UNIVERSITY OF SOUTHAMPTON

FACULTY FOR NATURAL AND ENVIRONMENTAL SCIENCES

Centre for Biological Sciences

Laboratory studies to investigate the efficacy and mechanism of action of copper alloys to kill a range of bacterial pathogens and inactivate norovirus.

by

Sarah Louise Warnes

Submission by published work (2009-2013) for the degree of Doctor of Philosophy

June 2014

‘The truth is rarely pure and never simple’

Oscar Wilde

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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Submission by published work for the degree of Doctor of Philosophy

LABORATORY STUDIES TO INVESTIGATE THE EFFICACY AND MECHANISM OF ACTION OF COPPER ALLOYS TO KILL A RANGE OF BACTERIAL PATHOGENS AND INACTIVATE NOROVIRUS

Sarah Louise Warnes

Contamination of dry surfaces with infectious pathogens can have a significant role in infection spread, particularly if the pathogen is resistant to environmental stressors and the infectious dose is low. The use of antimicrobial surfaces in high risk clinical and community environments could help to reduce cross contamination. Although copper alloys have been known to have medicinal properties for centuries it is only relatively recently that laboratory studies have demonstrated that alloys containing over 60% copper have antimicrobial properties.

This study encompasses work done 2008-2013 which has continued to investigate this premise, looking at efficacy of copper and copper alloys to kill newly emerging pathogens which are proving to be a significant risk to global healthcare and also determining the mechanism of pathogen destruction on copper surfaces. Stainless steel which is ubiquitous, partly because of resistance to corrosion, was used as a control surface throughout.

Initial work demonstrated that clinical isolates of vancomycin- resistant enterococci were rapidly killed on copper alloy surfaces within a few minutes to 2 hours dependant on the copper content of alloy, size of inoculum or aqueous content of the contamination (mimicking either wet droplet or dry fingertip touch contamination of fomites). In contrast, enterococci persisted on stainless steel for several months. Following increasing concerns about the emergence of infections caused by Gram-negative pathogenic bacteria these studies identified a rapid kill on copper alloys but not stainless steel of food-borne pathogens *Escherichia coli* O157 and *Salmonella,* and also multidrug-resistant *E. coli* and *Klebsiella pneumoniae* containing the β-lactamase genes *bla* $_{\text{CTX-M-15}}$ and *bla* $_{\text{NDM-1,}}$ respectively (which are responsible for a wide range of community and hospital acquired infections worldwide with diminishing effective therapies).

Further studies identified that release of Cu(I) and Cu(II) ionic species was requisite for copper surface antibacterial toxicity but significant differences in killing mechanism was observed between Gram-positive and Gram-negative bacteria related to their structural dissimilarities. Exposure to copper alloys inhibited respiration in all bacteria tested. Bacterial genomic and plasmid DNA was rapidly destroyed in Gram-positive cells but the cell membrane was not compromised immediately; however in Gram-negative cells the inner cell membrane was immediately depolarised on contact with copper alloys but the DNA breakdown occured more slowly. The outer membrane of Gram-negative bacteria remained intact upon initial contact with copper surfaces. Reactive oxygen species (ROS) are also generated so that in effect the bacteria 'commit metabolic suicide' on copper surfaces: hydroxyl radicals generated by Gram-negative bacteria suggested a role for a Fenton reaction although the importance varied between species. In enterococci short term production of superoxide was the principle ROS. The nucleic acid destruction observed in all bacteria tested could prevent the horizontal transfer of antibiotic resistance or virulence genes and allay concerns about the possibility of developing resistance to copper. This was supported when it was determined that transfer of β-lactamase genes from *E. coli* ST131 and *K. pneumoniae* to recipient *E. coli* did occur on stainless steel but not on copper dry surfaces and that transfer was immediate in the former. The incidence of carbapenemase gene $bla_{\text{NDM-1}}$ transfer increased with time on stainless steel, highlighting concerns that persistence of viable cells not only poses an infection risk but also that contamination of the environment with intact DNA also increases the risk of gene transfer. These results support the use of copper alloys as biocidal surfaces to kill pathogenic bacteria and prevent horizontal gene transfer (HGT).

The final investigation determined that copper alloys were efficacious in inactivating murine norovirus, a close surrogate for human virus, and exposure to copper surfaces destroyed the RNA genome. Copper ions were still responsible directly or indirectly for the inactivation but ROS were not part of the toxicity mechanism.

All the results suggest that copper alloys are effective at destroying a diverse range of pathogenic microorganisms although the mechanisms may be different and multi-faceted. Exposure to copper alloy dry surfaces also prevented the horizontal transfer of genes conferring drug resistance and virulence which has been responsible for the continuing evolution of some of the world's most dangerous pathogens. The results support the use of copper alloys as constantly killing surfaces in healthcare and community environments in conjunction with regular and efficient cleaning and decontamination regimes using non-chelating reagents that could inhibit the copper ion activity. Recent hospital trials now support this thesis.

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List of original publications from this study referred to in the text as an Appendices

Appendix 1

Warnes, S. L., Green, S. M., Michels, H. T. and Keevil, C. W. (2010). Biocidal efficacy of copper alloys against pathogenic enterococci involves the degradation of genomic and plasmid DNA. *Applied and Environmental Microbiology* **76**: 5390-5401.

Appendix 2

Warnes, S.L. and Keevil, C.W. **(**2011). Mechanism of copper surface toxicity in vancomycin-resistant enterococci following wet or dry surface contact. *Applied and Environmental Microbiology* **77**: 6049-6059

Appendix 3

Warnes, S. L., Caves, V. and Keevil, C. W. (2012). Mechanism of copper surface toxicity in *Escherichia coli* O157:H7 and *Salmonella* involves immediate membrane depolarisation followed by a slower rate of DNA destruction which differs from that observed for Gram-positive bacteria. *Environmental Microbiology* **14**: 1730-1743

Appendix 4

Warnes, S. L., Highmore, C and C. W. Keevil (2012) Horizontal transfer of antibiotic-resistance genes on abiotic touch surfaces: implications for public health. *mBio* **3**(6):e00489-12. doi:10.1128/mBio.00489-12.

Appendix 5

Warnes, S. L. and C. W. Keevil (2013). Inactivation of norovirus on dry copper alloy surfaces. *PLoS One* **8**(9): e75017

DECLARATION OF AUTHORSHIP

I, SARAH LOUISE WARNES

declare that the work presented in the 5 publications under consideration, which have been collated into this overview, was my own and generated by me as a result of my original research;

LABORATORY STUDIES TO INVESTIGATE THE EFFICACY AND MECHANISM OF ACTION OF COPPER ALLOYS TO KILL A RANGE OF BACTERIAL PATHOGENS AND INACTIVATE NOROVIRUS

I confirm that:

1. The work was done whilst I was an employee of the University of Southampton 2008-2013;
2. I have been responsible for writing the 5 scientific papers under consideration;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this submission is entirely my own work;
5. I have acknowledged all main sources of help;
6. The contributions of the co-authors on the publications were:
   Professor C. W. Keevil, my supervisor and advisor
   Dr S. M. Green, who provided the clinical isolates in the initial publication
   Dr H. T. Michels, who provided metal samples and metallurgical advice
   Miss V. Caves and Mr C. J. Highmore who were undergraduate students undertaking a laboratory project under my supervision

Signed: .....

Date:.....

Acknowledgements

Firstly, I would like to thank my supervisor Professor Bill Keevil who has been my mentor and provided constant advice, encouragement and support for many years. His faith in my abilities enabled me to get back to work following serious health issues for which I am very grateful. I would like to thank Dr Harold Michels from my industrial sponsor, the Copper Development Association, for keeping me on my toes and providing continuous support, invaluable advice on metal alloys and his knowledge on clinical applications of this research. I would like to thank everyone at the Environmental Healthcare Unit, past and present, who have all contributed to a friendly and constructive working environment. I would like to thank Dr Sandra Wilks for all her advice, help and patience over the years; Dr Rob Howlin for his unceasing kindness and respect, qualities that are often underestimated but are really among the most important. I would like to thank him for always being willing to help everyone, not just me, and for his tolerance of my untidy ways! I would like to say a special thank you to Dr Salome Giao for her enthusiasm and for being a joy to all who know her: I would not have completed this without her support. I would also like to thank Miss Caroline Duignan who encouraged me to collate my work for this thesis and to Dr Tom Secker for his help to format this thesis and for his cheerful advice on everything concerning the mysterious world of information technology and image acquisition. I would also like to thank Ms Lorraine Prout and Dr Jeremy Webb for their support and time and students Miss Vicky Caves and Mr Callum Highmore.

Dedication

I would like to dedicate this work to my wonderful husband Alan and my beloved children, John, David and Rachael.

‘**Thou, sun, art half as happy as we,**

In that the world's contracted thus.’ From ‘The Sunne Rising’ by John Donne, circa 1630

In memorium

I would like to acknowledge Martin Lee (1951-2012) who painstakingly hand-prepared all the metal coupons used in this study for many years. This was initially as an employee of the University and then as a private concern. He died on 20th January 2012 after a short but fierce battle with pancreatic cancer. He cut thousands of metal coupons for me which I am still using today.

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

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Definitions and Abbreviations

LIST OF ABBREVIATIONS

APF	Aminophenyl fluorescein, dye for the detection of ROS
ATP	adenosine triphosphate
BCS	bathocuproine disulfonic acid, chelator of Cu(I)
CA-MRSA	community-associated methicillin-resistant *Staphylococcus aureus*
CDA	Copper Development Association
CDC	Centers for Disease Control and Prevention (U.S.)
CRE	Carbapenemase-resistant *Enterobacteriaceae*
CS1	Copper sensor 1, 8-[*N*,*N*-bis(3′,6′-dithiaoctyl)-aminomethyl]-2,6-diethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene} a membrane permeable dye that increases red fluorescence 10 fold when bound to Cu(I)
CTC	5-cyano-2,3-ditolyl tetrazolium chloride, a redox dye
CTX-M	Extended spectrum β-lactamase (first isolate to hydrolyse cefotaxime in Munich)
DiOC2(3)	3, 3'-diethyloxacarbocyanineiodide, dye for the detection of ROS
DNA	deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid, a chelator of Cu(II)
EPA	Environment Protection Agency, U.S.
ESBL	extended spectrum- β-lactamase
GSH	glutathione
HAI	hospital acquired infection (nosocomial infection)
HGT	horizontal gene transfer
HIV	Human immunodeficiency virus, causative agent of AIDS (acquired immunodeficiency syndrome)

IMP metallo-β-lactamase (carbapenemase Class B)

KPC *Klebsiella pneumoniae* carbapenemase (Class A)

LPS lipopolysaccharide

MERS Middle East respiratory syndrome

MIC minimum inhibitory concentration

MNV-1 murine norovirus-1

MRSA methicillin-resistant *Staphylococcus aureus*

NADH nicotinamide adenine dinucleotide (reduced)

NADH-2 NADH (Nicotinamide adenine dinucleotide) dehydrogenase-2, a copper reductase

NDM-1 New Delhi metallo- β-lactamase (carbapenemase Class B)

OXA-48 oxacillinase group (carbapenemase Class D)

PBS phosphate buffered saline

PCR polymerase chain reaction

PG peptidoglycan, a constituent of bacterial cell walls

PVL Panton-Valentine Leukocidin, toxin commonly associated with CA-MRSA

RH relative humidity

RNA ribonucleic acid

ROS reactive oxygen species

RTqPCR reverse transcriptase real time PCR

SARS Severe acute respiratory syndrome

SEM/TEM scanning electron microscopy/ transmission electron microscopy

SHV β-lactamase (sulphahydryl variable) precursor of ESBL

SOD superoxide dismutase, an enzyme that dismutes superoxide radical to oxygen and hydrogen peroxide

ST131 sequence type 131

TBARS thiobarbituric acid reactive substances, from lipidbreakdown

TEM β-lactamase (named after the patient Temoniera) precursor of ESBL

VBNC refers to viable-but-non-culturable bacteria

VIM Verona–integrin-encoded metallo-β-lactamase (carbapenemase Class B)

1: Introduction

1.1 Uses of copper since ancient times

The discovery of copper, initially extracted from surface ores using stone hammering tools at approximately 6000BC, catapulted man out of the Stone Age. The discovery that the metal properties could be improved by heating and that the malleable nature of the metal meant that copper could be easily fashioned into tools, weapons and utensils. Further applications could be possible if copper was mixed with other metals that were being discovered at the time, such as tin, which started the Bronze Age proper in 4000BC. From 2000BC copper compounds were also being used for medicinal properties including emetics, astringents and in treatments for burns (1). Water was allowed to stand in copper vessels by the ancient Egyptians, a procedure which is still common in modern day rural India. Hippocrates, a physician in Ancient Greece in 400BC, referred many times to the medicinal properties of copper including the treatment of leg ulcers. In volume IX of his treatise he describes a method to relieve 'scabby and itchiness' of the eyelids by 'grinding a lump of flower of copper against a whetstone…..add strained juice of unripe grapes….pour into red copper vessel and continue to grind' this should be applied to eyelids. In addition wounds were dressed with copper salts (2). Over the centuries the importance of copper to all living things has continued to be explored. The metal itself is highly ductile, malleable and exhibits good electrical and thermal conductivity and resistance to corrosion. This has led to large scale use in industry, electrical and water systems and even the coins in our pockets. We need a specific amount of copper in our diets to enable numerous essential copper metallo-enzymes to function and likewise loss of the ability to metabolise copper or intake of too much can have serious repercussions.

1.2 Infectious diseases and the never ending race to develop effective antimicrobials

Infectious diseases have always plagued mankind; epidemics have been recorded throughout history and the discovery that microorganisms were the causative agents was made in 1876 by Robert Koch. This was followed by a wave of discovery of pathogenic bacteria, viruses and fungi and the race started to produce effective antimicrobials to treat pathogen related disease. The discovery of antimicrobial compounds in 1928 that disrupted the bacterial cell wall gave rise to the antibiotic era we know today. However continuous use, misuse and overuse of antibiotics have led to a constantly changing threat to healthcare as the pathogens evolve ways to survive (reviewed in 3-7). The current situation is that many commensals have acquired

resistance to antimicrobials and given rise to serious infections in vulnerable hospitalised individuals producing an explosion in hospital acquired infections (HAI) which occur when the pathogen gains access to the body via usual routes of ingestion, inhalation, exposure to mucosa and also via atypical routes including access to the bloodstream including following surgical intervention. This is exacerbated by the concomitant development of factors enhancing virulence that have also contributed to the development of highly pathogenic microorganisms. (Table 1)

Table 1 Summarising some of the current major infectious disease threats (References 3, 4)

Antibiotic-resistant pathogen	Current threat
Enterobacteriaceae (primarily *E. coli*, *K. pneumoniae* but increase in multidrug resistant *Salmonella* species) responsible for variety of nosocomial and community infections	Extended spectrum β-lactamase producers ESBL(still carbapenem sensitive) **Risk classed as 'serious' by CDC requiring prompt attention** Resistance to carbapenems (important antibiotics for the treatment of severe Gram-negative infections) continues to rise **Risk classed as 'urgent' by CDC requiring immediate attention**
Antibiotic resistance in non-fermenting bacteria: *Acinetobacter baumanii* *Pseudomonas aeruginosa* which cause healthcare-associated pneumonia and bacteraemia	Acquisition of multiple drug resistance **Risk classed as 'serious' by CDC requiring prompt attention**
Clostridium difficile resilient spores that are resistant to therapeutics Infection produces toxins that cause potentially life threatening diarrhoea	In the UK infection rates fell by 15%, in 2010/2011 There has been a marked change in the ribotypes causing severe disease and outbreaks in the UK there has been a reduction in O27 which has been associated with severe disease and increase in novel ribotypes. **Risk classed as 'urgent' by CDC requiring immediate attention**
Methicillin-resistant *Staphylococus aureus* (MRSA) Causes infections of the skin, wounds, pneumonia, and bloodstream.	In the UK MRSA bacteraemia cases dropped 22% in 2010/2011 compared to the previous financial year and MRSA as a cause of surgical site infection continues to show a decrease. PVL-MRSA – increasing numbers of these isolates continue to be submitted to the UK reference laboratory for investigation and a range of different clones have been found to be circulating. **Risk classed as 'serious' by CDC requiring prompt attention**
Antibiotic resistance in *Neisseria gonorrhoeae*	Gonococcal resistance – untreatable gonorrhoea becoming a possibility as third-generation cephalosporin resistance continues to rise. **Risk classed as 'urgent' by CDC requiring immediate attention**
Vancomycin-resistant enterococci responsible for variety of nosocomial and community infections *E. faecalis, E.faecium*	**Risk classed as 'serious' by CDC requiring prompt attention**
Multi drug resistant *Mycobacterium tuberculosis*, airborne pathogen causes tuberculosis (TB)	Concerns about reservoir in wild and domestic animals. **Risk classed as 'serious' by CDC requiring prompt attention**
Other pathogens that have acquired multiple drug resistance include *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., *Streptococcus pneumoniae*, *Candida* species	**Risk classed as 'serious' by CDC requiring prompt attention**
Emerging pathogens and acquisition of genes conferring increased pathogenicity	
Acquisition of toxin, colonisation and virulence genes by bacteria	Enteropathogenic *E. coli* strains producing shiga toxins.
Pathogenic RNA viruses: mutation, recombination and segment reassortment leads to evolution of viruses with increased viruence	Norovirus is one of the leading causes of viral gastroenteritis. Mortality is generally low but healthcare costs are extensive. Emergence of more virulent genotypes including GII.4. Increased risk of evolution of more virulent strains of many viruses, potentially from zoonotic sources, including respiratory pathogens such as influenza and coronavirus infections (SARS, MERS).
Human immunodeficiency virus (HIV)	Anti-retroviral resistance – careful and controlled use of drugs in combination for the treatment of HIV is leading to a reduction in rates of antiviral resistance.

Even before the advent of antibiotics, *Staphylococcus aureus* was responsible for a range of human and animal infections. Overuse of antibiotics provided a selection pressure that allowed the evolution of mechanisms to evade the action of antibiotics. Intrinsic penicillin resistance and then acquisition of methicillin resistance by *S. aureus* in the 1980s plus a variety of toxin genes led to a pandemic of infections worldwide. Initially these were often severe hospital acquired infections with a few virulent clones but these have now spread to the community. MRSA infections are the leading cause of death by a single infectious agent (8) in the U.S.

In the 1970s and 1980s, ubiquitous enterococci, previously considered as commensals of the digestive tract of humans and animals which were even used as pro-biotics, emerged as another serious threat to public health especially as a cause of HAI. Acquired resistance to vancomycin, considered as an antibiotic of last resort to treat the tide of MRSA infections, now presented a serious challenge. The two main pathogenic species are *E. faecalis* and *E. faecium*, which have a high propensity to acquire and transfer genetic information. Transposon mediated multi-drug resistance and the successes of a few clonal lineages have produced a resilient pathogen responsible for multiple pathologies to become a worldwide threat. It is estimated that 66,000 enterococcal infections occur each year in the US of which 22,000 are antibiotic-resistant (3).

Owing to multiple factors (including improved regimes of patient screening, isolation, decolonisation, disinfection and antibiotic treatment) the incidence of MRSA infection has decreased or remained unchanged: a reduction of 31% in invasive MRSA between 2005 and 2011 in US. Transfer of antibiotic resistance genes to Gram-negative bacteria of the gastrointestinal tract has now eclipsed the MRSA epidemic. The presence of extended spectrum β-lactamase producing *Enterobacteriaceae* has spread worldwide but treatment with carbapenems is still possible with these organisms. The evolution and transfer of carbapenemase genes from *Klebsiella pneumoniae* to other strains and species by promiscuous plasmid has now resulted in bacteria that are resistant to all generations of β-lactam antibiotics (Table 2).

More than half of patients who contract a carbapenem-resistant *Enterobacteriaceae* (CRE) blood stream infection do not survive. CRE organisms are classified by the Centre for Disease Control (US) as an urgent threat to health , as is antibiotic resistant *Neisseria gonorrhoeae* and *Clostridium difficile* (3). Further acquisition of genes conferring resistance to other antibiotic classes has produced pathogenic bacteria where a minor infection may escalate into serious systemic pathology that is untreatable. In addition, acquisition of virulence factors such as phage-mediated shiga toxin and plasmid encoded β-lactamase resistance has led to the evolution of *E.coli* O104:H4 2011 outbreak strain and other pathogens with serious pathologies.

As a consequence the number of effective antimicrobials is becoming limited, arousing fears that we are entering a pre-antibiotic era i.e. similar to the time **before** the discovery of

antibiotics. These problems are exacerbated by the acquisition of drug resistance genes by established pathogens such as *Mycobacterium tuberculosis* which now has a significant reservoir in wild and domestic animals. Infections caused by antibiotic resistant bacteria are becoming more prevalent in the community too (9).

