Appl. Environ. Microbiol. doi:10.1128/AEM.01725-15

Copper Reduction and Contact Killing of Bacteria by Iron Surfaces

Salima Mathews,[a] Ranjeet Kumar[b], and Marc Solioz[a,b]

Dept. Clinical Research, University of Bern, Berne, Switzerland[a]; Laboratory of Biochemistry and Molecular Biology, Tomsk State University, Russian Federation[b]

Running head: Contact Killing of Bacteria on Iron Surfaces

Address correspondence to Marc Solioz, marc@solioz-scientific.ch

Keywords: antimicrobial, contact killing, iron surfaces, redox, copper

The well-established killing of bacteria by copper surfaces, also called contact killing, is currently believed to be a combined effect of bacterial contact with the copper surface and the dissolution of copper, resulting in lethal bacterial damage. Iron can similarly be released in ionic form from iron surfaces and would thus be expected to also exhibit contact killing, though essentially no contact killing is observed by iron surfaces. However, we here show that exposure of bacteria to iron surfaces in the presence of copper ions results in efficient contact killing. The process involves reduction of Cu^{2+} to Cu^{+} by iron; Cu^{+} has been shown to be considerably more toxic to cells than Cu^{2+}. The specific Cu^{+} chelator, bicinchoninic acid, suppresses contact killing by chelating the Cu^{+} ions. These findings underline the importance of Cu^{+} ions in the contact killing process and infer that iron-based alloys containing copper could provide novel antimicrobial materials.

The killing of bacteria by metallic copper surfaces, so-called 'contact killing', is now well established and has explicitly been shown for many species (1). Bacteria are killed within minutes on surfaces of copper or copper alloys containing at least 60% copper. In contrast, cells can survive for days on surfaces of stainless steel, glass, or plastics. Copper and copper alloys have attracted attention as a means of creating self-sanitizing surfaces in the light of increasing nosocomial infections in Western hospitals. In a number of hospital trials, rooms have been fitted with copper alloy table tops, bedrails, door handles, light switches, bathroom fixtures, etc. in an effort to curb nosocomial infections (2-6). These copper surfaces resulted in a two- to three-log reduction of the microbial burden on a continuous basis. However, further data are needed to convincingly demonstrate that these measures also lead to a lasting reduction of nosocomial infections. But it appears clear that copper-containing materials can contribute to hospital hygiene and lower the bacterial burden also in other facilities where clean or aseptic working procedures are required (7).

The mechanism of contact killing of bacteria by copper-containing materials is of interest not only in connection to its use in hospitals, but also from a purely scientific point of view. Laboratory studies have shown that bacteria on copper surfaces suffer rapid membrane damage and DNA degradation, in addition to other less well-defined cellular damage (8-13). The importance and the order of the different processes leading to cell death may depend on the type of microorganism (10). One key element required for contact killing is the release of copper ions from the metal surface. Bacterial copper resistance systems appear unable to cope with the released copper (14-16). The second important requirement for contact killing is bacterial contact with the metal surface (17). Recently, we showed that bacteria are also killed effectively on iron surfaces if ionic copper ions are present (18). In this study, we also show that the reduction of Cu^{2+} to Cu^{+} by the iron surface plays a key role in the killing process. These findings underline

the greater toxicity of Cu^{+} when compared to Cu^{2+} and suggest novel antimicrobial materials based on iron alloys able to release copper.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The wild-type strain *Enterococcus hirae* (ATCC9790) wild-type strain was grown anaerobically by inoculating 10 ml of air-saturated N-media (18), followed by growth in sealed tubes to stationary phase at 37 °C. Cells were collected by centrifugation for 5 min at 5000 x *g*, washed twice with 20 ml of 100 mM Na-HEPES pH 7 or, where indicated, with 100 mM Tris-Cl pH 7, and resuspended in 10 ml of the same buffer. The average cell density was 2-8 x 10^8 cfu/ml. All handling of cells was performed aerobically.

Preparation of iron coupons. Iron plates of dimensions 10 x 20 x 0.5 mm, in the following called 'coupons' were >99% iron, <1% carbon, and were cleaned by ultrasonication in chloroform and ethanol for 10 min each, followed by air-drying. Following cleaning, all coupons used in this study were stored under nitrogen until used.

