survival, reported as the number of colony forming units (CFU), was determined by diluting culture samples in 0.85% NaCl and plating on LB agar. The results shown in Figs. 1, 5 are an average from three independent cultures done in duplicate. As anticipated, bacterial killing in Figs. 1, 5 of *S. aureus* and *P. aeruginosa* on the copper-containing alloys was biphasic – slow for the first 30 minutes and significantly more rapid after 30 minutes of exposure, the point at which the samples had dried on the alloy. In contrast, only one to two log of killing is seen on 304 steel surface. These results provide direct evidence that the rapid killing requires copper alloy. Cell death pattern after exposure to copper alloy showed similar results in *E. coli* [7] although the initial slow cell death rate was seen in first 15 minutes followed by increased levels of cell death. However the results of *B. subtilis* showed a slightly different cell death pattern. *B. subtilis* cells, in their vegetative state, show a similar cell death pattern. On the other hand, cells with spores show a different cell death pattern upon exposure to copper (data not shown here, manuscript in preparation). Overall, it seems like 30–60 minute exposure to copper seems to be a critical time period after which the rate of cell death seems rapid in the gram negative and positive bacterial strains tested in this study.

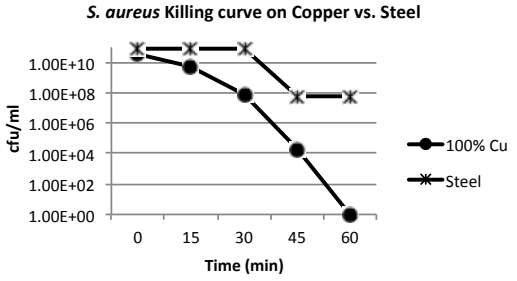


Fig. 1 Survival rate of *Staphylococcus aureus* on copper and steel surface over time

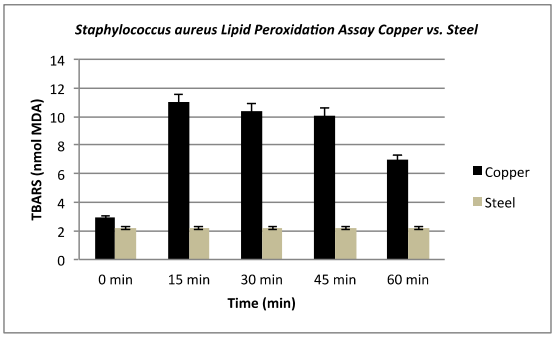


Fig. 2 Rate of lipid peroxidation as shown by TBARS assay on copper surface over time

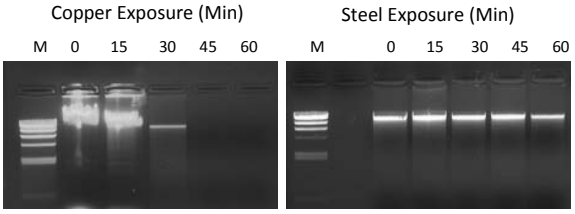


Fig. 3 Genomic DNA degradation in *Staphylococcus aureus* on copper and steel surface exposure over time. The size markers (M) are HindIII digested lambda phage DNA

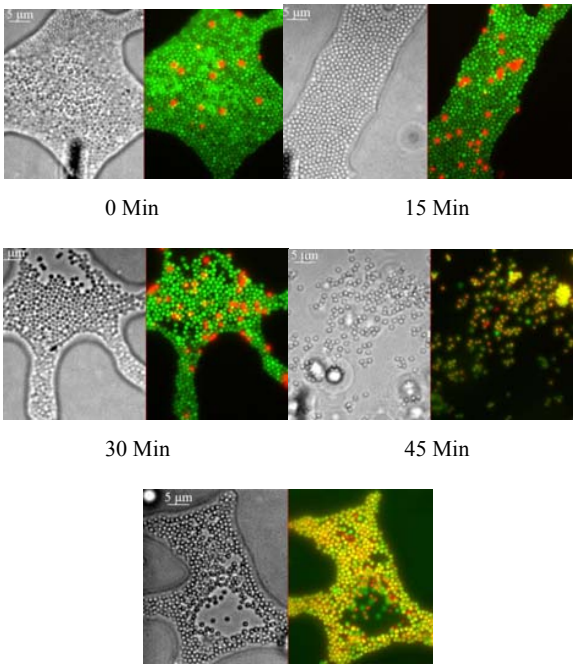


Fig. 4 Microscopic Live/Dead BacLight Assay® of membrane integrity in *Staphylococcus aureus* on copper surface over time