Table 2 Carbapenemases currently in circulation

Emerging carbapenemases amongst Enterobacteriaceae in the UK and internationally		
	UK and international distribution	Molecular epidemiology
NDM	Widespread in *Enterobacteriaceae* (especially *Klebsiella pneumoniae* and *Escherichia coli*) in India and Pakistan and often imported to the UK (and elsewhere) via patients with travel and hospitalisation/dialysis there. UK cases are scattered geographically, with little clustering; only isolated examples of UK cross-infection	Commonly encoded by labile and promiscuous plasmids belonging to diverse incompatibility types, although IncA/C types predominate; *bla*NDM has spread to a wide diversity of species
VIM	Globally scattered, mostly in *K. pneumoniae*. Endemic but being replaced by KPC in Greece and sometimes imported to the UK via patients previously hospitalised there. Clusters of *K. pneumoniae* with this enzyme are now occurring in Cheshire; these are partly clonal but with some plasmid spread amongst strains	In general, plasmid spread amongst strains is more important than clonal spread of strains
IMP	Scattered internationally, with little clear association to locale	Mostly spread amongst strains by plasmids; clonal outbreaks are rare
KPC	Recorded in the USA since 1997. Also now prevalent in Israel and Greece and rapidly expanding in Italy, with outbreaks elsewhere in Europe. Some UK cases imported via patient transfers, but significant local dissemination around Manchester	International dissemination greatly reflects the spread of epidemic *K. pneumoniae* ST258 with KPC-2 or -3 enzymes. In the UK, the greater problem is the spread of *bla*KPC-encoding plasmids amongst strains of *K. pneumoniae*
OXA-48	Widespread in *K. pneumoniae* in Turkey (where it originated), Levant and Maghreb. Some imports to the UK from these regions, but also unlinked cases and an outbreak in one London renal unit in 2008-2009	Mixture of plasmid and clonal spread; often encoded by ca. 70 kb plasmids with Tn1999 or Tn1999.2

Adapted from Livermore, 2012 (7)

1.3 Role of contaminated surfaces in the spread of infection and the evolution of pathogens via horizontal gene transfer

Diseases are transmitted by the transfer of infectious pathogens not only from person-person contact but by ingestion of contaminated food or water, inhalation of contaminated droplets and also touching contaminated surfaces (Figure 1). It has been estimated that the formation of biofilms, layers of microorganisms encased in a protective slime, contribute in the spread of 65% cases of hospital acquired infections. The general theories of biofilm development began in 1978 and initially referred to aquatic ecosytems and the natural environment. Further studies have revealed that the formation of complex, multispecies, bacterial biofilms is ubiquitous. The formation of bacterial biofilms *in vivo* often results in chronic conditions including endocarditis, otitis media and lung infections when biofilms form on heart valves, middle ear and in the lungs of cystic fibrosis patients (reviewed in10-12). Copper has been used in water pipes for centuries

and although biofilm formation is reduced compared to other pipe materials eventual biofilm formation can result in corrosion (13) and a recent study observed if water flow is slow the effects of copper may influence bacterial communities further along the pipes (14). However, there is no evidence for biofilm formation on dry copper surfaces.

The role of environmental contamination in the spread of disease has been found to be more significant than previously assumed (reviewed in 15-19). The use of constantly active biocidal solid surfaces could be useful, alongside rigorous disinfection protocols, to prevent the horizontal transmission of robust pathogenic microorganisms that have low infectious dose which is particularly relevant to those pathogens that originated from environmental sources (20).

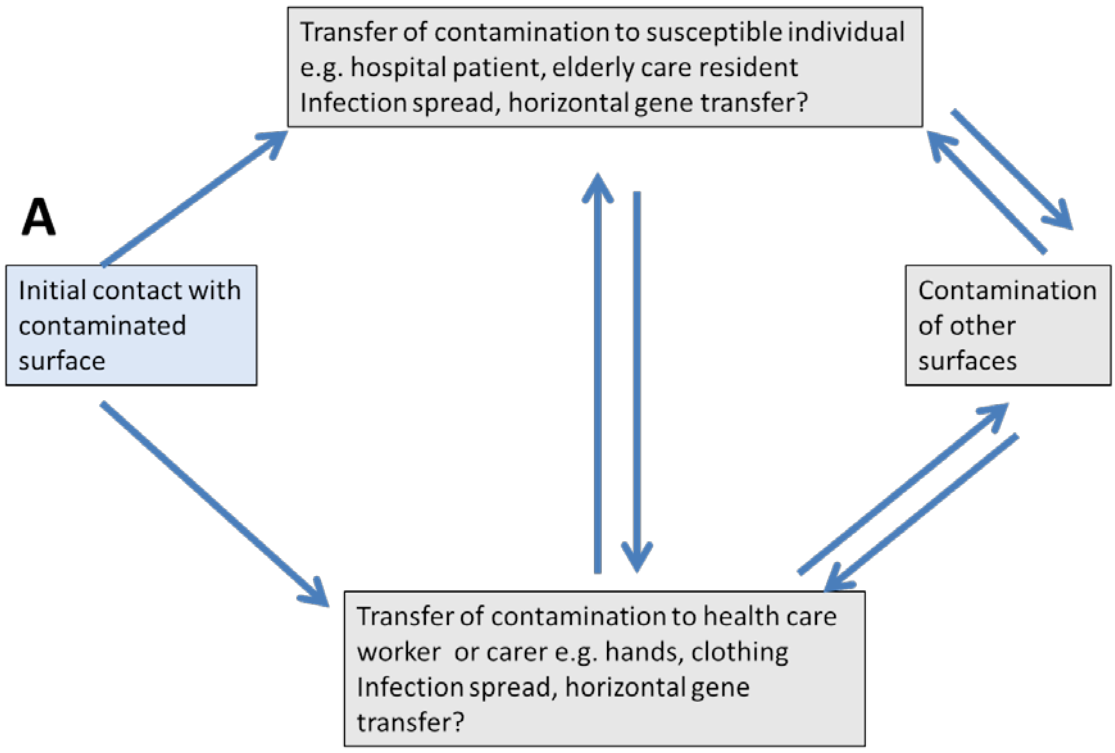
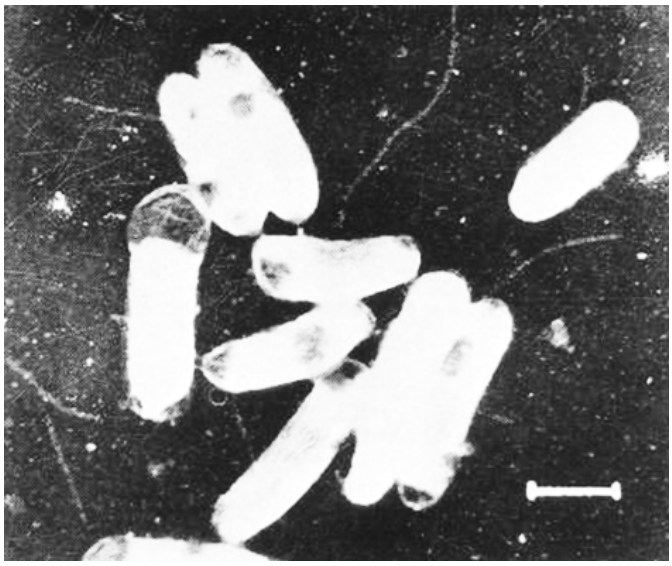


Figure 1 Potential routes for infection spread resulting from surface contamination

A flow diagram to demonstrate the effect a single contaminated surface can have in the spread of infection within a care facility. Contact with a single contaminated surface, A, by patients or carers could result in a chain of infection starting with themselves followed by transfer to other surfaces which could transfer infectious microorganism to others or direct transfer to other personnel. If the pathogen is robust and has low infectious dose, for example norovirus, infection spread can occur very rapidly.

Horizontal gene transfer (HGT) is the exchange of genetic information between organisms that may be taxonomically very different. The transfer of genetic material of different sizes, small gene fragments up to entire chromosomes, occurs rapidly resulting in genetic changes that could have taken millions of years if they were occurring by point mutation alone. HGT occurs via transformation, transduction and conjugation (21). Natural transformation is the uptake of naked DNA by a competent recipient cell and has been observed in the Gram-negative *Helicobacter pylori*. Generalised transduction describes the transfer of bacterial DNA, up to 45kb, via bacteriophages that inject the DNA into the recipient cell which may result in lysis of the host or integration into the host chromosome (lysogenic pathway). Replication of the phage inside the bacterial cell results in release of phage progeny carrying bacterial genes that are then spread to other bacteria. This method has been common in the spread of toxin genes in *S. aureus* and is significant because the transfer does not rely on direct contact between host and recipient. Conjugation involves the transfer of DNA to a recipient cell through a tube (pilus) or pore and requires host and recipient to be in close contact (Figure 2). However, very large pieces of genetic material can be transferred housing large number of genes. An example of this is the staphylococcal cassette chromosome *mec* (SCC*mec*) responsible for spread of methicillin-resistant *S. aureus* worldwide which has evolved to harbour additional genes conferring resistance to many other antibiotics including tetracycline, kanamycin, streptogramin, bleomycin, lincosamide and tobramycin (8, 21, 22). HGT of housekeeping genes, those affecting the ability to colonise the nares, as well as those affecting virulence and drug resistance also occurs. The acquisition of the cytotoxic Panton-Valentine-leukocidin (PVL) genes has resulted in an increase in community associated *S. aureus* infections (CA-MRSA). The current situation is a global ‘soup’ of genes encoding resistance to all in-use antibiotics originating from naturally occurring environmental bacteria that have now found their way into ubiquitous *Enterobacteriaceae*. HGT is known to occur in vivo and in the environment (Section 2.3).

A



B

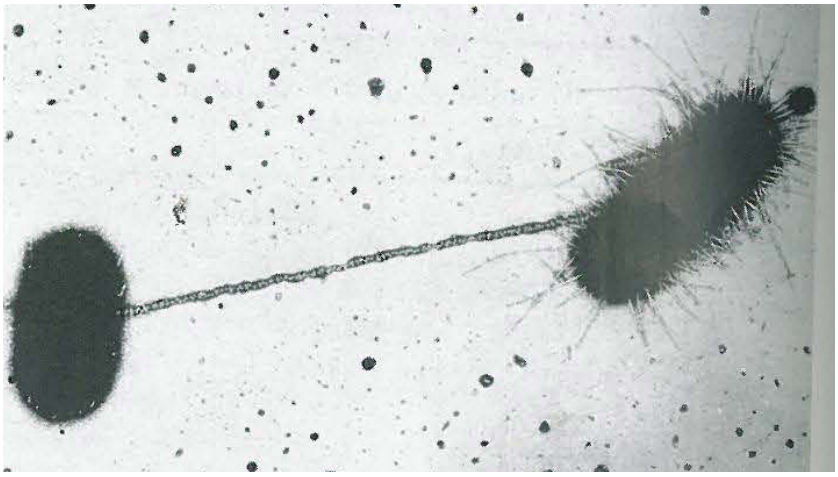


Figure 2 Horizontal gene transfer by conjugation

A: Electron micrograph of *E. coli* K12 stained with the negative stain osmium tetroxide. The F pili (conjugation) can be distinguished from other pili by the presence of adsorbed M12 phages which bind to sites along the length of the pilus. Bar =1µm. Image take from Brinton et al, 1964 (23)

B: Electron micrograph of bacterial conjugation occurring between two *E. coli* cells. On the right is F+ cell which is covered by small pili or fimbriae and a sex pilus extends and connects to the recipient cell on the left. The sex pilus is easier to see because bacteriophages are adsorbed along the length. Image taken from reference 24 and the photograph is credited to Charles C. Brinton, Jr. and Judith Carnahan

1.4 Copper chemistry

Copper is one of the transition metals which demonstrate good electrical and heat conductivity and have a high melting point. Most transition metals have incompletely filled d sub-orbitals allowing bonding with a variety of elements. However copper has the electron configuration $1s^2 2s^2 2p^6 3s^2 3p^6 4s^1 3d^{10}$ where an electron has transferred from the s to the d sub-orbital to retain stability. Under physiological conditions copper exists in two states. Cuprous Cu(I), derived from loss of the electron in the 4s sub-orbital, is highly unstable in aqueous, aerobic environments where it is disproportionates to cupric ion (which has a d^9 outer shell with one unpaired electron) and copper but forms stable complexes with a variety of ligands including sulphur. It is highly toxic, partly because of participation in Fenton and Haber Weiss reactions generating reactive oxygen species (see Section 2.5.i). Cupric Cu(II) is the predominate state in aqueous systems and the cycling between the two states allows for important redox reactions in biological systems with a potential range of -0.2 to +1V (reviewed in 25, 26)

1.5 Copper homeostasis

There are relatively few copper containing enzymes in prokaryotes, the most common being cytochrome oxidase and NADH dehydrogenases which are involved in the respiratory chain and superoxide dismutase for defence against oxidative stress. It is interesting that copper use by prokaryotes may be minimal but mechanisms to maintain copper homeostasis are universal and the P-type ATPase *copA* is present in 85% all bacterial genomes. These enzymes are located outside the cytosol suggesting copper ion transfer is not needed but all attempts must be made to remove copper ions to the outside of the cell. Copper is more toxic in the acidic, anaerobic conditions of intestine and bacteria resident here have evolved elaborate homeostasis mechanisms (27). Homeostasis is achieved by active efflux with ATPase expelling Cu(I) while bacterial copper oxidase reduces Cu(II) to Cu(I) which is then exported directly to outside of the cell in Gram-positive cells or into the periplasmic space in Gram-negatives (Figure 3, references 27-29). Within the cytosol or periplasmic space metallo-chaperones transfer copper to copper-requiring enzymes removing toxic free copper ions. Some bacteria have additional plasmid-encoded copper efflux systems, for example the *pco* determinant in *E.coli* and transferable copper resistance genes (*tcr*) in enterococci. This has been linked to copper salt supplementation of animal feed and developed as a mechanism to protect the bacteria from the increased copper concentrations encountered. A recent study shown that some bacteria that produce ROS in general metabolism or as virulence factors also have membrane bound metallo-chaperones for the removal of toxic copper ions (30). Autoregulatory copper sensors regulate transcription of homeostasis proteins.

1.6 Copper as an antimicrobial surface

The antimicrobial properties of copper however, although known for centuries, have only been explored in depth recently. For example, the use of copper salts in solution was used in the treatment of rheumatoid arthritis and connective tissue disease from the 1940's.

Phyllis Kuhn, a physician in the Unites States observed that brass doorknobs in her hospital showed a reduced number of bacteria compared to other materials in 1983 (31). However this was relatively overlooked until the millennia. Since then laboratory studies have shown that exposure to metal surfaces containing copper rapidly killed some of the main threats to public health including food borne pathogens *Escherichia coli* O157 (32), *Listeria monocytogenes* (33); major causes of HAI methicillin-resistant *Staphylococcus aureus* (MRSA) (34) and *Clostridium difficile* (35, 36); *Mycobacterium tuberculosis* (37), *Candida albicans* and other pathogenic fungi (38) and influenza A (39). This kill was not observed on stainless steel surfaces which are prevalent throughout the healthcare and community environment, primarily because of resilience and resistance to corrosion. In addition the smooth and often shiny appearance of stainless steel surfaces can give a false impression of cleanliness (discussed further in Section 5). 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 surfaces, and has recently been extended down to alloys with greater than 60% copper as a result of this ground-breaking work at the University of Southampton and elsewhere.

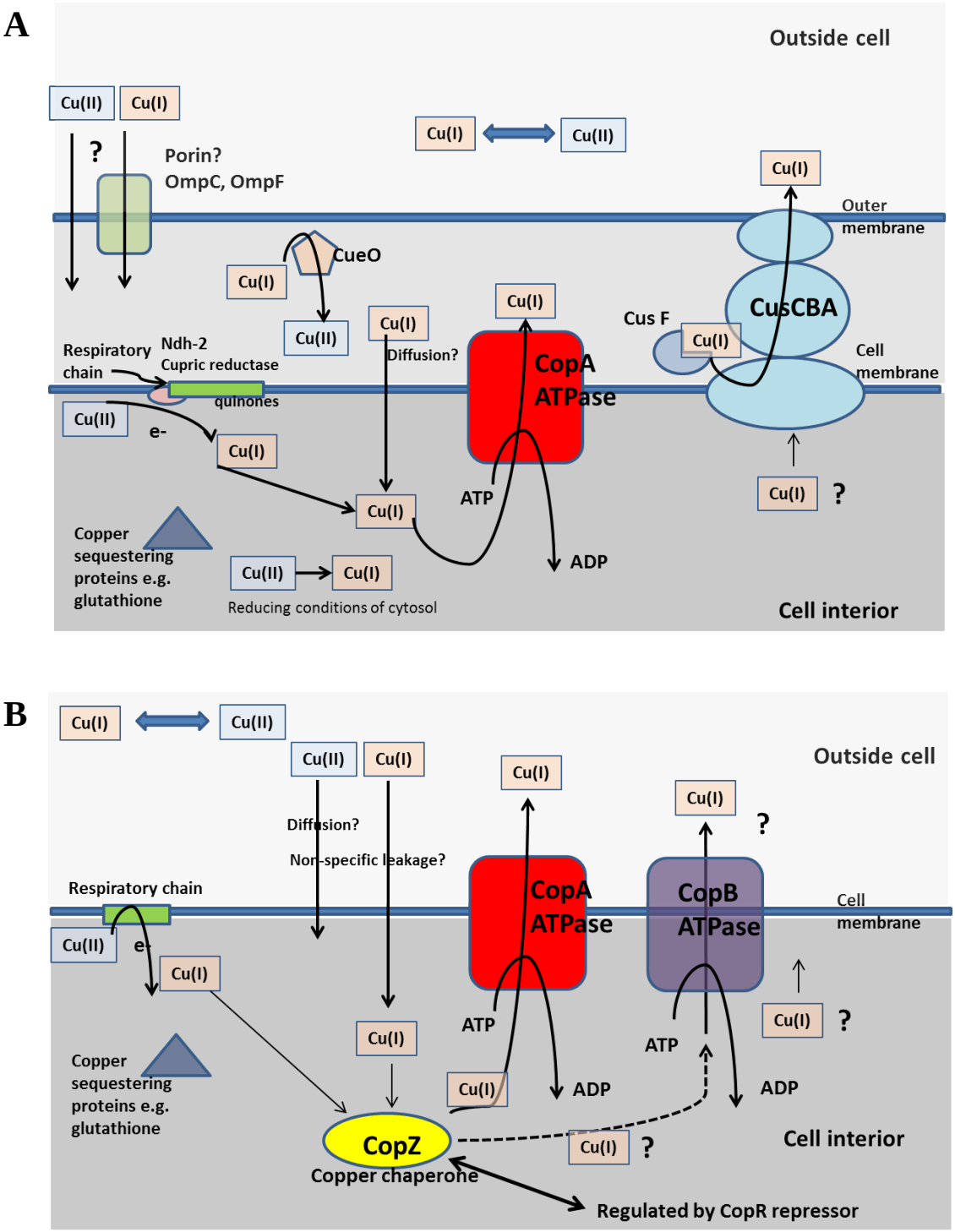


Figure 3 Copper homeostasis in bacteria

A: A simplified diagrammatic representation of copper homeostasis in Gram negative *E.coli* (reviewed in Rensing and Grass, 2003 (27))

The mechanism of uptake of copper ions into bacteria cell is largely unknown but thought to be via porins, other metal transporters or diffusion. In aerobic environments Cu(II) is the most prevalent ionic species which shifts to the more toxic Cu(I) under anaerobic conditions. Cu(II) is reduced by membrane reductases (NDH-2) to Cu(I) on uptake. There are two main mechanisms of copper homeostasis (regulatory genes not discussed here);

1. The proton driven *cus* (copper sensing) locus. CusF is a periplasmic copper binding protein which transfers Cu(I) to a transmembrane cation efflux pump (CusCBA) for export.

2. The Cue (copper efflux) system. CopA is a Cu(I) translocating P-type ATPase transports Cu(I) out of the cell into the periplasm using ATP hydrolysis. A periplasmic multicopper-oxidase, CueO, oxidises Cu(I) to less toxic Cu(II) in aerobic conditions

   Espirito Santo et al. (40), observed slight changes in kill times of *E.coli* mutants in CopA, Cus and CueO

B: A simplified diagrammatic representation of copper homeostasis in Gram positive *E.hirae* (reviewed in Solioz et al, 2010 (28))

Copper homeostasis in *E. hirae* is controlled by an operon which consists of 4 genes. *copA* and *copB* encode for copper-transporting ATPases, *copY* a repressor and *copZ* a chaperone. It is unknown how copper enters the cells but excess Cu(I) binds to CopZ which transfers it to CopA for export. It is believed that CopB may be important if very high concentrations of metal ions are present.

2: Aims of the research and methods employed 2008-2013

The aims of this research study, 2008-2013, was to investigate the efficacy of a selection of copper alloys (Figure 4) against other equally important and also newly evolving pathogens responsible for numerous community and hospital acquired infections (HAI) which are proving difficult to treat and control.

2.1 Efficacy of copper and copper alloys to kill Gram-positive pathogenic enterococci: effect on bacterial growth and respiration

Initial studies were on another major group of pathogens causing HAI, the enterococci. They have evolved from harmless intestinal commensal, originally administered as probiotics, into serious opportunistic pathogens particularly the vancomycin-resistant enterococci (VRE) of species *Enterococcus faecium* and *Enterococcus faecalis*, which are becoming almost impossible to treat in infected patients (they are intrinsically resistant to penicillin). Pathogenesis includes endocarditis, bacteraemia, urinary tract and particularly intra-abdominal infections following surgical procedures e.g. from contaminated lines and catheters. This problem has arisen primarily from widespread use of a vancomycin analogue in animal feed to reduce infections and enhance growth in the livestock and as a result many people are now colonised (reviewed in 41, 42). In the US enterococci are the second most common bacterium to be isolated from catheter associated infections. Since the 1970s *E. faecalis* was the most common enterococcal species to be isolated from HAI but, as predicted, the emphasis has now shifted towards *E. faecium* which is often associated with higher levels of antibiotic resistance and contains large plasmids which have also been discovered in commensal as well as clinical isolates. Enterococci are robust and capable of transferring antibiotic resistant genes to other pathogens, including other species, posing a particular threat to patients and staff in hospitals. It has been estimated that 25% of the entire genome may be connected with mobile elements (41). Initial work investigated the efficacy of a small representative group of copper alloys (Figure 4) to kill laboratory strains of vancomycin-sensitive and resistant enterococci and also, perhaps more importantly, from a selection of clinical isolated vancomycin-resistant enterococci. The effect of exposure on the bacterial cells to copper alloys was assessed by the ability to be cultured in bacteriological media and also the effect on bacterial respiration using the redox dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). This dye is reduced in actively respiring cells and accumulates intracellularly as insoluble red fluorescent formazan which can be observed with epifluorescence microscopy. It is important to determine if the bacteria are dead and not

existing in a sub-lethally damaged or viable-but-non-culturable state, VBNC, which could mean they could potentially return to actively growing state when environmental stresses are reduced and perpetuate the infection cycle (Appendix I). Further investigations attempted to ascertain the possible metabolic and structural targets of copper toxicity in bacteria and the mechanism of copper killing on dry surfaces (Appendix II).