Measurement of contact killing. To assess contact killing, a wet plating technique was used, essentially as previously described in (15). Briefly, 40 µl of cells suspended in 100 mM Tris-Cl or Na-HEPES, pH 7, and supplemented with 2 mM bicinchoninic acid (BCA) or 4 mM $CuSO_4$ were applied to the coupons. Following incubation at 25 °C for 0 to 300 min in a water-saturated atmosphere, 10 µl samples were withdrawn and serial dilutions in PBS were spread on N agar plates. Following growth for 24 h, survival was calculated from plate counts and expressed in colony forming units (cfu).

Copper and iron determinations. Copper or iron release from coupons during wet plating was assessed by removing 20 µl aliquots at 0 to 300 min, diluting them 50-fold with 0.065% HNO_3, and measuring the copper content by inductively coupled plasma atomic emission (ICP-AE) spectroscopy on a Jobin Yvon JY 24 instrument (HORIBA Jobin Yvon GmbH, Munich, Germany) at 324.754 nm for Cu or 259.940 nm for Fe.

Measurement of copper reduction. Cell suspensions as used to measure contact killing with 4 mM $CuSO_4$ (40 µl) were applied to iron coupons and at 0 to 300 min, 10 µl aliquots were withdrawn, mixed with 990 µl of 100 µM BCA in 0.1 M Tris-Cl pH 7. Formation of Cu^{+} was determined by measuring the concentration of the formed $Cu(BCA)_2$ complex at 354.5 nm, using an extinction coefficient $\varepsilon = 4.6 \times 10^4\ M^{-1}cm^{-1}$ (19).

RESULTS

Contact killing on iron coupons. Iron has similar redox properties as copper, yet it does not exhibit contact killing of bacteria. When 2 x 10^7 cells of *Enterococcus hirae were* applied to an iron surface, there was no significant reduction (<1 log) in the number of live bacteria is observed after 300 min, both under anaerobic and aerobic conditions (Fig. 1A). However, if 4 mM $CuSO_4$ is added to the cells, no survivors can be recovered after 300 min under anaerobic as well as aerobic conditions. After 100 min of exposure, a difference between anaerobic and aerobic conditions can be observed, with three logs of killing under aerobic and nearly six logs of killing under anaerobic conditions. The concentration of 4 mM $CuSO_4$ was chosen on the basis of previous findings which had shown that the rate of killing on iron is proportional to the copper concentration (17); 4 mM $CuSO_4$ provided an ideal time window for the present studies.

These experiments were conducted in Na-HEPES buffer which exhibits negligible complexing of copper. If these experiments were conducted in Tris-Cl buffer, which is known to complex copper strongly (20), contact killing was even more rapid, with complete killing observed already after 100 min, compared to 300 min in Na-HEPES, both under aerobic and anaerobic conditions (Fig. 1B). There was also a significant decline in viability in Tris-Cl buffer in the absence of copper. The cause of this loss in viability remains unknown, but may be connected to the membrane permeability of Tris-Cl in its non-dissociated state. Taken together, these experiments show that the addition of copper to cells on metallic iron induces contact killing and that the effect is accentuated by anaerobic conditions.

The role of copper reduction. The enhancement of copper-induced contact killing by anaerobic conditions led us to conclude that the oxidation state of the copper ions is important to the process. We thus determined the presence of Cu^+ in the course of contact killing experiments on iron. When cells were exposed to metallic iron in the presence of $CuSO_4$. there was significant generation of Cu^+ ions even at the shortest times measurable (1-2 s; Fig. 2). Overall, Cu^+ generation did not differ significantly between aerobic and anaerobic conditions and on average, remained at approximately 0.8 mM throughout the experiment. It has previously been shown that Cu^+ is considerably more toxic to cells than Cu^{2+} (21). The faster killing of *E. hirae* on iron in the presence of copper thus appears to be related to the generation of Cu^+.

Copper reduction by iron. The obvious source of electrons for copper reduction is the Fe(0) of the coupons. We therefore looked at iron release from the coupons in the presence of copper. There was substantial release of iron into the aqueous medium, regardless of the presence of either copper or cells (Fig. 3). After 300 min, 15 mM iron was released into the aqueous phase in the absence of copper. When 4 mM $CuSO_4$ was present, iron release was enhanced by about 30%. Iron is not very soluble under aerobic conditions at pH 7 and it must be assumed that most of the iron was present in the hydroxide form. In fact, the formation of a visible film, presumably of iron hydroxide, on the surface of the aqueous phase could be observed. Iron release was unexpectedly high, exceeding copper reduction almost 20-fold and this release

was unlikely to play an important role in contact killing by metallic iron in the presence of copper. The generation of Cu^{+} appears to be the key toxicity mechanism.