B. Increased Levels of Copper Surface Mediated Cell Death Correlates with Increased Levels of Lipid Peroxidation

We used the TBARS (Thiobarbituric Acid Reactive Substances) assay, a well-established assay for screening and monitoring lipid peroxidation by measuring the formation of malondialdehyde (MDA) [8] Results are shown in Figs. 2, 6. The error bars indicate standard deviation from three independent cultures assayed in duplicate. A 100 µl sample of a cell suspension containing approximately 10^9 – 10^{11} cells of *S. aureus* and *P. aeruginosa* was evenly spread over the surface of the metal coupons and allowed to air dry. During the 15-minute drying period the cells were exposed to the copper surface in moist rather than dry conditions. Results from the gram positive *S. aureus* are compared to gram-negative *P. aeruginosa* as well as previously published results from *E. coli* and *B. subtilis* (*data not shown, manuscript in preparation*). As can be seen by results in Figs. 2, 6 increased levels of lipid peroxidation was observed in both *S. aureus* and *P. aeruginosa* cells exposed to the 99.90% copper alloy

(C11000). No lipid peroxidation above initial levels was observed in cells exposed to 304 stainless steel. Similar results were observed before in *E. coli* as well as *B. subtilis* (data not shown). These results clearly indicate a strong correlation between increased levels of lipid peroxidation after exposure to copper surface.

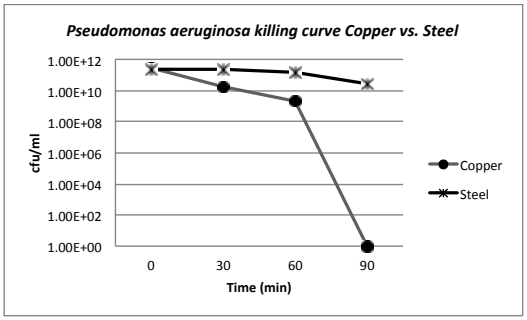


Fig. 5 Survival rate of *Pseudomonas aeruginosa* on copper and steel surface over time

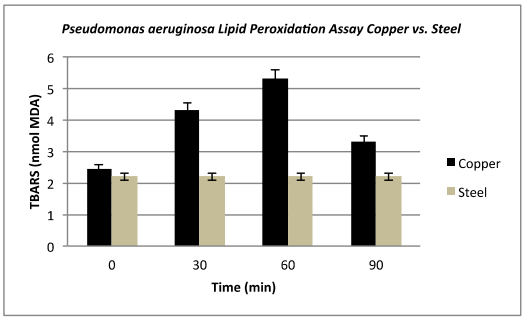


Fig. 6 Rate of lipid peroxidation as shown by TBARS assay in *Pseudomonas aeruginosa* on copper and steel surface over time

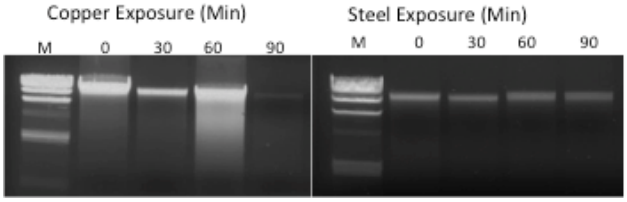
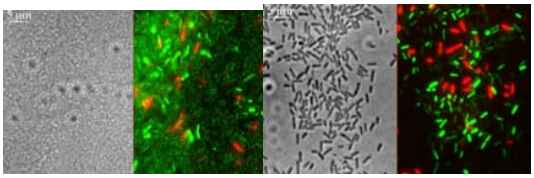
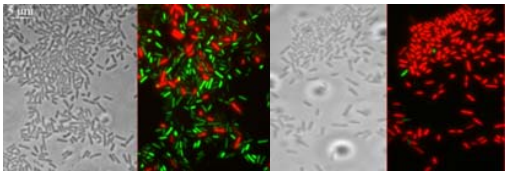


Fig. 7 Rate of genomic DNA degradation in *Pseudomonas aeruginosa* on copper and steel surface over time



0 Min 30 Min



60 Min 90 Min

Fig. 8 Microscopic Live/Dead BacLight Assay® of membrane integrity in *Pseudomonas aeruginosa* on copper and steel surface over time