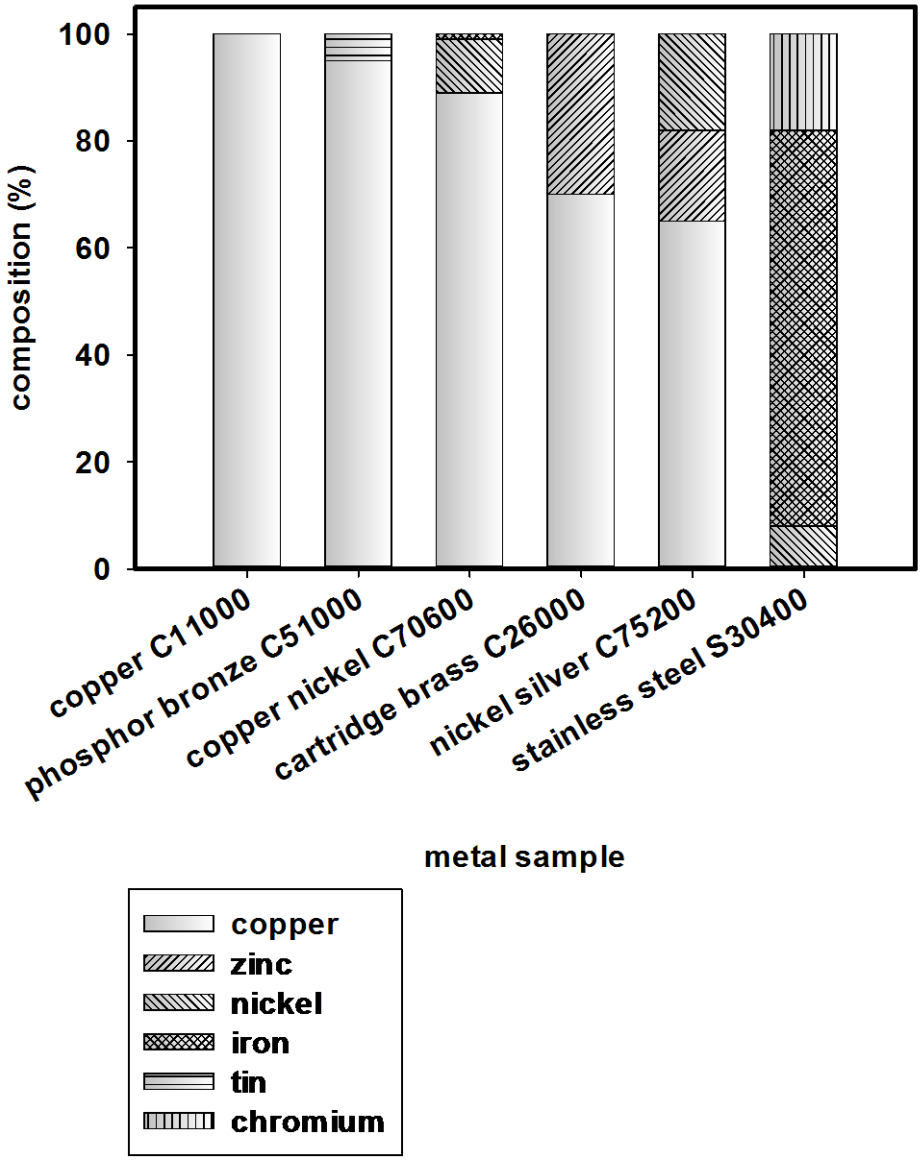


Figure 4 Composition of metals used throughout the study

Percentage composition of metals used for the study, which were a representation of the main copper alloy groups. They are classified by the Unified Numbering System.

2.2 Efficacy of copper alloys to kill Gram-negative bacteria: effect on bacterial growth and respiration

The increased use of antibiotics to treat the worldwide epidemic of MRSA, a Gram-positive bacterium, has been unavoidable and has successfully reduced the incidence of MRSA infections over the past 10 years by more than 85%. However the emphasis has now shifted to Gram-negative bacteria which have traditionally been harder to treat partially because uptake of antibiotics is hindered by the presence of an outer membrane (43). MRSA is found in the anterior nares and on skin and de-colonisation with antibiotics including mupirocin and neomycin combined with disinfectants such as chlorhexidine, can be done with patients admitted to hospitals to control the risk of MRSA infection spread. Initial studies investigated the ability of copper surfaces to kill food-borne pathogens *E. coli* O157 and *Salmonella* species (Appendix III). The great problem now is the acquisition of antibiotic resistance genes by the *Enterobacteriaceae*, some of the most ubiquitous microorganisms on the planet. Because these bacteria are found in the intestines of man and many animal species it is not possible to decolonise and the scale of environmental contamination via faecal wastes contaminating the land and water supplies increases the chances for cross contamination and infection spread cannot really be controlled (44). Commensal bacteria are essential for the welfare of our intestine but the acquisition of antibiotic resistance has turned them into potential killers especially as the armoury of effective antimicrobials is running out. Sometimes, gene acquisition that confers an advantage *in vivo*, such as antibiotic resistance, may affect the fitness of bacteria and in the ability to survive in the environment which is why these studies were undertaken on range of laboratory and clinical strains. Of course, the greatest concerns are when stress response genes are transferred concomitantly with drug resistance, producing a pathogen that is drug resistant and can survive in the environment. The evolution of relatively few high risk clones carrying antibiotic resistance genes can result in rapid worldwide spread of multi-drug resistant organisms (45).

The emergence of extended spectrum β-lactamase (ESBL) and metallo- β-lactamase producing *Enterobacteriaceae* which are resistant to all classes of β-lactams has rapidly spread worldwide (46, 47). The nature of this project has also evolved and developed to study each new threat as it appears. It is important that if copper alloy surfaces are to be employed in healthcare or community settings that they are efficacious to a wide range of pathogenic microorganisms and that they remain constantly killing surfaces, especially after the considerable expenses incurred in the cost of materials and installation. Studies were then carried out to investigate the efficacy of copper alloys to kill a rapidly spreading cephalosporin-resistant but carbapenem-sensitive uropathogenic serotype of ESBL *E. coli*, ST 131 and *Klebsiella pneumoniae* harbouring a

plasmid encoding NDM-1, conferring resistance to not only all β-lactams including carbapenems but also many other antibiotic classes (Appendix IV).

2.3 Efficacy of copper surfaces to prevent transfer of antibiotic resistance genes between bacteria

HGT, whether by conjugative transfer or integrative uptake, has been primarily responsible for the evolution of the world's most dangerous pathogens (21). This occurs *in vivo* as in transfer to bacteria of the same or different species in the gut that can occur within a single individual which has been observed with *bla* $_{\text{NDM-1}}$ (48). There are few reports of the significance of HGT occurring on environmental surfaces from bacteria to others in the immediate vicinity although Kruse and Sorum (49) observed gene transfer between food-borne pathogens on kitchen chopping boards in 1994. In this study experiments were performed to investigate this phenomenon by inoculating surfaces simultaneously with antibiotic resistant pathogens and recipient bacteria and determining if antibiotic resistant genes could be transferred on solid surfaces and if biocidal copper alloys could also influence the rate of gene transfer (Figure 5). Because of the multidrug resistant natures of *E. coli* and *K. pneumoniae* donors, selection of transconjugants is difficult. The kind donation of an azide resistant *E. coli* as a F- recipient strain enabled selection of transconjugants that were antibiotic and sodium azide resistant . (Appendix IV)

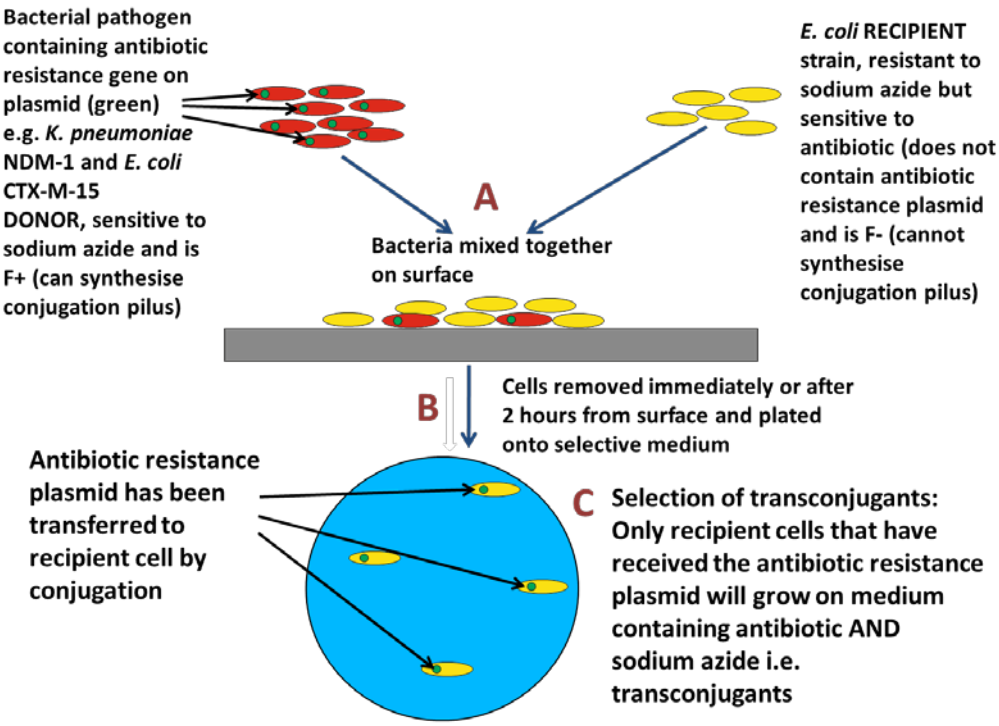


Figure 5 Diagrammatic representation of the method used to investigate gene transfer on solid surfaces

Simplified diagrammatic representation of laboratory experiments to see if antibiotic resistance plasmids can be transferred to recipient cells on surfaces. A: donor cells containing antibiotic resistance plasmid and recipient cells that were sensitive to antibiotic, but resistant to concentrations of sodium azide that kills the donor cell, were inoculated at the same time onto surfaces. B: at the required timepoint cells were removed from the surface by gentle pipetting. C: recovered bacteria were plated onto medium containing antibiotics and sodium azide. Only transconjugants, recipient cells that have received the plasmid, were able to grow on bacteriological medium containing antibiotic and sodium azide. After 24 hours growth the initial transconjugants were transferred to a fresh plate and incubated a further 24 hours to determine if the plasmid acquisition was stable.

2.4 Efficacy of copper alloys to inactivate pathogenic viruses

The other part of the project was to investigate the efficacy of copper alloys against pathogenic viruses. Noyce et al. (39) had shown, using a direct fluorescence method, that exposure to dry copper was efficacious against influenza A compared to exposure to stainless steel. In the studies reported here the efficacy of copper and copper alloys to inactivate norovirus, which is a main cause of viral gastroenteritis and estimated to cost at least 100 million pounds to the NHS

in the UK each year, was investigated (Appendix V)). This virus, which has a simple construction of positive strand RNA surrounded by icosahedral arrangement of capsid proteins compared to the complexities of bacterial structure, is extremely infectious owing to a very low infectious dose, robust structure, replication of sub-genomic RNA to increase the potential of the small genome and environmental contamination all of which plays a part in the spread of infection (50). This is primarily aided by the projectile vomiting and diarrhoea experienced by the sufferer which results in environmental contamination. In addition, the virus is resistant to many commonly used cleaning agents. Although there are numerous molecular techniques to detect human norovirus the fact it cannot propagate *in vitro*, in spite of numerous attempts to do so, makes the study of infectivity difficult unless human challenge studies are done. Studies were performed here using the closest surrogate for the human virus, the murine virus, MNV-1, which can infect a murine macrophage monocyte cell line, RAW 264.7, producing a cytopathic effect that can be visualised and enumerated using a plaque assay (51). The CW1 strain is an attenuated stain but similar to wild type MNV-1 and closer to the human virus than the feline norovirus which was previously used as a surrogate but causes a respiratory infection rather than gastro-intestinal infection in many species of Family *Felidae*.

2.5 Potential killing mechanisms of copper and copper alloy dry surfaces against microbial pathogens

For all pathogens under investigation several approaches were employed to investigate the kill rates and mechanism on copper and copper alloy surfaces

(i) Generation of reactive oxygen species

These studies not only investigated the efficacy of copper and copper alloys surfaces to kill or inactivate a diverse range of important pathogens that are a significant threat to all but also attempted to discover the mechanism and possible targets of the action of copper antimicrobial toxicity. It is well documented that soluble copper and iron can exert toxic effects by the synthesis of reactive oxygen species in aerobic systems via Haber-Weiss and Fenton reactions (52). In mammalian cells copper toxicity is thought to originate from redox cycling between Cu I and II oxidation states. In aerobic conditions this can lead to generation of reactive oxygen species (ROS) particularly hydroxyl ion ($\mathbf{OH}^{\cdot}$) and superoxide ($\mathbf{O_2}^{-}$) from reactions with dihydrogen peroxide (can be a by-product of electron transport chain of bacteria and also generated at copper surface in aqueous systems) which are known to inflict damage to many cellular components including structural proteins and DNA

Haber-Weiss reaction generation of ROS

$H_2O_2 + \mathbf{O_2}^{-} \rightarrow O_2 + OH^{-} + \mathbf{OH}^{\cdot}$ (equation 1)

Reduction of copper II to Copper I by superoxide:

Cu (II) + $\mathbf{O_2^-}$ → Cu (I) + O_2 (equation 2)

Fenton reaction between reduced metal ion and hydrogen peroxide resulting in generation of ROS

Cu (I) + H_2O_2 → Cu (II) + O_2 + OH^- + $\mathbf{OH^\cdot}$ (equation 3)

For all microorganisms studied the role of cuprous Cu(I) and cupric Cu(II) was investigated, the latter dominating in aqueous systems. This was done using chelators, bathocuproine disulfonic acid (BCS) and ethylenediaminetetraacetic acid (EDTA), to chelate Cu(I) and Cu(II), respectively, and observing the effect on pathogen survival following exposure to copper surfaces in culture. Likewise the role of individual ROS species was investigated using quenchers including D-mannitol to quench hydroxyl radicals; 4,5-dihydroxy-1,3-benzene disulphonic acid (Tiron) and superoxide dismutase (SOD) to quench or dismute superoxide. Observation of ROS synthesis in bacteria *in situ* on copper was done using carboxylated and fluorinated derivatives of fluorescein which are internalised into the bacteria cell where esterases remove acetate groups allowing oxidation by any ROS present which can be visualised using epifluorescence microscopy. The effect of copper ion chelators and quenchers on bacterial respiration and viral infectivity were also investigated.

(ii) The effect of exposure to copper and copper alloy dry surfaces on bacterial membranes

It seems logical to investigate the effect on bacterial membranes, responsible for influx and efflux of substances, conductivity, protection and maintaining equilibrium required for survival. The cytoplasmic membrane is a single membrane beneath a thick cell wall in Gram-positive cells which is shielded by an outer membrane and thinner cell wall in Gram-negative cells (Figure 6, reference 53). There is an electrical potential difference ($\Delta\Psi$) of approximately -100mV across this membrane which dissipates to 0 if the membrane is compromised and the bacterial cell dies as a consequence. The effect of exposure to copper and stainless steel surfaces on the membrane potential was investigated using cationic lipidophilic dyes, including rhodamine, which accumulate in the membrane and can be observed *in situ* using epifluorescence microscopy (54). Cells with intact membranes fluoresce but if the $\Delta\Psi$ dissipates this can be seen as a reduction in fluorescence. The carbocyanine dye, DiOC2(3) (3, 3'-diethyloxacarbocyanineiodide) accumulates in bacterial membranes in monomers (staining green) and a red shift in aggregates (stain red). If the membrane potential dissipates the intensity of red fluorescence diminishes as the aggregates separate.

A more usual way to look at the integrity of the bacterial membrane is to use intercalating nucleic acid stains of different sizes and affinities. SYTO 9 is a green fluorescent stain that exhibits brighter fluorescence when intercalated to double stranded DNA. It is a small molecule capable of passing through intact bacterial membranes and so stains live and dead cells. Propidium iodide (PI) is also an intercalating red stain for DNA but the molecules are much larger and can only traverse damaged bacterial membranes where the bound SYTO 9 can be displaced by the higher affinity of PI. Thus dead cells stain red. However, there are problems with this method including the fact that occasional temporary membrane fluxes in viable cells may allow entry of PI, giving misleading results (55). Another problem is that the method requires the nucleic acid to be unaffected by other parameters. Initial experiments gave poor results when studying bacteria exposed to copper alloys suggesting that the bacterial DNA was affected by exposure to copper which led to a series of experiments to investigate this premise.

The effect of exposure to copper on the outer membrane of Gram-negative cells was done by modifying a method described by Peng et al. (56). If the semi-permeable outer membrane is compromised, exposure to lysozyme, a large glycoside hydrolase found in tears and other bodily secretions, can then disrupt the peptidoglycan bacterial cell wall by binding and weakening the β 1-4 bonds between N-acetyl-D-glucosamine and N-acetylmuramic acid which then breaks resulting in cell lysis. This can be visualised using the Gram stain which stains intact cell wall with a crystal violet- iodine complex. Preliminary experiments using wheat germ agglutinin, a lectin that binds to peptidoglycan, were done to investigate the integrity on bacterial cell walls.

(iii) The effect of exposure to dry copper and copper alloy surfaces on bacterial nucleic acid

A series of methods were employed to investigate the effect exposure to copper and copper alloys had on bacterial DNA and the norovirus positive strand RNA genome. Bacterial plasmid and genomic DNA or norovirus RNA from organisms exposed to copper was extracted and purified. The size of fragments was studied using agarose gel electrophoresis. In addition bacteria recovered from copper surfaces were suspended in thin layers of agarose gel and permeabilised enough to allow genome fragments to protrude from the cells in a DNA fragmentation assay modified from the method described by Fernandez et al. (57). The cells were stained with sensitive nucleic acid stain SYBR Gold which stains single and double stranded DNA. This gentle analytical procedure allowed observations of intact or damaged genomes of individual cells. In addition to looking at the entire genomes primers were designed for specific regions of the bacterial genome or plasmids or viral genome to amplify and quantify microbial genes using real time PCR.

3: Results of research 2008-2013

The publications resulting from this work are referenced in Appendices I to V

3.1 Rapid death of Gram-positive and Gram-negative bacteria occurs on copper and copper alloy surfaces accompanied by inhibition of respiration

Results showed that the vancomycin-resistant enterococci, representatives of the two main pathogenic species, retained viability for over 60 days on stainless steel surfaces (Appendix I). In contrast the same inoculum, 10^6 colony forming units (CFU) per cm^2, were killed following 1-3 hours contact at room temperature. The inoculum concentration was a worst case scenario because reports of actual contamination of hospital surfaces are considerably lower at several hundred cells per 100 cm^2 (58). The kill rate was directly proportional to the percentage copper in the alloys although nickel silver occasionally out performed cartridge brass even though it contained 5% more copper. The results for this are unclear. Nickel is reported not to have any antimicrobial properties as a touch surface. Therefore other factors including surface finish of the metal, the presence of any surface oxides which could affect hydrophobicity and surface charge may be responsible but the effect was not large enough to affect the recovery of the bacteria with the method used at time 0. Reduction of the inoculum size decreased the kill time and virtually abolished the differences between the alloys although a minimum kill time of 20 minutes was achieved. The numbers of respiring cells enumerated by CTC staining were consistently higher than the culture results but this was not statistically significant suggesting enterococci do not remain on copper alloys as VBNC. Copper alloys were efficacious for laboratory and clinical strains of enterococci regardless of the presence of antibiotic resistance genes. *E. faecium* kill times were slightly longer which has been reported previously.

These experiments simulated wet droplet contamination of fomites from contamination with body fluids. However, a lot of contamination comes from touching surfaces with contaminated fingers. In this instance the aqueous content is low and dries very rapidly. Applying the same number of bacteria in 1/20 volume results in an inoculum that dries in seconds compared to 30-45 minutes and was found to be more reproducible than using a swabbing technique.

The kill times were considerably shorter with a 6-log reduction in viable enterococci within 10 minutes suggesting either a different mechanism of kill or the mechanism is the same and occurs up to 6x faster (Appendix II). The same results were seen for simulated dry touch

contamination of copper alloys with Gram-negative bacteria including *E. coli* pathogenic (including ESBL producers) and ancestral strains, *Salmonella spp.* and *Klebsiella pneumoniae* (Appendices III and IV). Espirito Santo et al (59) investigated this phenomenon and discovered that rates of copper ion uptake from surfaces were different in wet and dry conditions. In this interesting study, using inductively coupled plasma mass spectroscopy, they observed that in the first 5 seconds required to process the 'Time 0' sample for the dry test (the inoculum was applied by a swab and had dried by this time) bacteria had accumulated approximately 3.8×10^9 copper atoms per cell. This remained steady for the 2 minutes of the test, with a slight increase at 40seconds. Copper uptake in the dry test was 200,000 times greater than cells removed at 'Time 0' for the wet test. In the latter a steady increase of $1x\ 10^6$ atoms copper per cell per minute occurred for the 3 hours of the test reaching a maximum of 1.5×10^8 copper atoms per cell. The rapid drying of the inoculum may result in changes to the bacterial structure allowing increased influx of copper ions into the cells. This may also involve increased copper ion dissolution from the surface as the liquid evaporates. This massive uptake of copper ions in the dry test explains the rapid kill rate. However in the wet test the more gradual copper ion uptake was proportional to the rising kill rate.

No VBNC bacterial cells were detected on copper surfaces for all bacterial species tested in this study, suggesting culture results are accurately determining total kill times and supporting the use of copper surfaces as antibacterial surfaces. The kill times were not significantly different between Gram-positive and Gram-negative bacteria meaning that their structural differences do not affect efficacy. In addition, previous work has shown that bacterial spores of the anaerobic pathogen, *Clostridium difficile*, which may remain dormant in the environment until conditions allow them to germinate and carry on the infection cycle, are also killed on copper alloy surfaces (35).

The kill times can also be affected by the consistency and composition of the inoculating matrix itself. Initial studies by Noyce et al. (60) observed that death of the foodborne pathogen, *E. coli* O157, was delayed on copper surfaces in the presence of animal fat and meat juices.

Likewise, in this current study, if bacteria were inoculated in brain-heart infusion broth, a bacteriological medium high in lipid and salt content the kill times were longer than if inoculated in phosphate buffered saline (PBS). In addition Molteni et al. (61) described the dissolved copper ion release that was directly proportional to the kill rate was greater in neutral Tris-Cl buffer compared to PBS, presumably due to increased copper ion dissolution and the formation of Tris2Cu complexes.