Cu^{+} chelation suppresses copper toxicity on iron. Since the generation of Cu^{+} appears to be the toxicity mechanism of contact killing on iron in the presence of copper, specific chelation of Cu^{+} by BCA was investigated. Fig. 4 shows that contact killing in the presence of copper was massively reduced by BCA. After 300 min of exposure, only a two log (99%) reduction of cell survival was observed in the presence of 4 mM $CuSO_4$ and 2 mM BCA as compared to experiments without chelation, where survival was reduced by > 7 logs. BCA by itself had no significant effect on the survival of bacteria on iron or glass. This further supports that contact killing on iron surfaces in the presence of copper is due to reduction of Cu^{2+} to more toxic Cu^{+}.

Redox-inactive metal ions do not promote contact killing on iron. As a further test of the concept of copper reduction as the active principle in contact killing on iron plus copper ions, metal ions which are not redox-active were tested as to their effect on bacterial survival on iron. As shown in Fig. 5, neither Zn^{2+} nor Cd^{2+} had a significant effect on bacterial survival on iron (note that the ordinate of this Figure is greatly expanded for clarity). Clearly, redox reactions between iron and copper are the underlying mechanism of contact killing of bacteria on iron in the presence of copper ions.

DISCUSSION

We previously reported the augmentation of contact killing of bacteria on iron surfaces by copper ions (17). This study continued to evaluate copper contact killing gain insight into the biocidal mechanism. Solid iron by itself only marginally impairs bacterial survival. However, when cell suspensions are supplemented with Cu^{2+}, rapid bacterial killing is triggered. Key events in the process appear to be iron solubilization and copper reduction. Reduction of Cu^{2+} to Cu^{+} can be driven by metallic iron or Fe^{2+} serving as reductants. This process has been thermodynamically analyzed in detail by Matocha et al. (22). Here Cu^{+} can then form insoluble cuprite (Cu_2O), even under anaerobic conditions (22). Cu^{+} is considerably more toxic to bacteria than Cu^{2+}, due presumably to its greater membrane permeability than Cu^{2+} (21;23). Cu_2O was also found to be as toxic to bacteria as metallic copper (24). The copper-induced contact killing on iron shown here is more rapid than contact killing by copper surfaces, presumably due to the greater toxicity of Cu^{+} *versus* Cu^{2+}. However, there may also be synergistic effects of the simultaneous presence of Fe^{2+}, Fe^{3+}, Cu^{+}, Cu^{2+}, and Cu_2O (17). It was also observed that a copper-sensitive *E. hirae* mutant deleted in both copper ATPases and thus unable to expel cytoplasmic copper was completely killed on iron in the presence of copper in 100 min (> 6 logs), compared to 300 min for wild-type under the same conditions (data not shown). This further underlines the importance of copper ions in the contact killing process.

The buffer used to suspend cells had a significant effect on the rate of killing, with Tris-Cl buffer mediating faster killing than Na-HEPES. Tris is known to form complexes with copper, which could be more membrane-permeable than free copper ions (20). Tris was also shown to permeabilize the outer membrane of *E. coli* and may damage the cell membrane of Gram-positive organisms like *E. hirae*, but this will require further investigation (25). The chloride ions at the concentration present in Tris-Cl buffer stabilize the more toxic Cu^{+} ions (22).

Contact killing of bacteria by copper surfaces involves the following steps: damage of the outer and/or inner bacterial membrane, accumulation of copper ions in the cell, and degradation of the bacterial DNA (1). The order in which these events lead to cell death is an issue of debate and may vary with the organism (9-11;26;27). Copper can lead to the production of reactive oxygen species (ROS) in both Gram-positive and Gram-negative organisms by Fenton-type reactions. But cell death can only partially be suppressed by ROS quenchers like superoxide dismutase or catalase (14). ROS generation and lipid peroxidation by copper ions was also shown to occur in *E. coli* and *Salmonella* exposed to solid copper (10;28). A mutant strain with higher levels of unsaturated fatty acids and thus more sensitive to ROS exhibited an earlier rise in lipid peroxidation, higher sensitivity to contact killing, and an earlier onset of DNA degradation (28). Evidence for oxidative damage was also apparent from the proteome of *E. coli* exposed to metallic copper by the increased presence of oxidatively modified proteins (29). While ROS clearly cause cell damage in contact killing, it is probably an accompanying effect rather than the primary cause of cell death.