C. Copper Surface Exposure Causes Genomic DNA Degradation in Both Gram Positive and Gram-Negative Strains

Total genomic DNA was isolated from *S. aureus* and *P. aeruginosa* cells exposed to and recovered from coupons of 304 stainless steel (S30400), 99.90% copper (C11000). Results are shown in Figs. 3, 7. A minimum of three independent trials were done for each bacterial strain. Representative gels are shown. Exposure to 99.90% copper alloy (C11000) coupons caused rapid and extensive degradation of genomic DNA after the 30-minute time point in case of *S. aureus* while after 60 minute time point in case of *P. aeruginosa*. DNA degradation was complete by 60 and 90 minutes for each strain respectively and, reproducibly, no genomic DNA was found in the 60 or 90-minute samples. It should also be noted that no shorter DNA fragments could be observed in the gels suggesting that degradation produced random-sized fragments with no preferred nuclease target sites indicated. Our published results [7] with *E. coli* exposure to copper surface also gave similar DNA degradation pattern and complete DNA degradation in 60 minutes. These gel patterns do not indicate an apoptotic cell death pattern. Cell death is reported to occur via either programmed cell death (apoptosis) or via a catastrophic apparently irreversible process termed necrosis. A number of hallmarks distinguish the two processes. Most significantly, apoptosis is characterized by nuclear DNA fragmentation into non-random sized fragments occurring prior to plasma membrane lysis. The DNA breaks form between the nucleosomes and produces a ladder pattern in agarose gel electrophoresis. In contrast, necrosis begins with the swelling of the cytoplasm and intracellular compartments, loss of membrane integrity, and ends with total cell lysis. DNA fragmentation is a late marker of necrosis and randomly produces a DNA smear in agarose gel electrophoresis. Our results shown in Figs. 2, 7 indicate a necrotic cell death pattern. We will however explore the mode of cell death at a later time.

D. Microscopic Assay of Membrane Integrity After Exposure to 99.90% Copper Surface

We used Live/Dead staining to follow *S.aureus* and *P. aeruginosa* cell viability over this same time course shown in Figs. 4, 8. Strains were grown, harvested, and exposed to 99.90% copper coupon (C11000) surface, as described in Materials and Method. The cells were then washed from the coupon surface and the recovered cells stained as described by

the LIVE/DEAD kit manufacturer for observation by phase and fluorescent microscopy. This test kit monitors cell death indirectly by measuring membrane integrity. The presence of intracellular propidium iodide, which enters cells when the membrane ceases to function as a semi-permeable membrane, is used as an indicator of cell viability. The results in Figs. 4, 8 correlate with the survival curve shown in Figs. 1, 5. The percentage of dead cells or cells with incompetent membranes was approximately 50% at 30 minutes and increased to almost 100% at 90 minutes in *Pseudomonas aeruginosa*. In contrast, to 60 minutes in *S. aureus* (Fig. 4) and *E. coli* [12], few if any live cells can be observed either by this assay or by the cell titer assay. No evidence of cell death or loss of membrane integrity was observed in all the bacterial strains tested here exposed to 304 stainless steel (data not shown).

IV. Conclusion

Recent work done by several labs [9]-[13] indicate clearly that copper ions released by copper alloy surfaces are responsible for contact based killing of microorganisms. We decided to take a closer look in both gram negative strains like *E. coli* and *P. aeruginosa* as well as gram positive strains like *S. aureus* and *B. subtilis*. Cells exposed to metallic copper alloy surfaces, as shown in Figs. 1, 2, 5 and 6, demonstrate a clear correlation between exposure to copper and the time of onset of killing, the rate of cell death, and the kinetics of lipid peroxidation. Moreover, lipid peroxidation in cells exposed to 99.90% copper peaked at a time point that coincides with the loss of membrane integrity and the induction of genomic DNA degradation as shown in Figs. 3, 7. However important difference is seen in terms of the loss of membrane integrity in gram positive and gram negative strains as indicated starting at 45 minutes of copper exposure in Figs. 4, 8. This aspect needs to be explored further. These results shed a very important light on the fact that several non-spore forming gram positive as well as gram negative bacteria show similar patterns of cell death to copper exposure. We have currently undertaken a major effort to expose the genes involved in copper mediated cell death mechanisms. These studies are really important in our fight against hospital-acquired infections, which are prevalent due to steel clad surfaces where microbes can thrive.

Acknowledgment

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