3.2 The cytoplasmic membrane of Gram-negative cells is rapidly depolarised and compromised on contact with copper and copper alloy surfaces.

In *E. coli* and *Salmonella spp.* there was immediate depolarisation of the cytoplasmic membranes upon exposure to copper surfaces. This also occurred on copper alloy surfaces but was slightly delayed, presumably due to lower copper concentration. The outer membrane was also compromised but not immediately. Cations have an affinity for the lipopolysaccharide (LPS) in the outer membrane but do not appear to affect membrane viscosity. However it has been suggested that breakage of disulphide bonds in the ATPase responsible for K+ transport in cytoplasmic membrane, which increased the number of free-SH groups, may be responsible for changes in membrane potential (62). The unsaturated double bonds in eukaryotic membranes may undergo non-enzymic peroxidation by ROS, especially hydroxyl radicals. Hong et al (63) suggest this happens in bacteria. They observed immediate increase in the concentration of thiobarbituric acid reactive substances (TBARS) which are the breakdown products of oxidised lipids, including reactive aldehydes, in *E. coli* exposed to copper surfaces. In this study chelators of Cu(I) and Cu(II) were used to determine the role of copper ions in membrane depolarisation but results were inconclusive because of interference in the experimental procedure e.g., EDTA resulted in increased uptake of Rhodamine 123 compared to control cells, but the results did give partial protection suggesting copper ions are required for rapid depolarisation. This requires further work and the results were not included in the publications.

In enterococci the cytoplasmic membrane was not depolarised initially on contact with copper although respiration was affected. The bacterial nucleic acid had started to break down before the membrane was compromised. The reason for this is unclear but it highlights that copper surface toxicity is multi-faceted, complex and there is not a single universal mechanism. Lemire et al (26) offered a word of warning that bacterial membranes contain a lot of monounsaturated fatty acids which are unreactive in lipid peroxidation reactions *in vitro* and that it is unclear if loss of membrane integrity in bacteria exposed to copper is the cause of cell death or a consequence.

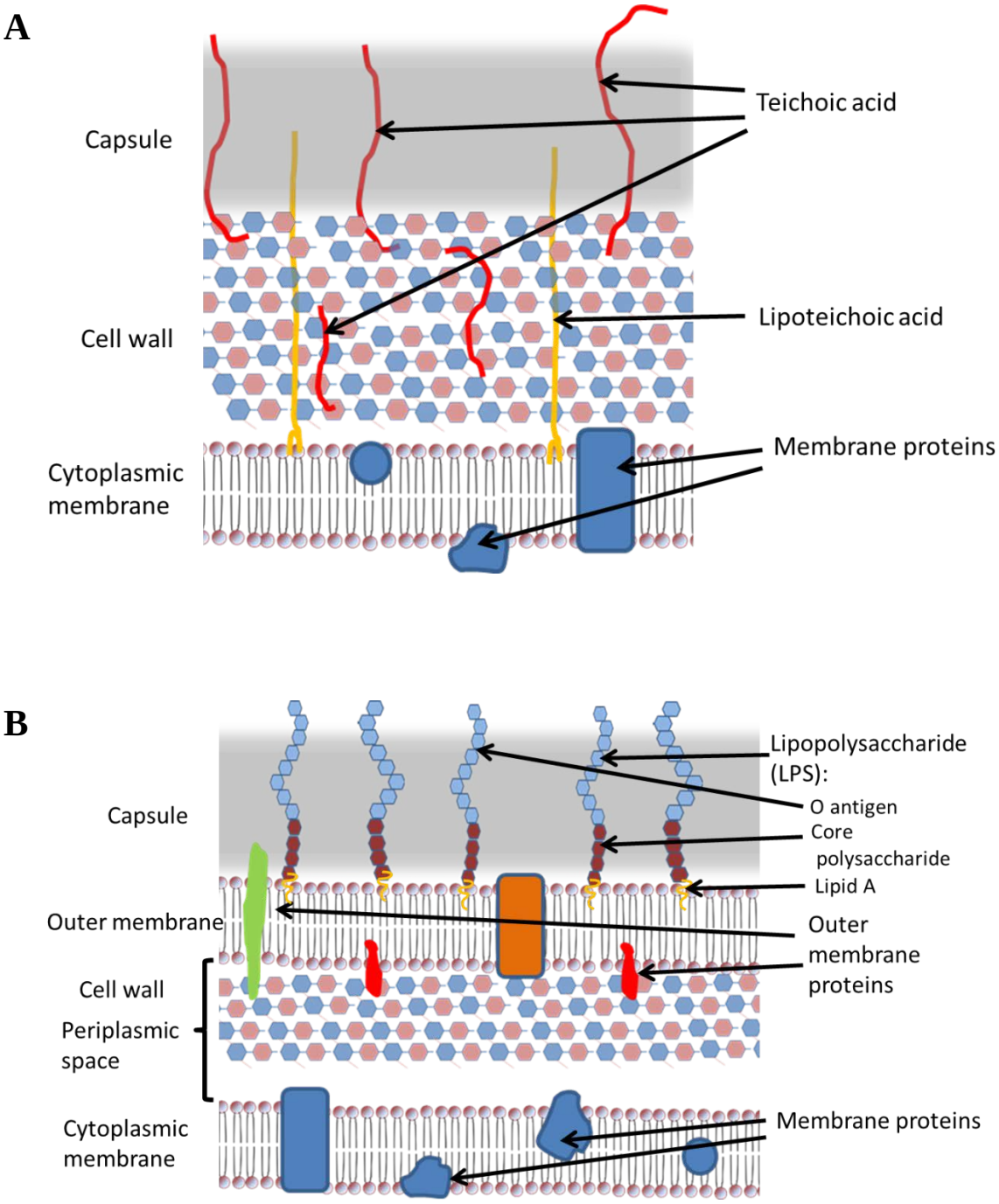


Figure 6 Outer structures of bacterial cell walls

A: The structure of the Gram-positive bacterial cell wall. The cell membrane is surrounded by a thick wall of amino acids and sugars ,peptidoglycan (PG) , which are sheets of repeating dimers of N-acetylglucosamine and N-acetyl muramic acid linked by short peptides. Teichoic acid associated with the wall or the phospholipid membrane are PG-linked polymers important in

maintaining cell shape, cell division, ion homeostasis and pathogenesis. A variety of proteins are located in the membrane including secretory proteins, respiratory enzymes and porins. A surface layer of polysaccharides for example teichuronic acid (a polymer repeating dimers of N-acetyl galactosamine and D-glucuronic acid) or polypeptide chains may be present forming a capsule or looser slime layer a glycocalyx.

B: The structure of the Gram negative bacterial cell wall. The cell has a double membrane structure. The outer membrane is semi-permeable and contains porins and transport proteins and vesicles 'bud' off frequently. The periplasm is the chamber between the two membranes and is an area of metabolic dynamic flux housing enzymes for cell wall growth and degradation. There is a thinner cell wall and lipopolysaccharide (LPS) chains extending from the outer membrane have endotoxic and immunogenic properties.

Structure reviewed in Swoboda et al., 2010 (53)

3.3 Copper ion release from the metal surface is essential to copper and copper alloy dry surface toxicity

Removal of Cu(I) and Cu(II) by chelators destroyed the efficacy of the copper and copper alloy dry surfaces to kill enterococci (in aerobic and anaerobic conditions) and *Enterobacteriaceae* suggesting release of these ionic species is essential, directly or indirectly, to the killing mechanism. This was also true for the simulated dry touch contamination which was a little unexpected because it was predicted that the copper ion release, which has been found to be proportional to the killing rate, is dependent on the aqueous nature of the matrix (61) and requires bacterial cell-surface contact (64) . Perhaps the ion release is extremely rapid as the inoculum dries in the dry touch contamination and may be affected by the drying process itself. Cu(I) appeared to be more significant in the kill of both laboratory and clinical strains *of E. faecalis* than Cu(II); however, it is unclear if this is due to the reduction of Cu(II) released from the metal surface to Cu(I) by the release of extracellular superoxide which is a virulence factor of this pathogen *in vivo* and the production of ROS *in vivo* also damages the DNA of gastric cells (65). The resulting toxicity from copper ion release may be from the direct effect of ions and/or the generation of ROS from the reduced ion.

3.4 Reactive oxygen species are generated by bacteria on copper and copper alloy dry surfaces

The addition of ROS quenchers also affected the kill times of bacteria on copper and copper alloy surfaces. In enterococci short term protection was observed if superoxide was quenched or dismuted but there was no evidence of hydroxyl radical generation (Appendix II); however, copper ion release was responsible for continued killing. Figure 7 summarises possible toxicity mechanisms in Gram-positive bacteria which is discussed further in Section 4

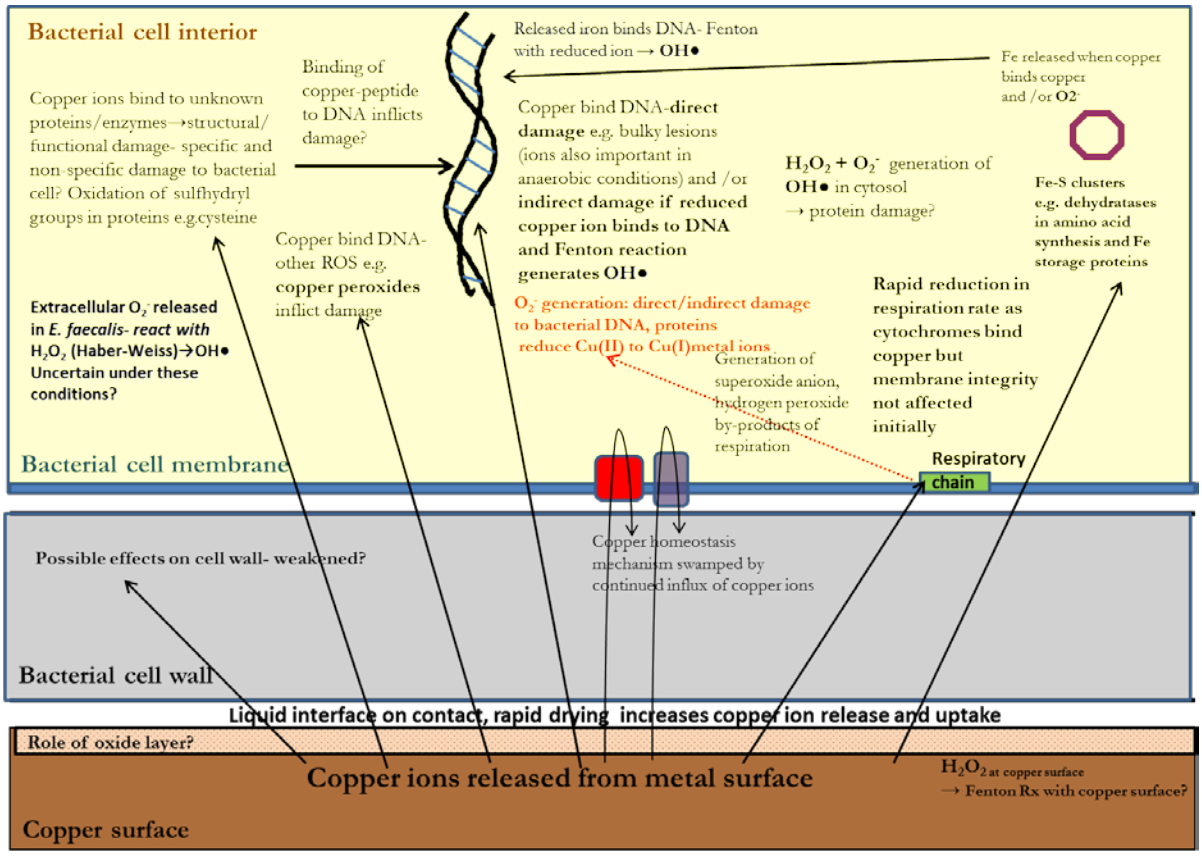


Figure 7 Summary of possible mechanisms of toxicity in Gram-positive bacteria

Diagrammatic representation of multi-site attack of copper on Gram-positive bacteria

Copper ion release from the surface is essential for efficacy which can be affected by the metal composition and arrangement of atoms i.e. other metal constituents of alloys, presence of oxide layers or other copper compounds, the composition and aqueous content of the matrix containing microorganisms (blood, sputum etc.). Homeostasis machinery is soon swamped by continual copper influx. Copper ions flood into cell and inflict damage to respiratory enzymes but membrane integrity is not compromised immediately. Copper ions directly or indirectly, through the generation of reactive oxygen species, especially superoxide anion, O_2^-, damage bacterial DNA causing it to disintegrate into small fragments. Fenton reaction generated hydroxyl radicals which are known to inflict nucleic acid damage were not detected in this study for Gram positive bacteria only for Gram negative cells. In the latter the presence of a periplasm resulted in accumulation of copper and immediate cytoplasmic membrane depolarisation. The continued influx of copper ions results in further multi-site damage to bacterial structural components and metabolism resulting in the death of the cell.

However in Gram-negative bacteria ROS were also important in copper surface toxicity but hydroxyl radicals were the dominant species, also observed by other groups (40); nevertheless, potentially other ROS may be involved because D-mannitol was only partially protective in some situations. The generation of ROS occurred throughout contact time and not just upon immediate contact. The bacterial cells continued to respire in the presence of chelators and ROS quenchers suggesting direct or indirect effect on respiratory metabolism by copper ions and ROS.

3.5 Copper ion release is also required for norovirus inactivation on copper and copper alloy surfaces

These studies have shown that murine norovirus is also rapidly inactivated on copper and copper alloy surfaces. Inactivation is once again even faster if the contaminating virus is applied as a dry touch contamination suggesting a synergistic effect of copper action and the rapid drying out of the virus suspension. The instrument of toxicity towards norovirus following prolonged exposure is generation of Cu(I) because specific Cu(I) chelators were protective in the long term. Interestingly, and in contrast to the results for bacteria, Cu(II) generation is more important short term, upon immediate and up to the first hour of contact. There was no evidence that ROS were important in the inactivation of norovirus (Appendix V) which perhaps is not surprising with the absence of respiratory machinery although ROS have been observed to play a part in inactivation of enveloped DNA virus exposed to soluble copper (66).

3.6 Destruction of nucleic acid of bacteria and viruses occurs on copper and copper alloy dry surfaces

All microorganisms under investigation exhibited degraded nucleic acid upon exposure to copper and copper alloy dry surfaces. The denaturation of the entire genome was observed *in situ* with DNA fragmentation assay and in DNA purified from bacterial cells exposed to copper alloy surfaces. It was especially interesting that genomic and plasmid DNA destruction in Gram-positive bacteria occurred immediately on contact but was delayed in bacteria with an outer membrane. It is possible to speculate that the periplasmic space provides spatial separation (40) initially as copper ions influx into the cell and delays contact with the nucleic acid. Coppersensor-1,CS-1, is a membrane permeable dye which is 10x more fluorescent when bound to Cu(I) and is used to locate the position of copper within eukaryotic and prokaryotic cells. Espirito Santo et al.,(59) observed high concentrations of Cu(I) accumulating over time in cells exposed to moist or dry copper surfaces using CS-1. However, it is unclear if the staining observed includes copper(I) in the periplasm as well as in the cytosol. Ramos et al., (67) observed accumulation of copper in the periplasm of the Gram-negative plant pathogen,

Xanthomonas campestris, exposed to high concentrations of cupric sulphate using electron spectroscopy imaging. The periplasm also contains the homeostatic machinery to remove excess copper which is presumably effective initially but is soon swamped by the continuous influx of ions which is as ineffectual as 'holding back the sea'. Hydroxyl radicals are highly toxic to a diverse range of biomolecules but only to those in the immediate vicinity because the range of activity is very short (68). In humans the long term effects of exposure to low levels of hydroxyl radicals is known to affect cellular DNA and this is a contributing factor to the aging process and in degenerative diseases including Alzheimer's. It is known that hydroxyl radicals generated in aerobic bacteria by Fenton reaction chemistry in solutions of copper salts destroys the bacterial nucleic acid by degrading the ribose ring (69). In this work it has been shown that the rapid influx of copper ions from a copper surface produces a massive disintegration of bacterial DNA that was observed in DNA fragmentation assay but also fragments are so small that copy numbers of individual genes are affected, indicated by the PCR results. Damage to the bacterial DNA could be protected by chelators of Cu(I) and Cu(II) suggesting copper ion release is an essential part of the killing mechanism which directly or indirectly damages the DNA. It is interesting that the predominant ROS generated varies between bacteria with different morphologies and that although hydroxyl radical generation was significant in *E. coli*, in *Salmonella* the effect was partial. This suggests the role for other ROS, including peroxides and singlet oxygen, may be important.

3.7 Summary of results

The results have demonstrated that copper and copper alloys are efficacious against a diverse range of pathogenic bacteria but the killing mechanism and targets of copper toxicity are influenced by morphological and physiological differences in bacterial species (summarised in Figure 8).

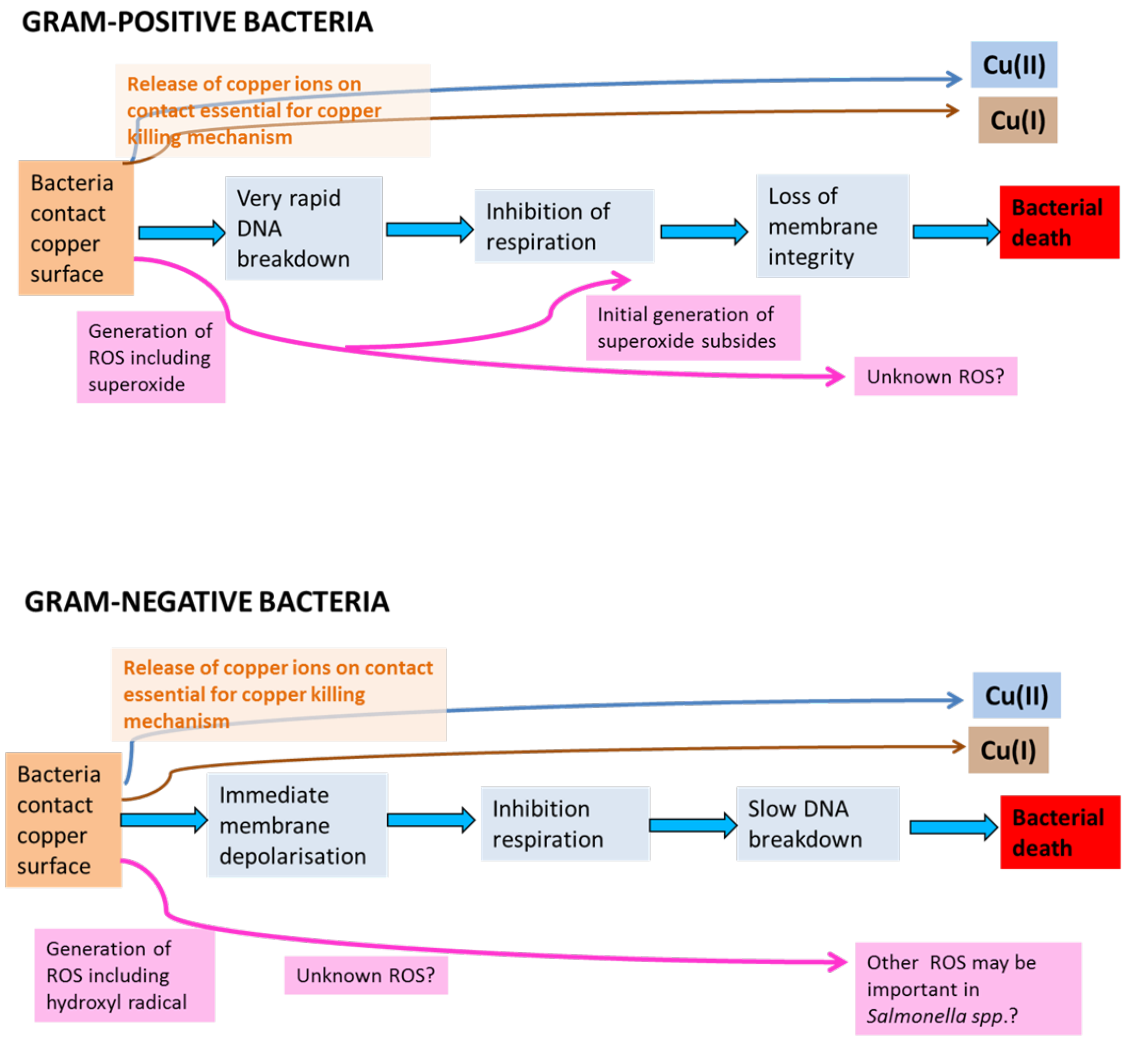


Figure 8 Summary of chronological order of bacterial cell death on solid copper surfaces

In Gram-positive bacteria uptake of copper ions and generation of reactive oxygen species (ROS) results in rapid disintegration of bacterial DNA and inhibition of respiration. Superoxide is the primary ROS although the hydroxyl radical, which is known to degrade DNA through Fenton chemistry was not detected. It is unclear if nucleic acid damage is a direct result of copper ions and superoxide or the indirect action of these moieties on other cell components. Although the respiratory machinery is rapidly affected by exposure to copper the single cell membrane is not depolarised immediately.

In Gram-negative bacteria copper ion release and generation of ROS is also part of the killing mechanism. However it is the hydroxyl radical that is the main ROS although other ROS may be important in species other than *E. coli*. The immediate depolarisation of the cytoplasmic membrane occurs on contact with copper and inhibition of respiration. Damage to the bacterial DNA occurs more slowly.

3.8 Statistical analysis

Data are expressed as mean ± standard errors of the mean (SEM) (or mean ± standard deviation (SD)) and are from multiple independent experiments. 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 and graphical representations were performed using Sigma Plot version 12.