In the experiments presented here, Cu^{+} is produced by the reduction of Cu^{2+} *via* oxidation of iron. Anaerobic conditions lead to more rapid killing than aerobic conditions, suggesting that ROS production as not a primary cause of cell death. Rather, anaerobic conditions stabilize Cu^{+}, leading to higher transient concentrations (cf. Fig. 2) and favor oxidation to Cu_2O rather than to CuO; Cu_2O has been shown to be as toxic to cells as unoxidized, metallic copper, while CuO is less toxic (24). Substantial amounts of iron are released from the coupons and copper stimulates this release by about 30%. Ionic iron can also induce killing of bacteria, as previously shown (30).

That Cu^{+} is a key player in contact killing in the experiments reported here is evident by the protective effect of the specific Cu^{+}-chelator BCA. How copper kills cells in contact killing is clearly different from the mechanism in growing cells. In culture, the toxic effect of copper on *E. coli* was shown to be the displacement of [4Fe-4S] clusters of dehydratases (31-33). Destruction of [4Fe-4S] clusters was also shown for Ag^{+}, Hg^{2+}, Cd^{2+}, and Zn^{2+} at concentrations which only marginally inhibited growth (33). In line with an attack of iron-sulfur clusters by these ions, their cytotoxicity was related to their thiophilicity. In our experiments, the redox inactive metals Cd^{2+} and Zn^{2+} were unable to elicit significant cell death on iron coupons. Also, zinc has previously been shown to display a death rate constant of contact killing < 1/20 of that of copper or silver (34), while cadmium has never been tested for contact killing. Taken together, these

observations suggest that displacement of iron from [4Fe-4S] clusters does not play a significant role in contact killing or, for that matter, the killing of cells on iron in the presence of copper.

Killing of various species of streptococci in solution by Fe^{2+} or Cu^{+} ions, but not by Fe^{3+} or Cu^{2+} ions, has previously been reported by Dunning et al. (30). These experiments were conducted under anaerobic conditions to prevent ROS production, but in the absence of metallic surfaces. Over four logs of killing in 50 min by 5 to 10 mM Fe^{2+} or Cu^{+} was observed for streptococci, but considerably slower killing occurred with *E. hirae* ATCC 9790, the strain used in this study. The authors concluded that killing was primarily due to inhibition of F-ATPase by Fe^{2+} or Cu^{+}, but we do not share this interpretation. While inhibition of cellular functions impairs growth, it does not necessarily lead to cell death. Killing by the simultaneous presence of iron and copper, as in the experiments reported here, was not investigated. Also, no measurements of residual oxygen or ROS production were reported, but this study underlines the importance of ionic species of iron and copper in the antibacterial activity of these metals.

In an atomic force microscopy study, it was found that the outer membrane of bacteria in contact with antibacterial stainless steel that contains 3.8% copper undergoes substantial changes, and suggests that membrane damage is a major event in contact killing (35). There was also release of copper ions by the copper-containing antibacterial stainless steel, thus providing an additional toxic component. It would be interesting to know if there was also generation of Cu^{+} under these conditions, but this question was not addressed. The study does, however, highlight the importance of bacteria-metal contact, as previously reported for copper and thus supports the current model of contact killing (17). Taken together, these and our findings suggest novel design criteria for antimicrobial, functional materials, based on combinations of iron and copper and show the greater potency of Cu^{+} compared to Cu^{2+} in contact killing.

ACKNOWLEDGEMENT

We thank Thomas Weber for expert technical assistance and Ilya Kublanov for suggesting the use of copper ions on iron coupons. This work was supported by Russian Federation Government grant to leading scientists (contract number 14.Z50.31.0011).

REFERENCES

1. **Grass G, Rensing C, Solioz M**. 2011. Metallic copper as an antimicrobial surface. Appl Environ Microbiol **77:**1541-1547.

2. **Rai S, Hirsch BE, Attaway HH, Nadan R, Fairey S, Hardy J, Miller G, Armellino D, Moran WR, Sharpe P, Estelle A, Michel JH, Michels HT, Schmidt MG**. 2012. Evaluation of the antimicrobial properties of copper surfaces in an outpatient infectious disease practice. Infect Control Hosp Epidemiol **33:**200-201.

3. **Schmidt MG, Attaway HH, Sharpe PA, John J, Jr., Sepkowitz KA, Morgan A, Fairey SE, Singh S, Steed LL, Cantey JR, Freeman KD, Michels HT, Salgado CD**. 2012. Sustained reduction of microbial burden on common hospital surfaces through the introduction of copper. J Clin Microbiol **50:**2217-2223.

4. **Casey AL, Adams D, Karpanen TJ, Lambert PA, Cookson BD, Nightingale P, Miruszenko L, Shillam R, Christian P, Elliott TS**. 2010. Role of copper in reducing hospital environment contamination. J Hosp Infect **74:**72-77.

5. **Laitinen K, Voutilainen P, Santala L**. 2010. Clinical trial on using copper and brass surfaces in a hospital in West-Finland using microbiological assessment. Internal report .

6. **Mikolay A, Huggett S, Tikana L, Grass G, Braun J, Nies DH**. 2010. Survival of bacteria on metallic copper surfaces in a hospital trial. Appl Microbiol Biotechnol **87:**1875-1879.

7. **O'Gorman J, Humphreys H**. 2012. Application of copper to prevent and control infection. Where are we now? J Hosp Infect **81:**217-223.

8. **Espirito Santo C, Lam EW, Elowsky CG, Quaranta D, Domaille DW, Chang CJ, Grass G**. 2011. Bacterial killing by dry metallic copper surfaces. Appl Environ Microbiol **77:**794-802.

9. **Espirito Santo C, Quaranta D, Grass G**. 2012. Antimicrobial metallic copper surfaces kill *Staphylococcus haemolyticus* via membrane damage. MicrobiologyOpen **1:**46-52.

10. **Warnes SL, Caves V, Keevil CW**. 2012. Mechanism of copper surface toxicity in *Escherichia coli* O157:H7 and *Salmonella* involves immediate membrane depolarization followed by slower rate of DNA destruction which differs from that observed for Gram-positive bacteria. Environ Microbiol **14:**1730-1743.

11. **Warnes SL, Keevil CW**. 2011. Mechanism of copper surface toxicity in vancomycin-resistant enterococci following 'wet' or 'dry' contact. Appl Environ Microbiol **77:**6049-6059.

12. **Warnes SL, Green SM, Michels HT, Keevil CW**. 2010. Biocidal efficacy of copper alloys against pathogenic enterococci involves degradation of genomic and plasmid DNA. Appl Environ Microbiol **76:**5390-5401.

13. **Weaver L, Noyce JO, Michels HT, Keevil CW**. 2010. Potential action of copper surfaces on meticillin-resistant *Staphylococcus aureus*. J Appl Microbiol **109:**2200-2205.

14. **Espirito Santo C, Taudte N, Nies DH, Grass G**. 2008. Contribution of copper ion resistance to survival of *Escherichia coli* on metallic copper surfaces. Appl Environ Microbiol **74:**977-986.

15. **Molteni C, Abicht HK, Solioz M**. 2010. Killing of bacteria by copper surfaces involves dissolved copper. Appl Environ Microbiol **76:**4099-4101.

16. **Elguindi J, Wagner J, Rensing C**. 2009. Genes involved in copper resistance influence survival of *Pseudomonas aeruginosa* on copper surfaces. J Appl Microbiol **106:**1448-1455.

17. **Mathews S, Hans M, Mücklich F, Solioz M**. 2013. Contact killing of bacteria on copper is suppressed if bacteria-metal contact is prevented and is induced on iron by copper ions. Appl Environ Microbiol **79:**2605-2611.

18. **Terzaghi BE, Sandine WE**. 1975. Improved medium for lactic streptococci and their bacteriophages. Appl Microbiol **29:**807-813.

19. **Brenner AJ, Harris ED**. 1995. A quantitative test for copper using bicinchoninic acid. Anal Biochem **226:**80-84.

20. **McPhail DB, Goodman BA**. 1984. Tris buffer--a case for caution in its use in copper-containing systems. Biochem J **221:**559-560.