4: Discussion and further work

4.1 Copper ion release from surfaces is essential to efficacy

This study observed that freshly cut copper or copper at various stages of oxidising tarnish were effective at killing bacteria and viruses, the kill time could be delayed in the presence of contaminating lipids and proteins and that copper ion release was essential to efficacy. Exposure to copper and copper alloy surfaces prevented bacterial growth, formation of biofilms, VBNC and inhibited the respiration of bacterial cells. Norovirus was irreversibly inactivated upon exposure to copper and copper alloys. Kill rates were affected by the percentage copper and many other parameters including composition of the matrix, aqueous content and drying time, temperature. The most important factor to retain a constantly killing surface is to ensure the absence of chelating agents in surface cleaning agents and frequent removal of contaminating substances to ensure continuous copper ion release. Studies on copper alloy coins revealed an array of bacteria able to survive in the dirty matrix, presumably because the bacteria were protected from copper ion influx (70). However the topography and finish of the metals tested was not investigated. Recent studies looking at the antimicrobial qualities of thermally sprayed copper coatings discovered that the increased surface roughness, with concomitant increased surface area, improved antimicrobial efficacy. This was also due to the formation of carbon-copper-phosphate nanoflowers because of the PBS matrix (71).

Further work is required to investigate the effect of different manufacturing polishing and buffing finishes to the metal which could also affect the degree of copper ion release and subsequently also the kill times for microorganisms. The extent and type of surface oxidation of copper and copper alloys could also have an effect.

Copper and copper alloys develop layers of oxide upon exposure to air and the composition and rate of deposition of which is affected by humidity, temperature and time. This primarily consists of a layer of cuprous oxide

$4Cu + O_2 \rightarrow 2Cu_2O$ (equation 4)

which is then oxidised as Cu^+ and electrons migrate away from the surface to form a layer of cupric oxide that occurs more slowly. The presence of these oxides could be affecting the results.

The surface testing experiments performed here have been performed using acetone-cleaned coupons which were then flamed in ethanol to sterilise them which will contribute to the oxide

layer. Hans et al (72) investigated the killing potential of heat generated oxide layers and found cuprous oxide had the same kill rate for *E. hirae* as pure copper but that cupric oxide was inhibitory, surmised to be from reduced ion release from this oxide. The predominant Cu^{+} released from cuprous oxide would be expected to disproportionate in aqueous environments. It seems logical to suggest that the rapid killing observed with dry touch simulated inoculation may be due to the presence of more toxic Cu^{+} but chelators of cupric and cuprous ions both provided long term protection to bacteria tested in this study for simulated dry touch as well as wet fomite contamination tests. However it may be that the increased role of Cu(I) that we have observed in the inactivation of norovirus may arise partially from surface oxidation layers.

Preliminary experiments at the beginning of this study (2008) compared fresh cut copper coupons and old coupons that had been through washing, drying, autoclaving and exposure to the air for several years. Those coupons displaying visible surface blackening, presumably due to cupric oxide formation, demonstrated the same total kill times for *E. faecalis* and *E. faecium* (referred to in Appendix II but results not shown). It was interesting that upon immediate contact the presence of an oxide layer resulted in a slightly slower kill rate for *E. faecalis* compared to freshly cut copper as observed by Hans et al. However, the opposite was true for *E. faecium* where the oxide layer resulted in a faster kill rate over the first 30 minutes of contact with 'wet' fomite inoculum and more rapid DNA degradation observed in agarose gel electrophoresis of purified DNA of bacteria removed from the two copper types described (not included in the publications). The mechanisms for this is unclear but may involve copper ion killing from the oxide rather than the metal surface and requires further work because the number of samples was relatively small. The effect of copper oxides on bacterial kill rates may not be significant when comparing survival rates on copper surfaces and non- copper containing surfaces in real life situations where the development of oxide layers occurs more slowly and surfaces will have been subjected to numerous cleaning protocols.

Copper surfaces exposed to the indoor and outdoor atmosphere which may contain high level of pollutants may also develop layers of black copper sulphides. Numerous studies have investigated the footprints of air pollution and the changing environment on our built environment structure (Reviewed by Kumar 73). Copper foils exposed to artificially high concentrations of sulphur dioxide (0.5%), 75% RH for 90 minutes in glass test chamber observed copper I oxide, copper I sulphide and sulphuric acid formation occurred which led to pitting of the surface observed using SEM. However, they suggested that the copper sulphides are unstable and oxidised with prolonged exposure to air (74).There have been many studies investigating the effect of atmospheric pollutants on indoor surfaces, particularly on museum artefacts and art treasures throughout the world. The most damaging substances include hydrogen sulphide but also carbonyls with carbonyl sulphide, often emitted from historical wool

containing textiles, being one of the most critical (75). The atmospheric sulphide concentrations indoors are usually low and any sulphide films that do form on a copper surface do not uniformly cover the surface. However this could affect the antimicrobial properties and further investigations are required. The blackening of old copper coupons used in this study may have copper sulphide deposits.

Many studies have investigated the bioavailability of copper in aqueous systems, copper-based anti-fouling treatments in the marine industry and *in vivo* uses of copper such as dental amalgam's and intrauterine devices which is affected by water characteristics such as pH, concentrations of salts and dissolved organic matter. There is a balancing act between the requirement for copper to reduce fouling for example on the base of ships and limiting the release of copper into the marine environment to prevent damage to natural ecosystems present therein. (76). Copper employed as dry outdoor surfaces on buildings is exposed to the atmosphere where the rate of copper corrosion is affected by pollutants and weather conditions. Many studies have investigated copper run off from building surfaces in rain water which enters soil and rivers (reviewed by Hedberg 2014, 77). There is very little information on corrosion of copper surfaces indoors. Copper corrosion occurs up to 100 times faster outdoors due to the higher relative humidity. It is difficult to determine the degree of corrosion, for example, of a brass door handle which may be in place for decades and would require measurements of loss of overall mass. It is unlikely that environmental release of copper will be a significant issue from dry copper alloy surfaces indoors. However, sluicing of contaminated copper surfaces with cleaning agents may release copper into water supplies and this warrants further study. Also the nature of contaminant may affect copper corrosion, for example, regular contamination with acidic vomitus may result in a higher corrosion rate.

These investigations should be incorporated into clinical trials determining the efficacy of copper containing surfaces to reduce bioburden and infection spread.

4.2 The role of copper ions in bacterial killing and virus inactivation on dry copper biocidal surfaces

4.2.1 Indirect effect of copper ions

It is surmised that copper toxicity in biological systems is primarily due to the generation of ROS from reactions of cuprous ion and respiratory intermediates, as described earlier. In this study the short term generation of superoxide in enterococci and hydroxyl radicals in Gram-negative pathogens were important but not the whole story. In the absence of oxygen copper ion

release alone was responsible for the death of enterococci exposed to copper surfaces in this study.

The ROS quenchers used in this study have some limitations. The lack of specificity has been described by others. The rapid DNA degradation observed in Gram-positive bacteria that did not appear to be a result of hydroxyl radical generation requires further work. It is unclear if hydroxyl radical generation IS occurring but it is not being detected. This may be due to conformational changes in nucleic acid and/or cellular proteins induced by free copper ions preventing D-mannitol access to the radical. If the generation of hydroxyl radicals is a short term intermediate stage in the generation of other radicals it may be very difficult to detect. The partial quenching by D-mannitol in *Salmonella* species supports this premise. Some experiments were performed using 3'-(p-aminophenyl) fluorescein (APF). APF is a more stable fluorescein derivative which is non-fluorescent until it reacts primarily with the hydroxyl radical but also peroxynitrite and hypochlorite anion. Upon oxidation, APF exhibits bright green fluorescence (excitation/ emission maxima ~490/515 nm) however this dye was oxidised on control stainless steel surfaces, presumably due to chromium oxide layer, making it unsuitable for our studies. It is highly probable that other ROS, including singlet oxygen and copper peroxides are generated in aerobic conditions when bacteria contact solid surfaces containing copper.

4.2.2 Direct effect of copper ions

Bacteria have an anionic surface with an isoelectric point pH 2-4 which means that metal cations can bind and affect the surface charge of the cell to become more electropositive. Beveridge and Fyfe (78) observed that Gram-positive bacteria had a larger charge capacity and bound 30 times more Cu(II) than Gram-negative cells. This was found to be due to binding to teichoic acid and carboxyl group of glutamic acid in the peptidoglycan cell wall forming large deposits close to the cross linking chains. Gram-negative bacteria have an outer membrane covering a much thinner cell wall surrounding the plasma membrane (Figure 6). Copper ions are known to react with the polar head groups of phospholipids, acidic group polypeptides and create patches in the lipopolysaccharide protrusions.

Preliminary studies using wheat germ agglutinin in this study suggested that copper ions may affect the bacterial cell wall itself although the cytoplasmic membrane is not an immediate target in Gram-positive bacteria tested here. This appears to be contradictory because it may be supposed that the peptidoglycan is affected and ‘mopping up’ an influx of copper ions but the genomic and plasmid DNA is immediately under attack presumably from copper ion activity. Perhaps differences in the composition of the membrane in Gram-positive and Gram-negative

cells and involvement of lipopolysaccharides in the latter play a role. Perhaps the cell wall is rapidly weakened destabilising the bacterial cells but further studies are required.

In specialised copper binding proteins such as SOD and cytochromes copper can be displaced by higher affinity metals. Leterlier et al (79, 80) observed for the first time, in eukaryote microsomal fractions, indiscriminate binding of Cu(II) (with a limited number of binding sites) to many other thiol containing proteins that could not be displaced even as copper influx increases. This did not occur with iron. They observed increased binding of free copper ions as other metals were added due to conformational changes that exposed further thiol groups. They observed that binding affected the structure and function of the proteins involved including cytochrome P450 and GSH transferase in liver cell extracts (81). This indiscriminate copper binding may reduce free copper for ROS generation creating a 'Cu^{2+} sink' and subsequent lipid peroxidation by ROS was reduced as all free copper ions were bound. They observed that the indiscriminate binding occurs very rapidly but ROS generation occurred slowly. Perhaps in our system the dry copper surface toxicity is a result of a combination of ROS generation and free copper ion binding to biomolecules. Metal ions can induce strand breaks and conformational changes directly in nucleic acid or enzymes involved in DNA replication and repair. Perhaps the extensive nucleic acid damage to bacteria in this study is due to combinations of bound metal ions, metal-peptide complexes and generated ROS including some species that have not been investigated in this study because breakdown of naked DNA was faster in viable compared to dead cells. The influx of copper ions into Gram-negative cells is initially very rapid into the periplasmic space providing temporary protection of the bacterial nucleic acid. Here homeostasis mechanisms are soon swamped and the fact the ions have now accumulated to a high concentration result in a rapid burst of ROS that produce peroxidation of membrane lipids compromising the membrane. Indiscriminate binding of copper ions may now affect a range of biomolecules within the cell resulting in death but the absence of free ions means ROS generation is now reduced. In enterococci the lack of a periplasmic space and binding of copper ions to the cell wall may mean accumulation of copper ions into the cytosol is slower but this is enough to produce a burst of superoxide which is overridden in time as indiscriminate copper ion binding takes over. In anaerobic conditions toxicity must be entirely due to copper ion binding (82).

Macomber et al proposed that ROS were not essential in copper killing process (83, 84). Copper oxidises exposed thiol groups and they suggested that copper ions displace iron from iron-sulphur clusters in metalloproteins and dehydratases involved in amino acid synthesis, thereby altering the conformation and functionality of these proteins. Possibly in the presence of oxygen it is the displaced iron that participates in Fenton chemistry (although the Fenton reaction is 60x faster with copper) (68). In this current study experiments were done using the chelator,

deferoxamine mesylate, but the lack of specificity for iron gave inconclusive results because of concerns that cupric ions may also be chelated so this work was not included in the publications from this study.

Copper specific dyes can be used to trace copper ions. Espirito Santo et al (85) also demonstrated intracellular copper ions in Gram-positive *Staphylococcus haemolyticus* exposed to dry copper surfaces using Coppersensor I (CSI) but suggested that ion uptake was slowed by the thick cell wall unlike their results with *E. coli* (52). The studies on enterococci suggested Cu(I) is more important than Cu(II) in dry copper surface toxicity in *E. faecalis* compared to *E. faecium*. It would be interesting to determine if CSI could be used to trace Cu(I) in these species on a longer timescale.

Nandakumar et al (86) did an extensive proteomic study investigating gene expression in *E. coli* exposed to copper surfaces which observed upregulation of genes involved in DNA regulation and repair. More information into the differences in mechanism of copper surface killing in Gram-positive and Gram-negative bacteria that has been observed may be obtained by similar proteomic studies looking at expression of individual genes.

4.3 Inactivation of norovirus on copper and copper alloy surfaces

These studies have demonstrated the rapid inactivation of norovirus on copper and copper alloy surfaces. Further studies are required to investigate a larger range of alloys and preliminary new work (unpublished and ongoing) has identified a small change in percentage copper can have a significant effect on viral infectivity. This study has demonstrated that the viral RNA genome is degraded by exposure to copper but further work is required to look at the effect on the capsid. Reports have described capsid peroxidation in human norovirus exposed to chlorine using biotinylated virions. It is proposed to try this methodology in murine norovirus exposed to copper alloys and also determine any morphological changes using transmission electron microscopy. Alloys containing a lower concentration of copper rapidly inactivated the virus initially but after 60 minutes the rate slowed. This may be due to sub-populations of norovirus with different susceptibilities, perhaps reflecting the integrity of the capsid at different stages in replication. Investigations into capsid integrity are required using protease and RNases which can access nucleic acid if the capsid is damaged or incomplete. Therefore the viral genes will only be amplified in viruses with intact capsids and protected viral RNA. Rather like the classification of bacteria into 2 major categories dependant on outer structure of the bacterial cell wall viruses can be roughly sorted into non-enveloped and enveloped varieties. The latter have an outer membrane derived from the host studded with proteins, often with highly antigenic domains, surrounding the basic structure of nucleic acid core and multimer protein

capsid. Norovirus does not have an envelope and is consequently more resistant to environmental stress with a low infectious dose. Preliminary experiments with influenza A and other respiratory viral pathogens are investigating efficacy of copper alloys and differences in the mechanisms of copper surface virus inactivation.

4.4 Concerns about copper resistance developing in bacteria exposed to copper and copper alloy dry surfaces

Concerns are regularly expressed that if copper resistance develops (which may be concomitant with drug resistance, virulence or stress response genes) copper biocidal surfaces may no longer be effective. An example of this is the transferable copper resistance genes, *tcrB*, in enterococci which also have antibiotic 'resistance' genes located on the same conjugative plasmids. These have developed from the feeding of cattle with copper sulphate, macrolides and tetracycline as growth enhancers (87). The results from this current study suggest this is unlikely because extensive nucleic acid degradation observed in all bacteria tested resulted in rapid kill and prevented HGT. The effect of different temperatures, exposure times, matrices on the efficacy of copper alloys to prevent HGT needs to be investigated. Mutations in genes responsible for copper homeostasis that are lethal upon exposure to elevated soluble copper concentrations have been shown to affect kill times on solid copper surfaces. However the bacteria containing copper 'resistance' genes still die rapidly on copper surfaces compared to ubiquitous surfaces like stainless steel (88) suggesting a tolerance rather than true resistance. Perhaps the continuous copper ion release exhibited on dry copper surfaces overwhelms the chaperones and ATPases involved in copper homeostasis. Further studies investigating the survival of these strains and copper extremophiles, which have a high minimum inhibitory concentration against copper (MIC 800 mM) (89), should be performed on copper surfaces.

Copper alloys have been shown by this and other studies to be very efficacious against bacteria, viruses and fungi. The efficacy of copper to kill more complex pathogens including protozoa including the food and waterborne pathogen *Cryptosporidium parvum* and other parasites which are responsible for millions of infections and deaths worldwide per year should be investigated.

4.5 The next step: clinical trials in high risk environments

There is now a considerable amount of evidence to suggest implementation of copper alloy surfaces could have a significant effect to reduce the spread of infection. Many laboratory studies have observed rapid killing of bacterial, fungal and viral pathogens.The natural progression of this work and other laboratory studies is clinical trials where copper alloy surfaces are incorporated in real life situations. Casey et al., 2010 (90) were the first to report a

significant reduction in bioburden in a busy ward of a hospital in the UK which had incorporated copper fitments. To follow on from this several clinical trials worldwide have shown a reduction in bioburden if copper alloy surfaces are incorporated into health care facilities and care homes (91-93). Weber's review (20) on self-disinfecting surfaces describes the pros and cons with a notable lack of data demonstrating reduction of infection rate in most clinical trial data. However, a recent study, incorporating only 6 surfaces within ICU in 3 hospitals in the USA, has reported > 60% reduction in infection rate (94). Although extremely encouraging this is just the tip of the iceberg. This pioneering study highlighted statistical flaws which can only be addressed by further, large scale studies worldwide where the results can differentiate between individual bacterial species and also investigate the effect on non-bacterial pathogens, while accounting for geographic differences in healthcare hygiene practices.

Many people are beginning to take notice, including health care authorities, industrial organisations, care facilities and the general public and wondering about the practicalities of installing copper alloy surfaces in their institutions or homes. This substantial progress could now be hampered by confusion about antimicrobial claims and the enduring problem of the lack of standardisation of the methodology worldwide. The world is a smaller place with more accessible travel and we have seen rapid worldwide spread of emerging pathogens which have no regard for border controls. The methodology used in one standard test should produce similar results to others regardless of country of origin although differences in ambient temperatures and relative humidity (RH) must be taken into account. In particular, caution should be expressed in interpreting the results for antimicrobial metal ions incorporated into inert matrices which may appear more attractive in terms of costs but must be supported by good scientific evidence. Michels et al, (2009, 95) observed that the Japanese testing protocol, JIS Z 2801, produced promising results (6-log reduction viable bacteria) for silver ion impregnated surfaces which encompasses >90% RH, achieved by a plastic film covering, and an exposure temperature of 35^{o}C. However, if the same samples were tested using the same bacterium spread over a dry surface, uncovered, at 20^{o}C or 35^{o}C as a dry inoculum there was only 0-0.2 log reduction i.e. the surface was only effective at very high RH . Other differences between recognised standard methods in Europe and U.S. in the preparation of the inoculum and test surfaces used for the tests, test microorganisms themselves, method of removal of bacteria from test surfaces, presence of chelating substances in the diluents used to halt action of copper ions and bacteriological media for culture methods. Standardisation in the use of molecular methods and choices of primers and probes and further tests for viral pathogens should also be incorporated.

In a real life health care situation surfaces are routinely contaminated with varying amounts of organic matter including vomitus, faeces, blood, sputum and other body fluids. Heavy

contamination is often manually removed, and then the surface is cleaned with detergent based reagents to remove grease, lipid and other soil and include surfactants that helps wet the surface and aid soil removal. The area is then washed to remove traces of detergents before being disinfected with single or a combination of substances such as quaternary ammonium compounds, alcohols and bleaches with proven effectiveness against a variety of microbial pathogens. The problems arise if this regime is ineffectual leaving residual viable pathogens on the surface. Of course even if the cleaning is effective it depends on the intervals between cleaning and the extent of re-contamination that could occur during this time to determine the infection risk. In addition many disinfectants, including quaternary ammonium compounds, are bacteriostatic, arousing fears that further contamination of the same area with body fluids could re-initiate growth of the bacteria.

The results from this study suggest that implementation of copper alloy surfaces could provide a surface that continuously kills pathogens which could be very beneficial in reducing infection spread from contaminated surfaces. Copper surfaces would have to be used with rigorous and effective cleaning, disinfection and hygiene regimes, including other surfaces that would not be rendered antimicrobial with copper. The results from this study have highlighted the importance of continued ion release for copper alloy surfaces to retain their antimicrobial activity. Concerns have been expressed that conventional cleaning reagents could react with the copper producing harmful by products that could be injurious to the cleaning staff. In addition some disinfectants contain chelators, including EDTA, to prevent precipitation of calcium and magnesium metal salts in hard water used to dilute them.

Sodium hypochlorite is one of the most common disinfectants because it is effective against a range of microorganisms and relatively cheap. It is inactivated in the presence of soil, does not wet surfaces effectively and concentrated solutions are corrosive, particularly to metals such as copper, nickel and chromium (96) which catalyse to metal salt and water and may result in the release of oxygen gas. Contact between hypochlorite and acids must be avoided to avoid the release of chlorine gas. The robust nature of norovirus and the spores of pathogenic bacteria such as *Clostridium difficile* mean that this disinfectant is recommended by many authorities at 1000-5000ppm, a higher concentration than is required for many vegetative bacterial pathogens (reviewed in 97). The lack of infectivity assay for norovirus has had to resort to the use of surrogates for investigations into the inactivation of this troublesome gastrointestinal pathogen. Many early studies used the respiratory feline virus, FCV, because of the availability of a plaque assay. FCV, however is more sensitive to many disinfectants than human norovirus which is a problem because the majority of standard disinfection protocols and disinfection claims by manufacturers have used FCV infectivity assay. The discovery of murine norovirus which is a gastrointestinal pathogen, genetically similar to human norovirus, resistant to low pH conditions

and less than 1000ppm hypochlorite and the development of tissue culture assay in 2003 has been hailed as a better surrogate for the human virus and was used in this current study (Appendix V),although it is more sensitive to alcohols than human strain (98). Now several formulations are screened for efficacy against MNV whilst the search goes on for suitable cell lines for *in vitro* assay for human virus, other methods to assess infectivity such as the ability to identify damaged virions combined with RT PCR detection of viral nucleic acid or to identify a closer surrogate other than MNV.

The Copper Development Association recommends removal of soil on copper alloy surfaces with detergent which is then washed away and dried before disinfection as for any surface. Disinfection with the majority of common reagents including alcohols, quaternary ammonium compounds, phenols, hydrogen peroxide and steam will not harm the copper or affect efficacy (quaternary compounds have been observed to act synergistically against biofilms with copper sulphate solutions (99)). Hypochlorite and ammonium chloride used at high dilution recommended by manufactures for short contact times (often 5 minutes) should minimise corrosion even to C26000 brass which is one of the more sensitive alloys (100). Schmidt et al., 2012 (58), successfully used several proprietary quaternary disinfectants and the reduction in bioburden on copper items was >80%.

Sodium hypochlorite and other disinfectants may also be incorporated into disposable wipes, which may be very advantageous for low dose highly infectious pathogens although high monetary and environmental costs also have to be taken into account. The use of wipes and may reduce the corrosive effects on copper alloys because of rapid drying. Other factors such as percentage active ingredients, wetness, force used are all important (101).

The use of copper polishing products however is not recommended because of residual film that is left behind after cleaning. If a shiny appearance is required dilute citric –acid based cleaning products can be used.

Eventually the potential for copper biocidal surfaces to be used in community high traffic areas e.g. public transport, education facilities as well as clinical facilities should be investigated. Advances in the preparation of copper nanoparticles raise important questions about toxicity for use *in vivo* as therapeutic agents but surface applications to flexible and irregular surfaces may increase the gamut of surfaces in our living environment that could be rendered antimicrobial.

5: Conclusion and contribution to field

My studies have followed on from the ground-breaking work of earlier colleagues who observed copper alloys were efficacious at killing laboratory strains of certain pathogens, in the laboratory. The results suggested copper alloys may potentially be used in areas where surface contamination plays a role in infection spread such as in clinical environments to prevent nosocomial infections. I have followed on from this addressing more recent healthcare concerns. My initial studies investigated the efficacy of copper alloys against clinical isolates of vancomycin-resistant enterococci (VRE) which had evolved from intestinal commensal to become a significant health risk acquiring resistance genes primarily because of overuse of antibiotics and the hardy and persistent nature of enterococci on surfaces. It has been known for many years that soluble copper can result in damage to biomolecules by the generation of reactive oxygen species and I investigated if the copper killing mechanism of enterococci and other pathogenic bacteria on dry copper alloy surfaces was the same. I discovered that although copper surfaces were effective at killing many bacterial pathogenic species the killing mechanism was variable and dependant on bacterial structure.

Horizontal gene transfer is responsible for spread of antibiotic resistance between bacteria of the same or even to other species. This and other factors including more accessible global travel has led to worldwide spread of antibiotic resistance and concerns that we are entering a pre-antibiotic era. I have shown that genes conferring resistance to all penicillin-like antibiotics, which are prevalent in the natural environment in certain areas of the world, can be transferred to other bacteria on stainless steel surfaces but this transfer could be prevented on copper alloys.

This publication (Appendix IV) was featured as one of the most significant papers and discussed in American Society of Microbiology (ASM) This Week in Microbiology podcast http://traffic.libsyn.com/twimshow/TWiM047.mp3

More recently I have concentrated on efficacy of copper alloy dry surfaces against norovirus, a highly infectious and costly pathogen. The published article has attracted a great deal of media attention worldwide highlighting the huge problems faced during norovirus outbreaks including closure of care facilities, hospital wards and other partially contained environments.

I feel that my work over the past 5 years has made a significant contribution to healthcare research and attracted complimentary attention from other scientists and the media to publicise scientific research at the University of Southampton.

Although I have worked on a diverse range of pathogenic microorganisms the overall conclusions are that copper alloy surfaces could be employed as effective antimicrobial surfaces, in conjunction with good hygiene and cleaning regimes, in the fight to reduce reinfection of individuals from contaminated surfaces potentially in hospitals and other high risk community environments.

The relatively simple premise of 'copper kills' is actually a multi-faceted and complex mechanism that is still not fully understood. However the stages appear to be dissolution of copper ions from copper surface or from oxide layers which affect specific targets such as depolarisation and peroxidation of membrane lipids of the plasma membrane of Gram-negative bacteria and membrane bound respiratory enzymes and amino acid biosynthesis pathways. The copper homeostasis mechanisms are subsequently overwhelmed with continuous influx of ions which build up in the cytosol and results in ROS generation damaging nucleic acid and other cell compartments. This is followed by indiscriminate binding of copper ions to the exposed thiol groups of numerous proteins and biomolecules. This multi-site attack now results in irreversible cell death.

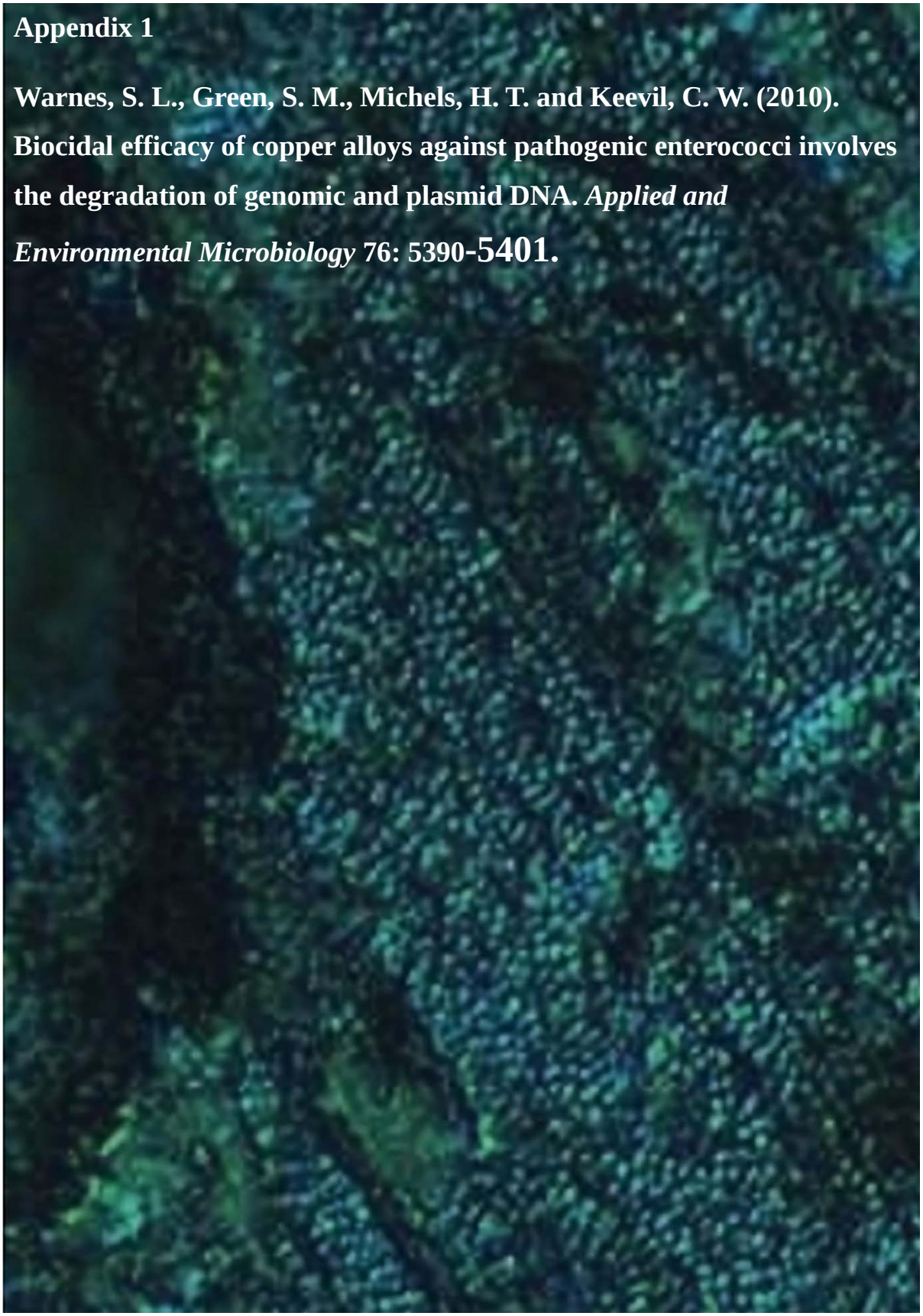
Further laboratory studies are necessary to investigate the efficacy of copper surfaces to kill other pathogens, not addressed here, that may contaminate surfaces and spread infection to others including protozoa. More studies are required on viral pathogens, possibly newly emerging zoonotic and recombinant variants including investigations into inactivation mechanisms and targets. If copper alloy surfaces are to be employed it is hoped that the biocidal activity will be constantly active for years and so long term effects of surface oxidation and cleaning materials have to be evaluated.

One cannot underestimate the aesthetic requirements that may play a role in the selection of copper alloys for use as antimicrobial surfaces. Currently the fashion dictates a preference is for silver coloured alloys in the more minimal interior designs with the golden finishes of copper alloy that were prevalent for centuries regarded as 'old fashioned'. In addition, the smooth and shiny surface of stainless steel incorporated into industrial and domestic environments may give a false impression of cleanliness because it is judged by appearance only. It is known that many pathogenic microorganisms survive in an infectious state on stainless steel and even after cleaning microscopic striations and scratches on the surface can provide a protective sanctuary for pathogens to reside. Unfortunately the opposite misapprehension is true for copper alloys where a discolouration or tarnish may be considered 'dirty'. Ironically, copper alloy surfaces are the ones that are 'clean' providing constantly killing surface that are effective against many pathogenic microorganisms where it doesn't matter if the surfaces are scratched or uneven (in fact this may increase kill rate because of increased surface area).

New alloys are currently under development that contain enough copper to be antimicrobial but are more silvery in appearance and this may be important when the general public begins to express an interest in antimicrobial fitments for the home.

Appendix 1

Warnes, S. L., Green, S. M., Michels, H. T. and Keevil, C. W. (2010). Biocidal efficacy of copper alloys against pathogenic enterococci involves the degradation of genomic and plasmid DNA. *Applied and Environmental Microbiology* 76: 5390-5401.



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Biocidal Efficacy of Copper Alloys against Pathogenic Enterococci Involves Degradation of Genomic and Plasmid DNAs▽

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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 regimens; 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 h at an inoculum concentration of $\leq 10^4$ CFU/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 that mutation is less likely to occur. It is therefore highly unlikely that genetic information can be transferred to receptive organisms recontaminating the same area. A combination of effective cleaning regimens and contact surfaces containing copper could be useful not only to prevent the spread of viable pathogenic enterococci but also to mitigate against the occurrence of potential resistance to copper, biocides, or antibiotics and the spread of genetic determinants of resistance to other species.

Enterococci are an important cause of nosocomial infections worldwide (34), and in the United States, approximately one-third of enterococcal infections in intensive care units are caused by vancomycin-resistant enterococci (VRE) (35).

There is now a significant problem of enterococci acquiring resistance to clinically important antibiotics, particularly aminoglycosides; glycopeptides, including vancomycin; and quinolones (18, 23, 24, 45, 47, 50). 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, 12, 29).

Enterococci have a propensity for genetic transfer via transposons and plasmids which has resulted in the dissemination of antibiotic resistance genes (28, 41, 54). In addition, a recent report has highlighted the gap between the development of new antibiotics and an increasing number of infections caused by multiantibiotic-resistant organisms (14).

Enterococci are intestinal commensals and can withstand the 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 (36), upholstery, and floor and wall coverings (27), and can exhibit resistance to some routinely used cleaning agents, including sodium hypochlorite (10, 25). 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-colonized patients as after touching the patients themselves (9, 22, 40).

Contaminated surfaces are known to contribute to infection spread (4, 8, 45); therefore, the use of bactericidal surfaces along with rigorous disinfection protocols could potentially reduce the incidence of horizontal disease transmission.

The biocidal properties of copper have been known for centuries (3). More recently, the potential use of copper alloys as microbicidal surfaces has been described. Rapid killing of *Escherichia coli* O157 (38, 52), *Listeria monocytogenes* (53), methicillin-resistant *Staphylococcus aureus* (MRSA) (37), *Clostridium difficile* (48, 51), *Mycobacterium tuberculosis* (32), *Candida albicans* and other pathogenic fungi (49), and influenza A virus (39) has been observed on copper compared to the stainless steel surfaces which are prevalent throughout the health care environment. In the United States, Environmental Protection Agency approval has been granted for the use of alloys containing >65% copper as bactericidal surfaces (7) and has recently been extended down to alloys containing >60% copper. It is estimated that biofilm formation on surfaces is a

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TABLE 1. Characteristics of the *Enterococcus* clinical isolates used in this study

Isolate	Source	Sample type	Antimicrobial resistance[a]
E. faecalis 1	Surgical ward	Wound swab	VAN, ERY, CHL, TET
E. faecalis 2	Surgical ward	Feces	VAN, ERY, CHL, TET
E. faecium 1	Intensive care unit	Ascitic fluid	VAN, PEN, ERY, CHL, TET, AMP
E. faecium 2	Surgical ward	Intraabdominal drain swab	VAN, PEN, ERY, CHL, AMP
E. faecium 3	Leukemia ward	Blood culture	VAN, PEN, ERY, CHL, TET, AMP
E. faecium 4	Neonatal unit	Gastric aspirate	VAN, PEN, ERY, CHL, TET, AMP
E. faecium 5	Intensive care unit	Central venous catheter swab	VAN, PEN, ERY, CHL, TET, AMP
E. gallinarum	Surgical ward	Feces	VAN, PEN, ERY, CHL, TET, AMP

[a] Antimicrobial abbreviations: VAN, vancomycin; PEN, penicillin; ERY, erythromycin; CHL, chloramphenicol, TET, tetracycline; AMP, ampicillin.

contributing factor in 65% of cases of nosocomial infections (43). There is no evidence that pathogens can form biofilms on dry copper surfaces (37), and the effectiveness of copper alloys as bactericidal agents is not affected by temperature and humidity, unlike that of silver (33).

At present, trials of copper surfaces used along with conventional cleaning regimens in the clinical environment are under way. In the United Kingdom, 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) (5, 6). In addition, plastic toilet seats 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 (5, 6). Other trials are in progress worldwide, including the United States, Germany, Chile, Japan, and South Africa (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.

MATERIALS AND METHODS

Bacterial strains. Vancomycin-resistant control strains *Enterococcus faecalis* ATCC 51299 (VanB phenotype) and *Enterococcus faecium* NCTC 12202 (VanA phenotype) were supplied by Oxoid.

Clinical isolates of vancomycin-resistant *E. faecalis* ($n = 2$), *E. faecium* ($n = 5$), and *E. gallinarum* ($n = 1$) were obtained from patients (aged 11 days to 70 years) between June 2004 and June 2006 at Southampton General Hospital, Southampton, United Kingdom (Table 1). The enterococcal genomes exhibited 54 to 93% similarity (Dice similarity coefficient) by pulsed-field gel electrophoresis of SmaI-digested DNA (data not shown).

Vancomycin-sensitive *E. faecalis* NCTC 775 was supplied by a local water authority.

Culture preparation. Bacteria were maintained on Glycerol Protect beads (Fisher Scientific) and also in 1-ml aliquots of VRE broth (VREB; Oxoid) containing 15% (wt/vol) glycerol at −80°C. For each experiment, one bead or vial of enterococci was inoculated into 15 ml sterile brain heart infusion broth (Oxoid) or VREB and incubated aerobically at 37°C for 18 ± 2 h.

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

Inoculation. For each exposure time, duplicate coupons were analyzed using either culture methods or staining methods; 20 μl of bacterial culture was spread evenly over the surface of each coupon, dried in a sterile airflow in a class II microbiological safety cabinet, and incubated on the bench at room temperature (21 ± 2°C) for various time periods. The use of bacteriological medium as an inoculation matrix was included as a "worst-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 s. (Preliminary experiments using PBS with 20 mM EDTA to chelate free copper ions gave no significant difference in any of the viability testing methods used.) Serial dilutions were prepared, and 10 or 100 μl of each dilution was spread over 45- or 90-mm agar plates in triplicate. Slanetz and Bartley agar (Merck) and Columbia blood agar (CBA; bioMérieux) were used for the recovery of vancomycin-sensitive strains, and VRE agar containing 6 mg/liter vancomycin (Oxoid) and CBA were used for VRE strains (but with no meropenem added, as only pure cultures were investigated). Plates were allowed to dry before inversion and aerobic incubation at 37°C for 24 and 48 h. Colonies on plates were counted by eye, and the concentration per coupon was calculated and recorded as CFU per coupon (1 cm^2).

Staining protocols used to detect actively respiring bacterial cells and membrane integrity *in situ* on metal surfaces. (i) SYTO 9 and CTC (5-cyano-2,3-ditolyl tetrazolium chloride). A 30-μl volume of CTC (Sigma-Aldrich) at a final concentration of 5 mM was pipetted onto the surface of each inoculated coupon for the final 2 h of required contact time. Coupons were placed in a humid chamber and incubated in the dark at 37°C for 2 h. To stain all of the cells present on coupons, SYTO 9 (7 μM; Invitrogen) was pipetted onto the coupons and they were incubated at room temperature in the dark for the final 30 min.

(ii) *Bac*Light (SYTO 9 and propidium iodide [PI]). A staining solution containing 7 μM SYTO 9 and 40 μM PI (L7012; Invitrogen) was prepared in filter-sterilized deionized water (2 μl/ml each stain); 50 μl of this solution was pipetted onto the coupons, and they were incubated at room temperature for the final 30 min of required contact time in the dark.

After staining with SYTO 9/CTC or *Bac*Light, coupons were tipped to remove the stain and 1 drop of sterile deionized water was gently pipetted onto the

TABLE 2. Compositions of the alloys tested in this study

UNS[a] no.	% of total composition that was:					
	Cu	Zn	Sn	Ni	Fe	Cr
C11000	100					
C26000	70	30				
C28000	60	40				
C51000	95		5			
C70600	89			10	1	
C75200	65	17		18		
S30400				8	74	18

[a] UNS, unified numbering system.

coupon from a disposable Pastette and the coupon was tipped to remove the remaining excess stain.

Episcopic differential interference contrast (EDIC) microscopy combined with epifluorescence microscopy was used to scan the coupons directly (Nikon Eclipse ME600; Best Scientific, Swindon, United Kingdom) (26). A minimum of 10 fields of view were photographed using a digital camera (Coolsnap CF; Roper Industries) connected to a computer with digital image analysis software (Image-Pro Plus, version 4.5.1.22; Media Cybernetics). Total cells (SYTO 9 stained), respiring cells (CTC stained), and membrane-damaged/intact cells (PI/SYTO 9 stained) were enumerated.

(iii) Genomic DNA assay. The protocol used is described in detail elsewhere (17) and allows analysis of whole bacterial genomes. Briefly, approximately 10^7 bacterial cells left 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 pretreated with lysozyme (4 mg/ml [approximately 30,000 U]) for 15 min at 37°C. The cell membrane was permeabilized with a lysing solution (2% sodium dodecyl sulfate, 0.05 M EDTA, 0.1 M dithiothreitol, pH 11.5) for 5 min at 37°C before drying in ethanol baths and overnight baking in a 65°C oven. Dried slides were heated in a microwave oven (4 min, 750 W) before staining with the sensitive nucleic acid stain SYBR Gold (Invitrogen), which detects single- and double-stranded DNA, for 5 min at room temperature in the dark. Epifluorescence microscopy was used to analyze the DNA fragments produced.

(iv) 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 pretreatment with lysozyme), and preparations were separated on a 1, 2, or 3% (wt/vol) agarose gel containing the DNA stain SYBRsafe (Invitrogen) exposed to a current of 300 mA for 90 min. Plasmid DNA was extracted using the QIAprep Spin Miniprep kit (Qiagen), and preparations were 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 means ± the standard errors of the means. Differences between duplicate samples were assessed using the Mann-Whitney rank *t* test. Group comparisons were analyzed using the Mann-Whitney U test, where statistical significance was considered $P < 0.05$. Statistical analyses were performed using Sigma Stat version 3.5, and graphical representations were performed using SigmaPlot version 10.

RESULTS

Culture analysis. The survival time of all of the strains of enterococcal species tested was significantly less on copper and copper alloys containing 60 to 95% copper than on stainless steel for all of the inoculum concentrations studied (Fig. 1 to 4).

On stainless steel using the highest inoculum concentration of 10^6 CFU/cm^2 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 of both species were still present at 60 days (Fig. 3).

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

Although all of the 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 of copper in the alloys (Fig. 1). The most rapid killing occurs on pure copper and alloys containing >90% copper (C51000 and C70600), with increased survival times on the remaining alloys containing 60 to 70% copper (C28000, C75200, and C26000) (Fig. 1). Alloys containing >95% copper (C51000, C70600) were as effective as pure copper for isolates 2 and 3, resulting in cell death at 1 h, but small numbers of cells of isolates 1and 4 remained viable (86% similarity) at up to 2 h after contact. Viable cells of all of the isolates were detected following 2 h of 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 h of 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* (Fig. 2), with rapid killing achieved with all of the copper alloys compared to stainless steel. However, unlike *E. faecium*, no viable cells were detected on alloy C28000 at 2 h of contact. It is interesting that alloy C75200 is once again a more effective bactericidal surface than alloy C26000 for the *E. gallinarum* isolate tested (Fig. 2, graph 4). This species, although not as prevalent as *E. faecium* and *E. faecalis*, was reported to be isolated in 2.6% of the enterococcal bacteremia infections in the United Kingdom in 2007 (23). More-rapid killing was observed with the vancomycin-sensitive *E. faecalis* strain (Fig. 2, graph 5), all of the cells of which were killed on pure copper and copper alloys at 30 and 80 min, respectively (C28000 not tested), but it is difficult to assess the significance of this unless further sensitive strains are tested, and the results may not be clinically relevant.

Survival time is also dependent on the inoculum concentration. If the inoculum was increased to 5×10^7 CFU/cm^2, low numbers of viable *E. faecalis* cells could be recovered after 2.5 h of contact with alloys C26000 and C70600, but not C75200, after 2.5 h of contact (data not shown). Reducing the inoculum concentration of the *E. faecium* control strain to 10^5 CFU/cm^2 and below minimized the discrepancy between pure copper and the copper alloys, as all of the alloy surfaces were even more effective at killing enterococcal cells (Fig. 4). All of the cells were killed in 60 min, except for those on alloy C70600, where a few cells remained viable from an inoculum concentration of 10^5 CFU/cm^2. However, contact for 20 min on pure copper killed all of the cells at an inoculum concentration of 1,000 cells/cm^2.