21. **Abicht HK, Gonskikh Y, Gerber SD, Solioz M**. 2013. Non-enzymatic copper reduction by menaquinone enhances copper toxicity in *Lactococcus lactis* IL1403. Microbiology **159:**1190-1197.

22. **Matocha CJ, Karathanasis AD, Rakshit S, Wagner KM**. 2005. Reduction of copper(II) by iron(II). J Environ Qual **34:**1539-1546.

23. **Chaturvedi KS, Henderson JP**. 2014. Pathogenic adaptations to host-derived antibacterial copper. Front Cell Infect Microbiol **4:**3.

24. **Hans M, Erbe A, Mathews S, Chen Y, Solioz M, Mücklich F**. 2013. Role of copper oxides in contact killing of bacteria. Langmuir **29:**16160-16166.

25. **Irvin RT, MacAlister TJ, Costerton JW**. 1981. Tris(hydroxymethyl)aminomethane buffer modification of *Escherichia coli* outer membrane permeability. J Bacteriol **145:**1397-1403.

26. **Tian WX, Yu S, Ibrahim M, Almonaofy AW, He L, Hui Q, Bo Z, Li B, Xie GL**. 2012. Copper as an antimicrobial agent against opportunistic pathogenic and multidrug resistant *Enterobacter* bacteria. J Microbiol **50:**586-593.

27. **Espirito Santo C, Lam EW, Elowsky CG, Quaranta D, Domaille DW, Chang CJ, Grass G**. 2011. Bacterial killing by dry metallic copper surfaces. Appl Environ Microbiol **77:**794-802.

28. **Hong R, Kang TY, Michels CA, Gadura N**. 2012. Membrane lipid peroxidation in copper alloy mediated contact killing of *Escherichia coli*. Appl Environ Microbiol **78:**1776-1784.

29. **Nandakumar R, Espirito Santo C, Madayiputhiya N, Grass G**. 2011. Quantitative proteomic profiling of the *Escherichia coli* response to metallic copper surfaces. Biometals **24:**429-444.

30. **Dunning JC, Ma Y, Marquis RE**. 1998. Anaerobic killing of oral streptococci by reduced, transition metal cations. Appl Environ Microbiol **64:**27-33.

31. **Macomber L, Rensing C, Imlay JA**. 2007. Intracellular copper does not catalyze the formation of oxidative DNA damage in *Escherichia coli*. J Bacteriol **189:**1616-1626.

32. **Macomber L, Imlay JA**. 2009. The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. Proc Natl Acad Sci USA **106:**8344-8349.

33. **Xu FF, Imlay JA**. 2012. Silver(I), mercury(II), cadmium(II), and zinc(II) target exposed enzymic iron-sulfur clusters when they toxify *Escherichia coli*. Appl Environ Microbiol **78:**3614-3621.

34. **Kawakami H, Yoshida K, Nishida Y, Kikuchi Y, Sato Y**. 2008. Antibacterial properties of metallic elements for alloying evaluated with application of JIS Z 2801:2000. ISIJ Intl **48:**1299-1304.

35. **Nan L, Liu Y, Lu M, Yang K**. 2008. Study on antibacterial mechanism of copper-bearing austenitic antibacterial stainless steel by atomic force microscopy. J Mater Sci Mater Med **19:**3057-3062.

FIGURE LEGENDS

FIG 1 A. Contact killing of *E. hirae* on iron. Cells suspended in Na-HEPES buffer and 4 mM $CuSO_4$ were incubated on iron coupons under either aerobic (●) or anaerobic (○) conditions. Controls without copper under anaerobic (▲) or anaerobic (Δ) conditions. B. As in A, but cells suspended in Tris-Cl buffer. The error bars indicate the standard deviations of three independent experiments.

FIG 2 Measurement of Cu^+ production. Iron coupons were incubated with cell suspensions containing 4 mM of $CuSO_4$ under either aerobic (●) or anaerobic (○) conditions. At the times indicated, samples were withdrawn and the Cu^+ formed was complexed with BCA, followed by spectrophotometric determination of Cu^+ as described under Materials and methods. The error bars indicate the standard deviations of three independent experiments.

FIG 3 Determination of iron release. Iron coupons were incubated with cell suspensions under the following conditions: minus cells minus copper (○), plus cells minus copper (●), plus cells plus copper (▲), and minus cells plus copper. Copper was always 4 mM $CuSO_4$. At the times indicated, samples were withdrawn and the iron content was determined by ICP-AE spectroscopy as described under Materials and methods. The error bars indicate the standard deviations of three independent experiments. * $P < 0.006$, ** $P < 0.03$ by Student's *t* test.

FIG 4 Contact killing in the presence of BCA. Survival of cells in suspension was determined in the presence of both 4 mM $CuSO_4$ and 2 mM BCA on either iron (■) or glass (□), or in the presence of only BCA on iron (•) or glass (○). The experiment was conducted as described in the legend to Fig. 1. The error bars show the standard deviations of three independent experiments.

FIG 5 Exposure to iron in the presence of Zn^{2+} or Cd^{2+}. Cells in the presence of 4 mM $ZnSO_4$ were exposed to either iron (■) or glass (□), or were exposed in the presence of 4 mM $CdSO_4$ to either iron (▲) or glass (Δ). Also shown are controls without metal ions on iron (•) or glass (○). Other details of the experiment are as described in the legend to Fig. 4. The error bars show the standard deviations of three independent experiments.