Analysis of respiring cells (CTC) and membrane integrity (SYTO 9/PI) following contact with copper and stainless steel. To determine if noncultivable cells were indeed dead and to investigate the potential mechanism of copper's antibacterial action, copper and steel coupons were inoculated with vancomycin-resistant *E. faecalis* control strain ATCC 51299 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 visualized *in situ* using epifluorescence microscopy and a long-working-distance objective lens. Figure 5A demonstrates CTC staining of the cells after 4 h of contact with copper and steel at room temperature. Actively respiring bacterial cells can be seen on stainless steel coupons (Fig. 5A, part 1b CTC) but not on copper (Fig. 5A, part 1a CTC). The membrane-permeating DNA stain SYTO 9 was used to determine the total bacterial count, but the staining intensity on copper (Fig. 5A, part 1a SYTO 9) appeared greatly diminished relative to that on stainless steel (Fig. 5A, part 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 (Fig. 5B) and C70600, as well as stainless steel, when a higher inoculum concentration of 5×10^7 CFU/cm^2 was used for 2 h of contact time but not on copper alloys C51000 and C75200 (data not shown; alloy C28000 not tested). At this higher concentration, the cells are

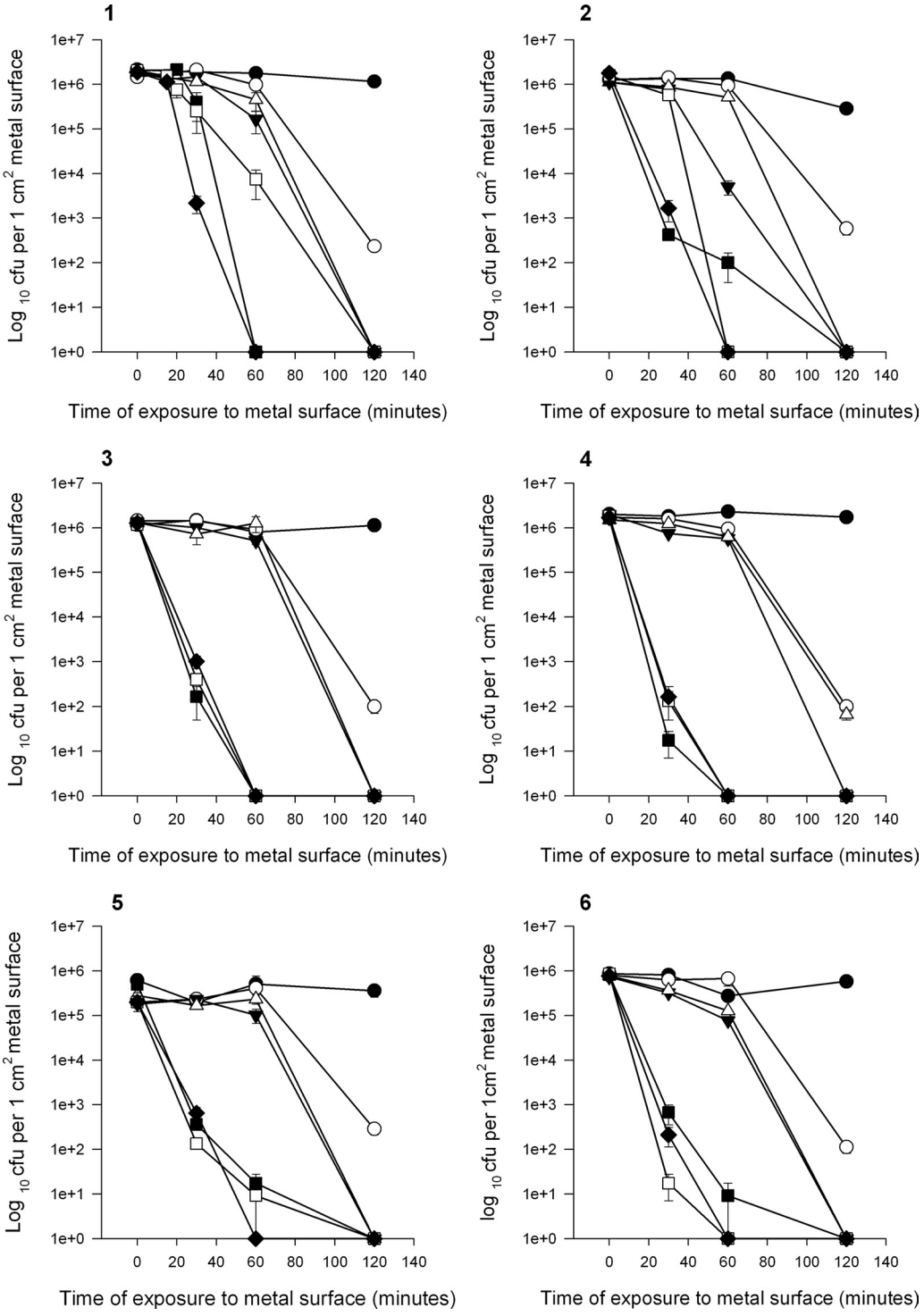

FIG. 1. Survival of vancomycin-resistant *E. faecium* NCTC 12202 (graph 1) and clinical isolates 1 (graph 2), 2 (graph 3), 3 (graph 4), 4 (graph 5), and 5 (graph 6) on stainless steel, pure copper, and copper alloys (S30400 [●], C28000 [○], C75200 [▼], C26000 [△], C70600 [■], C51000 [□], and copper C11000 [◆]) at 22°C.

piled on top of each other and are not necessarily in direct contact with the alloy surface (Fig. 5B). This appears not to be a problem for pure copper and alloys C75200 (65% Cu) and C51000 (95% Cu) but may be important if a particular alloy has a reduced copper release rate.

Enumeration of CTC-positive cells on stainless steel gave consistently higher counts than results obtained from culture, but the difference was not statistically significant.

*Bac*Light comprises two nucleic acid stains, SYTO 9 and the higher-affinity stain PI. SYTO 9 can permeate the membrane

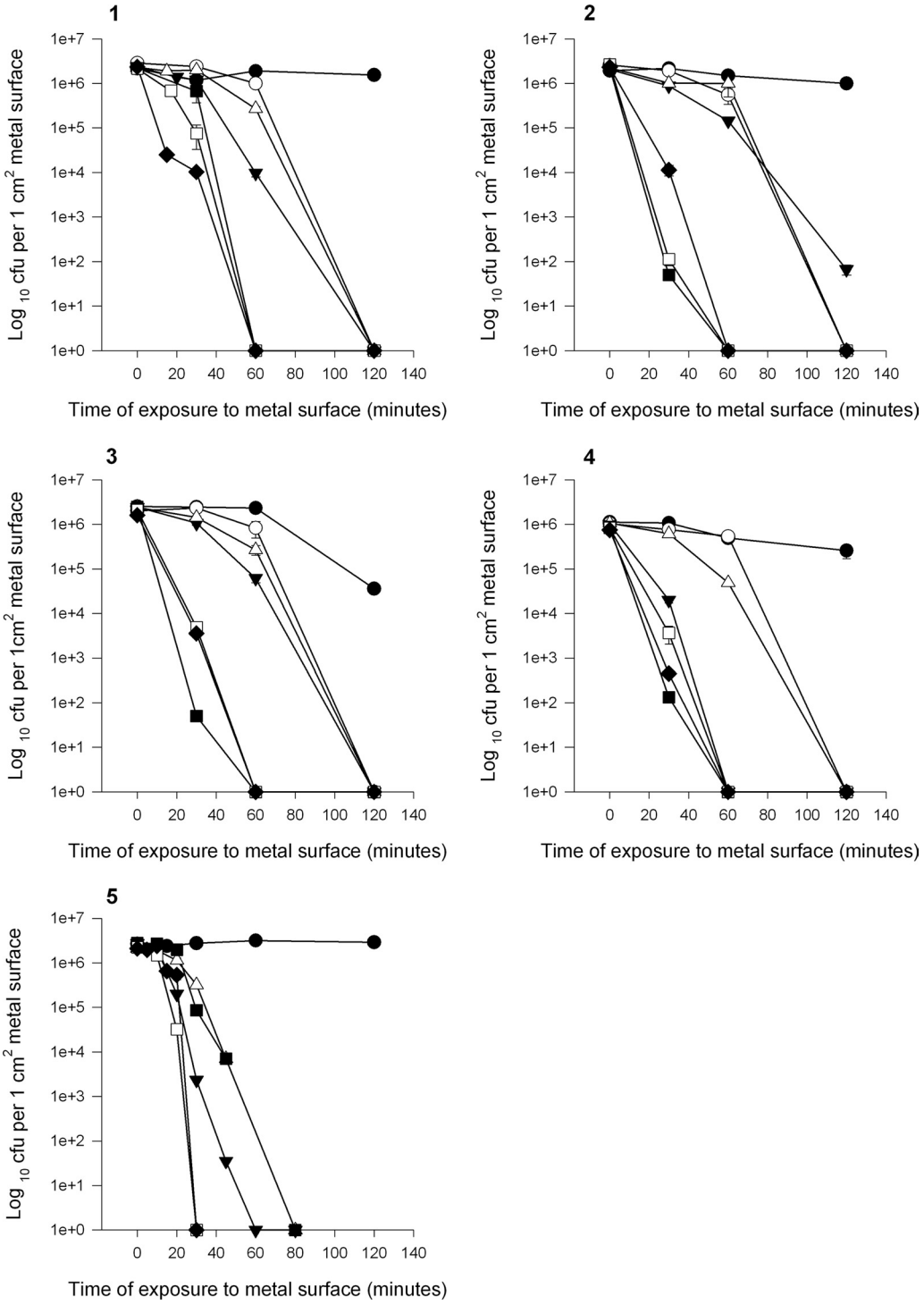

FIG. 2. Survival of vancomycin-resistant *E. faecalis* ATCC 51299 (graph 1), *E. faecalis* clinical isolates 1 (graph 2) and 2 (graph 3), *E. gallinarum* (graph 4), and vancomycin-sensitive *E. faecalis* NCTC775 (graph 5) on stainless steel, pure copper, and copper alloys (S30400 [●], C28000 [○], C75200 [▼], C26000 [△], C70600 [■], C51000 [□], and copper C11000 [◆]) at 22°C.

and will stain all of the cells regardless of viability. If the cell membrane is damaged, PI can enter the cell and displace the lower-affinity stain SYTO 9. As the cells die, the ratio of cells staining green (membranes intact) changes to predominantly red (dead cells with compromised membranes). The vancomycin-resistant *E. faecalis* control strain was inoculated onto all of the metal samples. At the earliest time point of 30 min, green staining *in situ* revealed a large number of viable cells and few red-staining cells with damaged membranes in all of the samples. After 4 h of contact with stainless steel, the number of

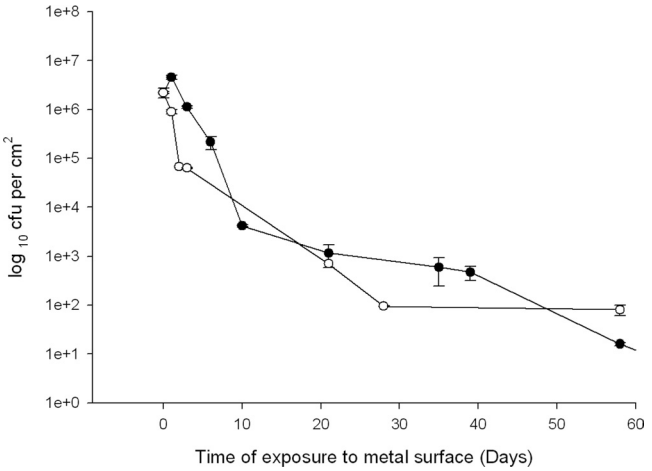

FIG. 3. Survival of vancomycin-resistant *E. faecalis* (ATCC 51299) (●) and *E. faecium* (NCTC 12202) (○) on stainless steel at 22°C.

bright SYTO 9-stained cells was reduced, while red PI staining had increased, suggesting that cells die slowly on stainless steel and their cytoplasmic membranes are compromised (Fig. 5A, part 2b). It was not possible to enumerate viable cells *in situ* beyond 30 min on copper and copper alloys because preparations were negative for both stains. (Fig. 5A, part 2a). If the cells were removed from the coupons prior to staining, there was still no uptake of PI, suggesting that membranes were not damaged. A similar finding was demonstrated for MRSA (L. Weaver et al., unpublished data), where contact with copper resulted in respiratory failure but not damage to the bacterial cell membrane.

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

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

Analysis of bacterial DNA following exposure to copper and stainless steel surfaces. (i) DNA size separation by agarose gel electrophoresis. The reduced SYTO 9 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 obtained with DNA purified from the vancomycin-resistant *E. faecium* control strain are shown in Fig. 6A, and those obtained with DNA purified from *E. faecium* clinical isolate 5 (lanes 7 and 8) and *E. faecalis* clinical isolate 2 (lanes 9 and 10) are shown in Fig. 6B. DNA purified from cells that had 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 (>10 kb), remaining at the site of loading. The same pattern is seen with DNA purified from cells exposed to stainless

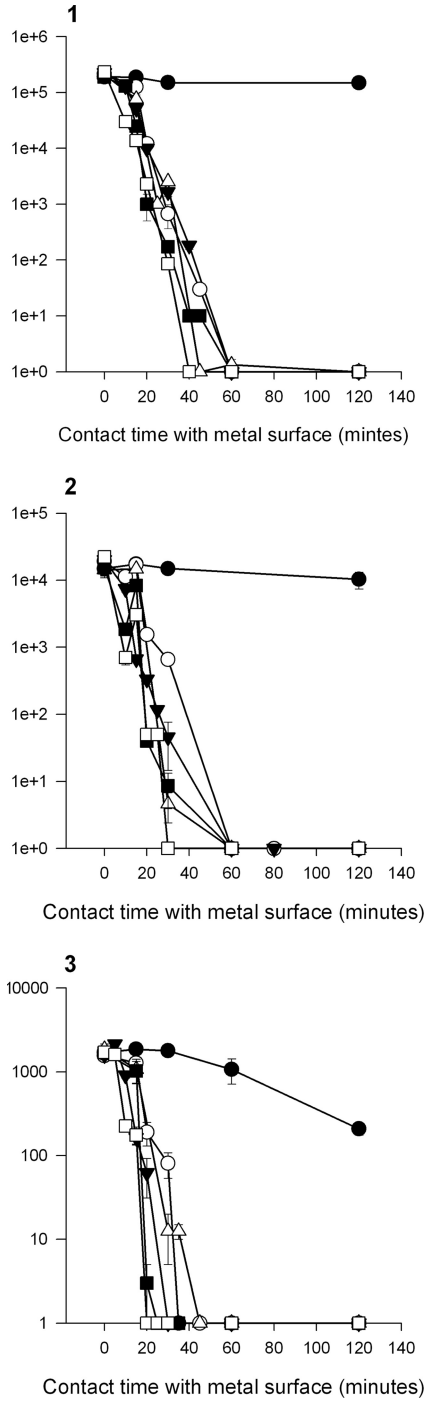

FIG. 4. Effect of inoculum concentration on survival of vancomycin-resistant *E. faecium* (NCTC 12202) on stainless steel, pure copper, and copper alloys (S30400 [●], C75200 [○], C26000 [▼], C70600 [△], C5100 [■], and C11000 [□]) at 22°C. Inoculum concentrations tested: 10^5 CFU/cm^2 (graph 1), 10^4 CFU/cm^2 (graph 2), and 10^3 CFU/cm^2 (graph 3).

steel for 2 h (Fig. 6A, lane 3, and B, lanes 7 and 9). However, the DNA from all of the strains tested, when exposed to copper for 2 h, exhibit a pronounced smearing effect characteristic of nucleic acid degradation (Fig. 6A, lane 4, and B, lanes 8 and 10). This suggests that the DNA is being broken down but not

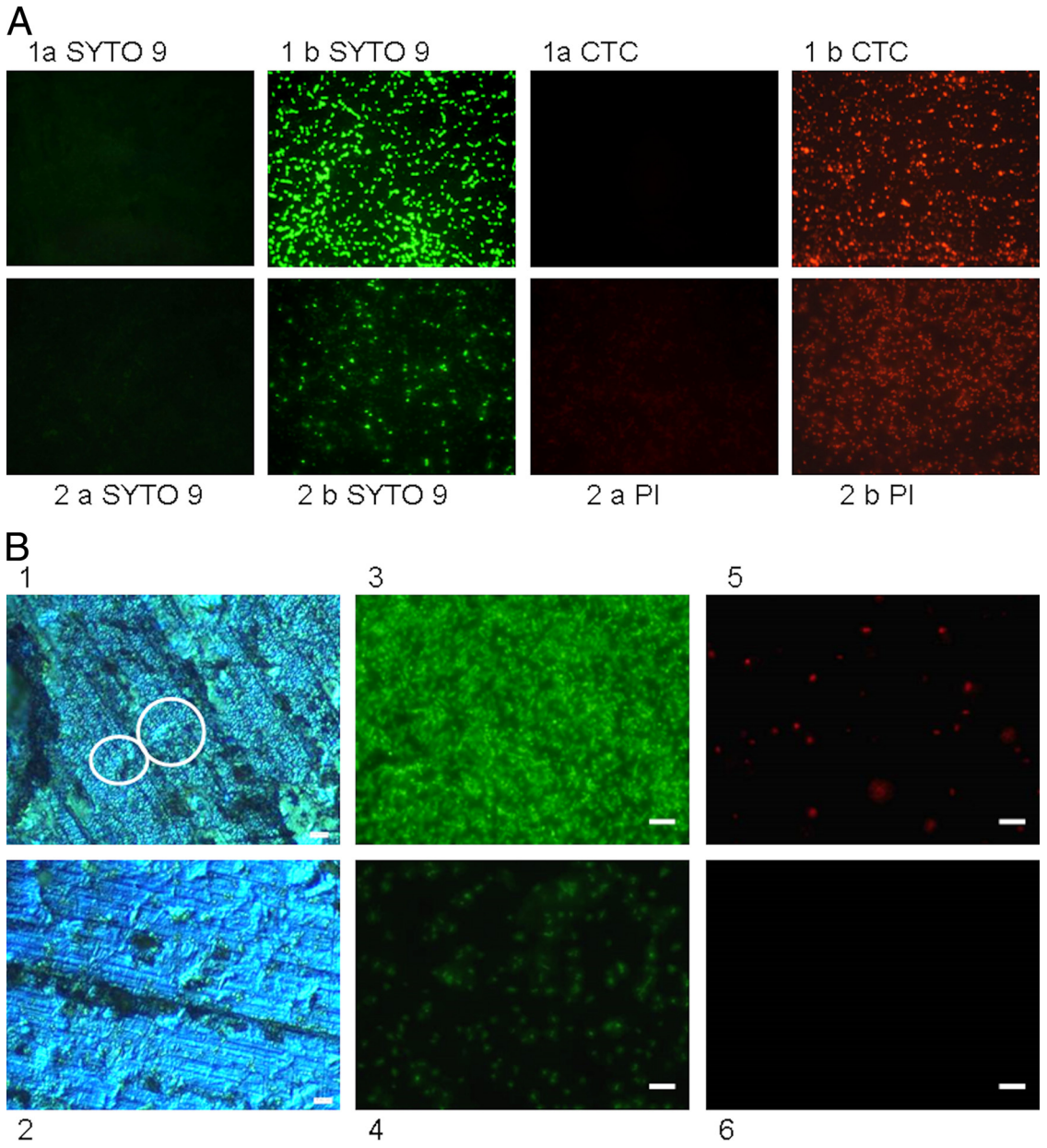

FIG. 5. (A) Assessment of viability of *E. faecalis* (ATCC 51299) on copper (a) and stainless steel (b) surfaces using the redox dye CTC (positive for respiring cells) and SYTO 9 (total cell count, regardless of viability) (row 1) or *Bac*Light (row 2) to detect bacterial membrane integrity. (B) Effects of inoculum concentration and cell stacking on the susceptibility of alloy C26000 to inhibit respiration. The alloy was inoculated with VRE strain *E. faecium* NCTC 12202 at inoculum concentrations of 5×10^7 CFU/cm^2 (images 1, 3, and 5) and 5×10^6 CFU/cm^2 (images 2, 4, and 6) for 2 h. Images 1 and 2 were captured using EDIC microscopy. Circled in image 1 are areas where bacterial cells at the higher inoculum concentration may not be in direct contact with the metal surface, as they are stacked on top of each other. At the lower inoculum concentrations (image 2), the cells are spread in small clumps and are all exposed to the alloy directly (this spreading of individual cells is also clearly visible in epifluorescence image 4). Epifluorescence images 3 and 4 represent SYTO 9 total cell staining. CTC-positive staining of respiring cells is present at the higher (image 5) but not at the lower (image 6) inoculum concentration. Bars, 10 μm.

at specific points because there is no accumulation of particular-sized fragments. If cells are exposed to copper for a longer time, 4 h, 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 visualized and may have run straight through the gel. Increasing the agarose concentration did not allow further visualization of the small DNA fragments generated on contact with copper surfaces, suggesting that extensive denaturation of the DNA has occurred (Fig. 6A and B show 2% agarose gels).

No viable cells were present at either time point on copper surfaces when they were assessed by any of the viability testing 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

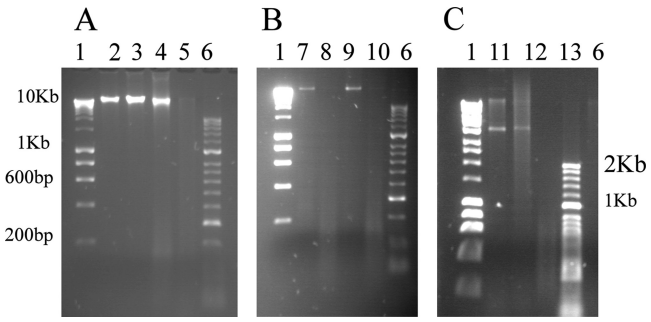

FIG. 6. Agarose gel electrophoresis of purified enterococcal DNA. (A) Purified genomic DNA of *E. faecium* NCTC 12202. Lane 2, cells not exposed to metal surfaces; lane 3, cells exposed to stainless steel for 2 h; lane 4, cells exposed to copper for 2 h; lane 5, cells exposed to copper for 4 h. (B) Purified 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 h at 22°C. (C) Purified plasmid DNA of *E. faecium* NCTC 12202 not exposed to metal (lane 11) or exposed to stainless steel (lane 12) or copper (lane 13) for 2 h at 22°C. Control lanes are Bioline Hyperladder I (lanes 1) and Hyperladder II (lanes 6). Genomic DNA was purified using the Qiagen DNeasy Blood and Tissue kit (2% agarose) and the Qiaprep Spin Miniprep kit for plasmid DNA (0.9% agarose).

from the *E. faecium* control strain exposed to copper and stainless steel (Fig. 6C). Lanes 11 and 12 demonstrate plasmid DNA present in untreated cells and those exposed to stainless steel for 2 h. 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 it includes supercoiled and linear fractions of the same plasmids. However, the results suggest that both genomic and plasmid DNAs are denatured upon exposure to copper surfaces.