339

Fig. 1A

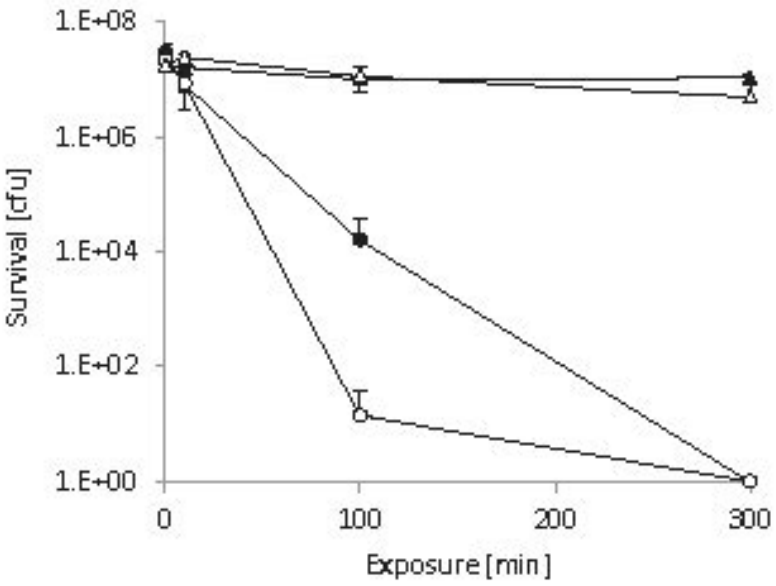

Fig. 1B

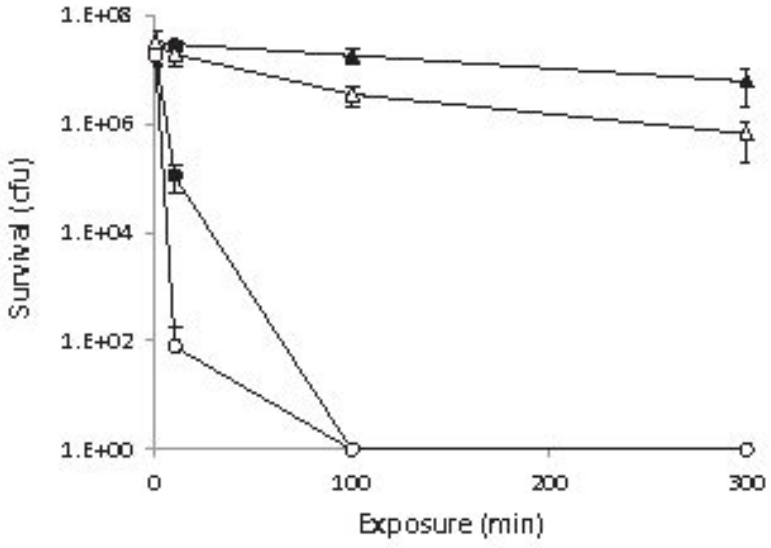

Fig. 2

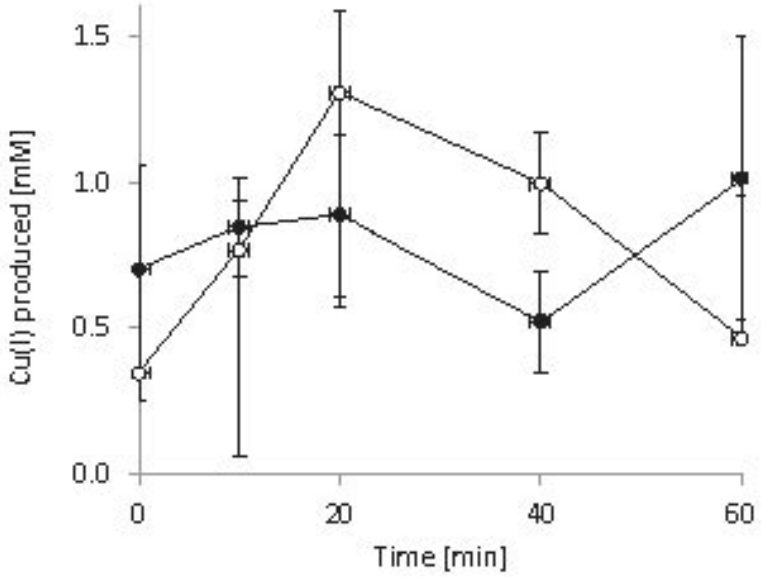

Fig. 3

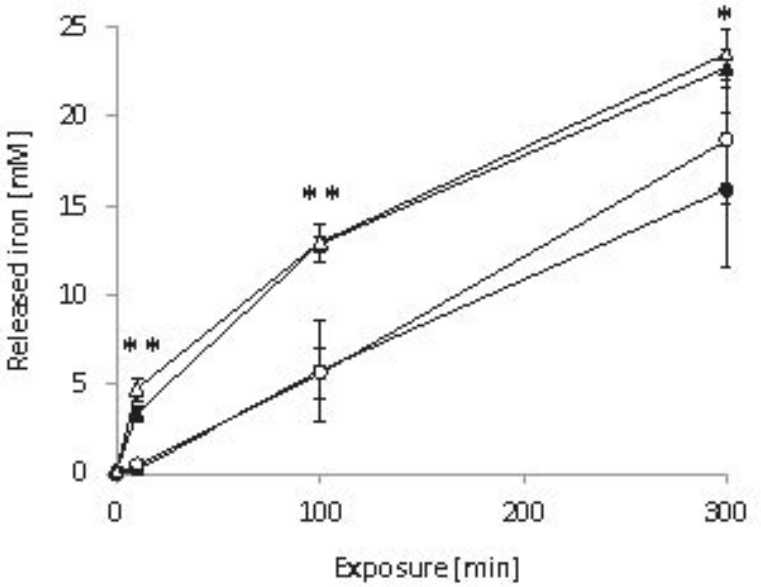

Fig. 4

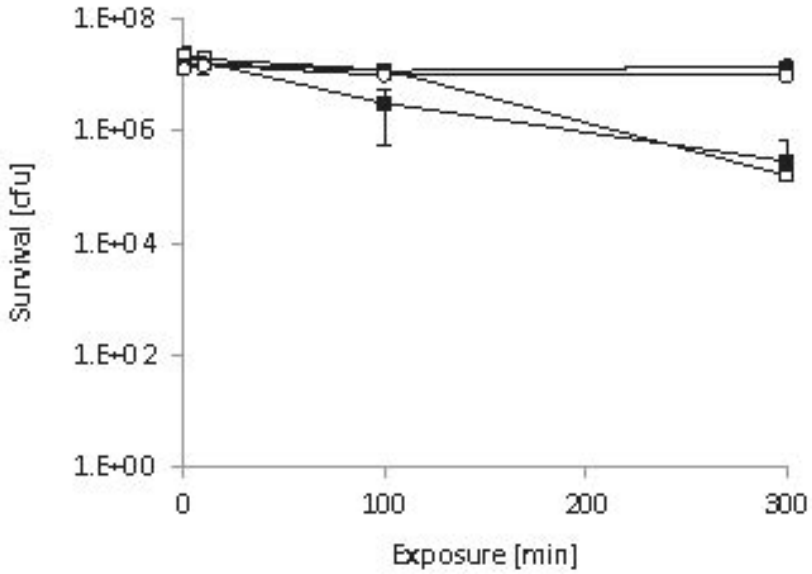

Fig. 5