(ii) Genomic DNA fragmentation assay. To determine if DNA disintegration into very small fragments was due to copper alone and not a preparation artifact, the DNA fragmentation assay was used to gently visualize the entire genome of individual cells *in situ* (Fig. 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 (Fig. 7, panels 3 and 4, respectively), characteristic of an intact genome. Following 2 h of contact with stainless steel, the pattern of DNA staining was similar but there were more discrete fragments that had diffused away from the bacterial cells (Fig. 7, image 2).

The pattern of DNA staining after 2 h of contact with copper surfaces was very different (Fig. 7, image 1). The absence of

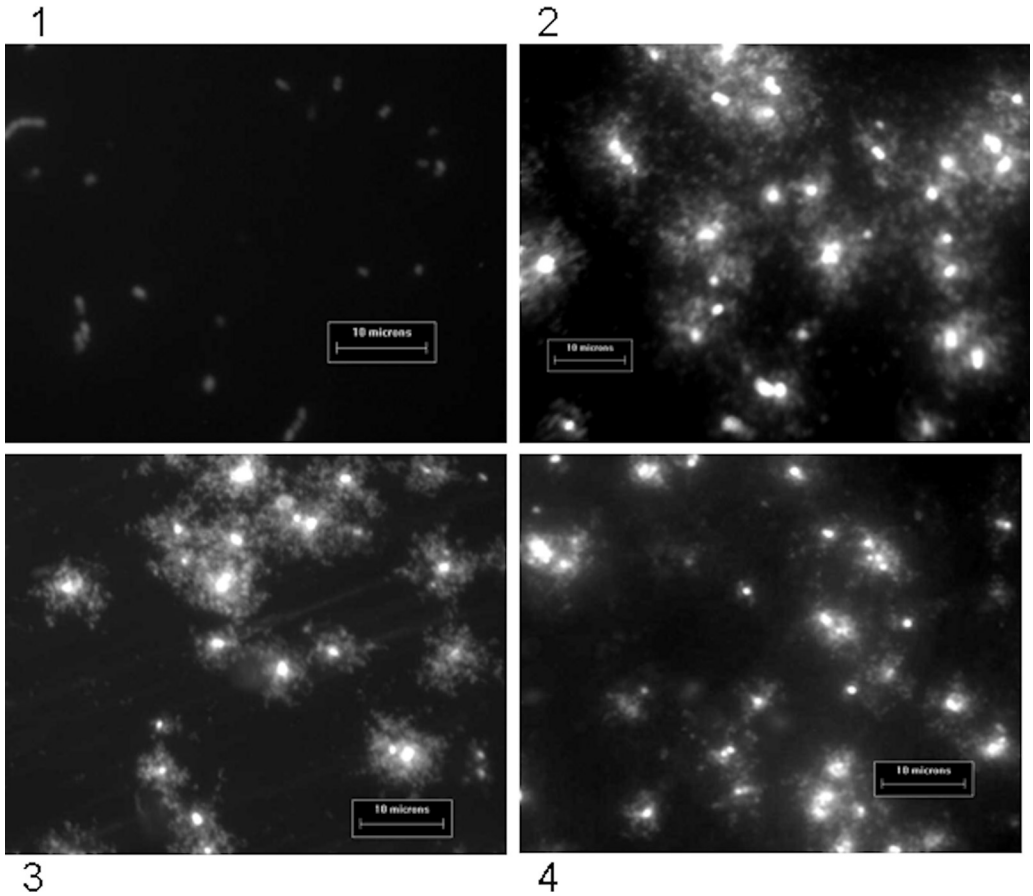

FIG. 7. Analysis of genomic DNA of *E. faecalis* ATCC 51299 *in situ* by DNA fragmentation assay following 2 h of exposure to copper (image 1) or steel (image 2). Images 3 and 4 show bacterial cells not exposed to metal surfaces and are of live and dead (heat-killed) cells, respectively, which were then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

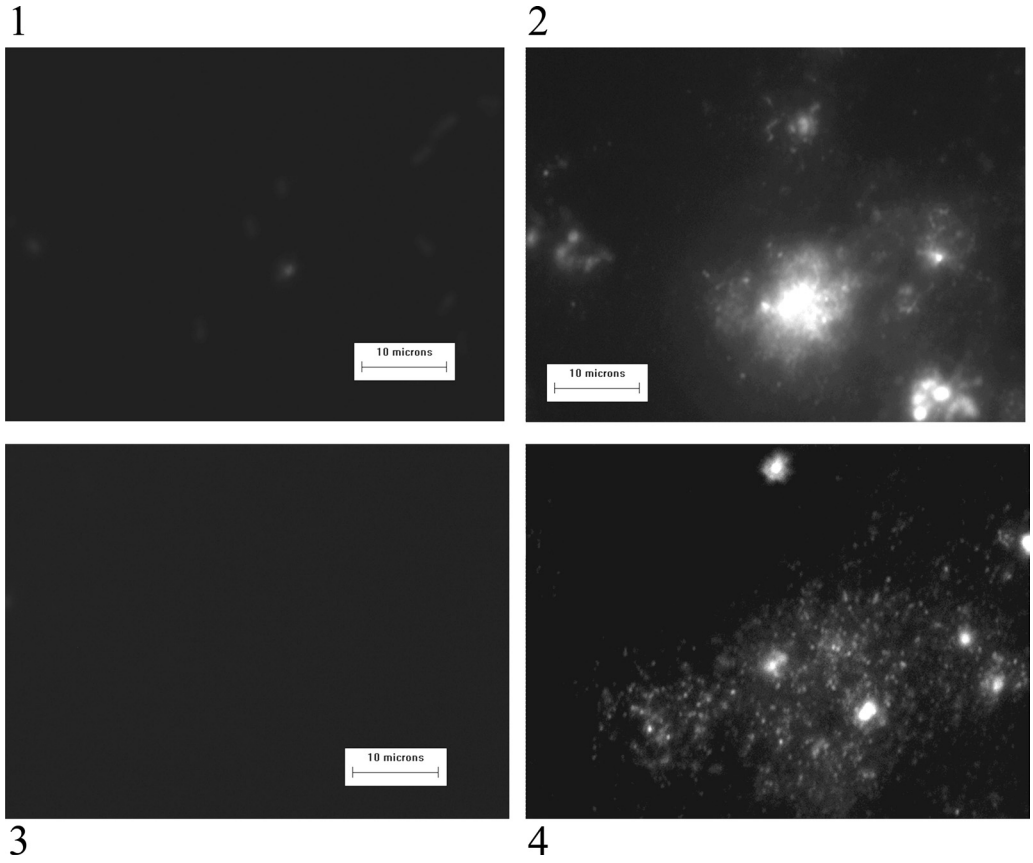

FIG. 8. Analysis of genomic DNA of clinical isolates *E. faecalis* 2 (images 1 and 2) and *E. faecium* 5 (images 3 and 4) *in situ* by DNA fragmentation assay following a 1-h exposure to copper (images 1 and 3) or steel (images 2 and 4). No DNA loops are visible on cells exposed to copper, whereas DNA fragments are visible emanating from cells isolated from stainless steel surfaces.

visible DNA strands suggests that extensive damage to the nucleic acid has resulted in fragments that are too small to be visualized and have dispersed throughout the slide.

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

DISCUSSION

Contamination of hospital surfaces with pathogenic microorganisms contributes to reinfection and spread of disease. Not only are enterococci hardy and able to survive on many types of "touch" surfaces (27, 36) for several weeks, but the ability of these organisms to easily transfer antibiotic resistance means that it is essential that any contamination of the environment from infected individuals with viable cells be effectively destroyed (41). 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 the 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 the reinfection of personnel and especially of vulnerable patients.

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

In general, alloys containing >90% copper were as effective as pure copper, with all of the isolates of *E. faecalis* and two of *E. faecium* achieving complete cell death by 1 h (with a few cells of the remaining *E. faecium* isolates remaining viable for another hour but still with a 3- to 4-log reduction in number). For both species, no viable cells were detected after 2 h of contact with alloys containing 60 to 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 the bactericidal activity reported.

However, at bacterial contamination levels of $\leq 10^5$ CFU/cm^2, all of the copper alloys tested were virtually indistinguishable from pure copper and were very effective at killing pathogenic enterococcal cells within 1 h and in 20 min at $<10^3$

CFU/cm^2. This reinforces the potential for the use of these alloys in the clinical environment, because a recent study in the United States determined VRE contamination on surfaces to be, at most, a few hundred cells/100 cm^2 at three 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 mechanism of *Staphylococcus aureus* and *E. coli* O157 killing by copper, respectively (38, 46). Recent work in our laboratories has also determined the importance of chelating substances on bacterial survival on copper surfaces (unpublished data). Experiments with a rapidly drying swabbed inoculum in PBS or water suggest that killing is 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 state on copper surfaces under these conditions.

Assessment of the viability of cells recovered from stainless steel using *Bac*Light SYTO 9/PI staining also demonstrated no significant difference from results obtained by culture but suggested that some damage to the cell membrane does occur on this surface. However, staining with *Bac*Light does not always produce distinct "live" and "dead" populations, particularly because cells with an intact membrane are not necessarily alive (2). The *Bac*Light staining method was difficult to interpret for enterococci exposed to copper alloys because of the diminished and frequently absent staining with SYTO 9 and PI, respectively, except at the 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 that damage to the 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, and 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 under 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 (30). Macomber et al. (31). 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 gene-specific PCR assay. The reasons suggested were compartmentalization 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 the sites of hydroxyl radical generation are spatially separated from the nucleic acid. 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, i.e., dihydrogen peroxide, hydroxyl radicals, and superoxides.

Exposure to relatively low soluble copper concentrations, described by Macomber et al. (31), is very different from continual contact with copper and copper alloy surfaces. In our system, we have reported extensive disintegration of the genomic and plasmid DNAs 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 SYTO 9 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 the efficacy of specific anticancer agents on patient DNA. Fernández et al. (17). 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 the access of copper(I)/(II) and generated ROS to the DNA directly and rapidly inflict damage.

The ligands, described by Macomber et al. (31), 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 are saturated with copper ions.

Espírito Santo et al. (13) determined that ROS are generated when *E. coli* is exposed to copper surfaces. They identified hydroxyl radicals generated under aerobic conditions, presumably by Haber-Weiss and Fenton reactions of reduced copper ions [supplied by redox cycling of copper(I) and copper(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. (31) 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 do affect survival times on copper alloys (11, 13), 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 (16) has summarized 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 favor 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 (41). The dissemination of these mobile elements, their genetic organization, 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 (15, 16, 44). The plasmid-localized copper resistance *tcrB* gene has been identified in *E. faecium* and *E. faecalis* thought to originate from pigs fed with copper sulfate-supplemented food (19). The Tn*1546* element and *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 coselected for antibiotic resistance (20) except under experimental conditions in piglets fed a high concentration of copper sulfate (21). However, continued use of copper sulfate was not able to maintain high levels of antimicrobial resistance (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 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 resistance genes.

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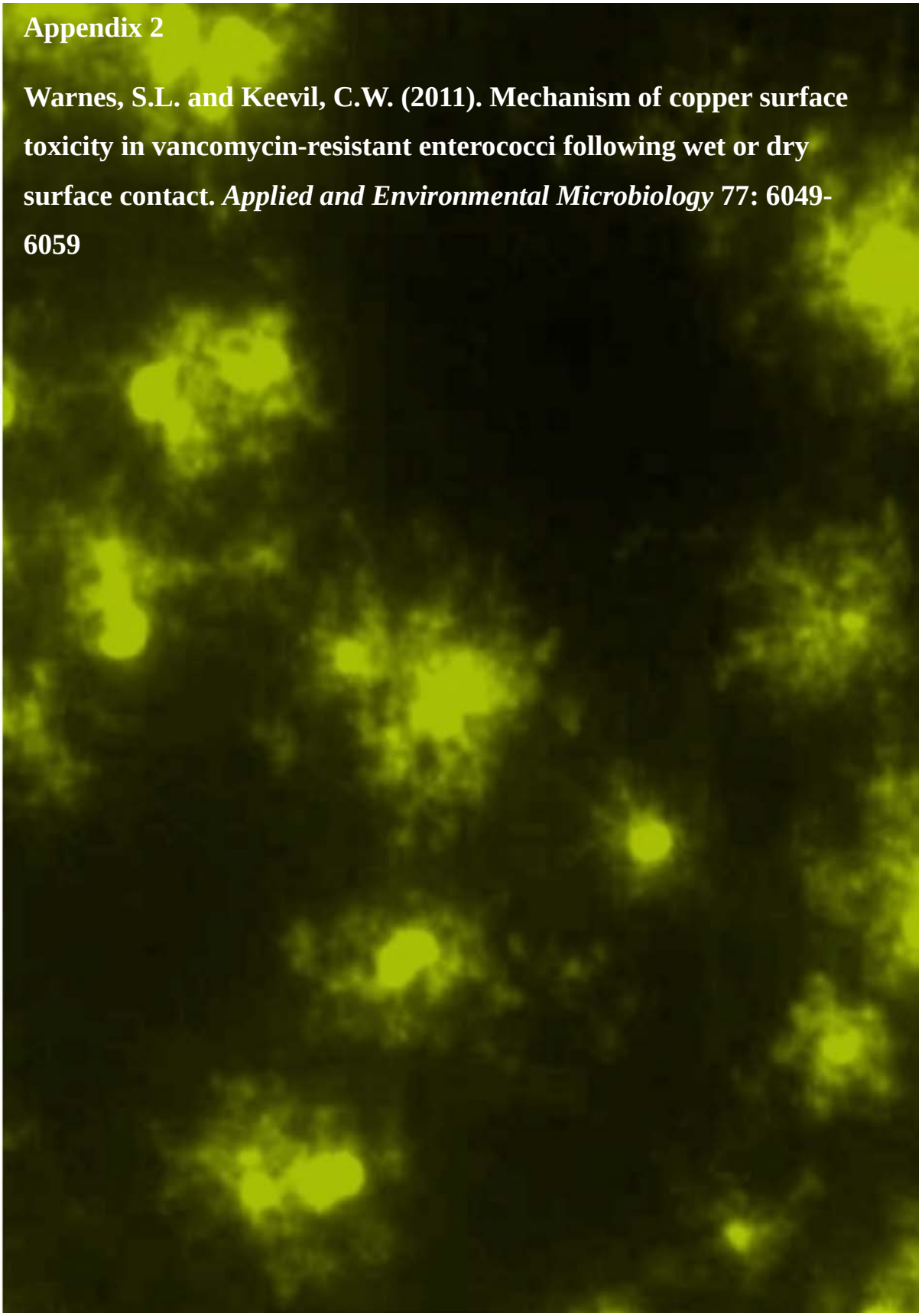
REFERENCES

1. **Amyes, S. G. B.** 2007. Enterococci and streptococci. Int. J. Antimicrob. Agents **29:**S43–S52.
2. **Berney, M., F. Hammes, F. Bosshard, H.-U. Weilenmann, and T. Egli.** 2007. Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight kit in combination with flow cytometry. Appl. Environ. Microbiol. **73:**3283–3290.
3. **Borkow, G., and J. Gabbay.** 2004. Putting copper into action: copper impregnated products with potent biocidal activities. FASEB J. **18:**1728–1730.
4. **Boyce, J. M.** 2007. Environmental contamination makes an important contribution to hospital infection. J. Hosp. Infect. **65**(Suppl. 2)**:**50–54.
5. **Casey, A. L., P. A. Lambert, L. Miruszenko, and T. S. J. Elliot.** 2008. Copper for preventing microbial contamination, poster K-4121, p. 202. 48th ICAAC. American Society for Microbiology, Washington, DC.
6. **Casey, A. L., D. Adams, T. J. Karpanen, P. A. Lambert, B. D. Cookson, P. Nightingale, L. Miruszenko, R. Shillam, P. Christian, and T. S. J. Elliot.** 2010. The role of copper in the reduction of contamination of the hospital environment. J. Hosp. Infect. **74:**72–77.
7. **Copper Development Association.** 2010. Reducing the risk of healthcare associated infections: the role of copper touch surfaces. CDA publication 196. Copper Development Association, New York, NY. http://www.copperinfo.co.uk/antimicrobial/downloads/pub-196-reducing-risk-hcais-report.pdf.
8. **Drees, M., D. R. Syndman, C. H. Schmid, L. Barefoot, K. Hansjosten, P. M. Vue, M. Cronin, A. Nasraway, and Y. Golan.** 2008. Prior environmental contamination increases the risk of acquisition of vancomycin-resistant enterococci. Clin. Infect. Dis. **46:**678–685.
9. **Duckro, A. N., D. W. Blom, E. A. Lyle, R. A. Weinstein, and M. K. Hayden.** 2005. Transfer of vancomycin-resistant enterococci via health care worker hands. Arch. Intern. Med. **165:**302–307.
10. **Eckstein, B. C., D. A. Adams, E. C. Eckstein, A. Rao, A. K. Sethi, G. K. Yadavalli, and C. J. Donskey.** 2007. Reduction of *Clostridium difficile* and vancomycin-resistant *Enterococcus* contamination of environmental surfaces after an intervention to improve cleaning methods. BMC Infect. Dis. **7:**61.
11. **Elguindi, J., J. Wagner, and C. Rensing.** 2009. Genes involved in copper resistance influence survival of *Pseudomonas aeruginosa* on copper surfaces. J. Appl. Microbiol. **106:**1448–1455.
12. **Eliopoulos, G. M.** 2009. Microbiology of drugs for treating multiply drug-resistant Gram-positive bacteria. J. Infect. **59:**S17–S24.
13. **Espírito Santo, C. E., N. Taudte, D. H. Nies, and G. Grass.** 2008. Contribution of copper ion resistance to survival of *Escherichia coli* on metallic copper surfaces. Appl. Environ. Microbiol. **74:**977–986.
14. **European Centre for Disease Prevention and Control and European Medicines Agency.** 2009. ECDC/EMEA joint technical report. The bacterial challenge: time to react. A call to narrow the gap between multidrug-resistant bacteria in the EU and the development of new antibacterial agents. http://www.ema.europa.eu/pdfs/human/antimicrobial_resistance/EMEA-576176-2009.pdf.
15. **European Commission.** 2002. Opinion on triclosan resistance. European Commission Health & Consumer Protection Directorate-General, Brussels, Belgium. http://ec.europa.eu/food/fs/sc/ssc/out269_en.pdf.
16. **European Commission.** 2009. Assessment of the antibiotic resistance effects of biocides. Scientific Committee on Emerging and Newly Identified Health Risks, Brussels, Belgium. http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_0_021.pdf.
17. **Fernández, J. L., M. Cartelle, L. Muriel, R. Santiso, M. Tamayo, V. Goyanes, J. Gosálvez, and G. Bou.** 2008. DNA fragmentation in microorganisms assessed in situ. Appl. Environ. Microbiol. **74:**5925–5933.
18. **Gould, I. M.** 2008. The epidemiology of antibiotic resistance. Int. J. Antimicrob. Agents **32**(Suppl. 1)**:**S2–S9.
19. **Hasman, H., and F. M. Aaresstrup.** 2002. *tcrB*, a gene conferring transferable copper resistance in *Enterococcus faecium*: occurrence, transferability and linkage to macrolide and glycopeptide resistance. Antimicrob. Agents Chemother. **46:**1410–1416.
20. **Hasman, H., and F. M. Aaresstrup.** 2005. Relationship between copper, glycopeptide, and macrolide resistance among *Enterococcus faecium* strains isolated from pigs in Denmark between 1997 and 2003. Antimicrob. Agents Chemother. **49:**454–456.
21. **Hasman, H., I. Kempf, B. Chidaine, R. Cariolet, A. J. Ersboll, H. Houe, H. C. B. Hansen, and F. M. Aaresstrup.** 2006. Copper resistance in *Enterococcus faecium*, mediated by the *tcrB* gene, is selected by supplementation of pig feed with copper sulfate. Appl. Environ. Microbiol. **72:**5784–5789.
22. **Hayden, M. K., D. W. Blom, E. A. Lyle, C. G. Moore, and R. A. Weinstein.** 2008. Risk of hand or glove contamination after contact with patients colonized with vancomycin-resistant enterococcus or the colonized patients environment. Infect. Control Hosp. Epidemiol. **29:**149–154.
23. **Health Protection Agency.** 2008. Antimicrobial resistance and prescribing in England, Wales and Northern Ireland, 2008. Health Protection Agency, London, United Kingdom.
24. **Health Protection Agency.** 2008. Health protection report 2, no. 42. Health Protection Agency, London, United Kingdom.
25. **Kearns, A. M., R. Freeman, and N. F. Lightfoot.** 1995. Nosocomial enterococci: resistance to heat and sodium hypochlorite. J. Hosp. Infect. **30:**193–199.
26. **Keevil, C. W.** 2003. Rapid detection of biofilms and adherent pathogens using scanning confocal laser microscopy and episcopic differential interference contrast microscopy. Water Sci. Technol. **47:**105–116.
27. **Lankford, M. G., S. Collins, L. Youngberg, D. M. Rooney, J. R. Warren, and G. A. Noskin.** 2006. Assessment of materials commonly utilized in health care: implications for bacterial survival and transmission. Am. J. Infect. Control **34:**258–263.
28. **Launay, A., S. A. Ballard, P. D. R. Johnson, M. L. Grayson, and T. Lambert.** 2006. Transfer of vancomycin resistance transposon Tn*1549* from *Clostridium symbiosum* to *Enterococcus* spp. in the gut of gnotobiotic mice. Antimicrob. Agents Chemother. **50:**1054–1062.
29. **Livermore, D. M.** 2009. Has the era of untreatable infections arrived? J. Antimicrob. Chemother. **64**(Suppl. 1)**:**i29–i36.
30. **Lloyd, D. R., and D. H. Phillips.** 1999. Oxidative DNA damage mediated by copper(II), iron(II) and nickel(II) Fenton reactions: evidence for site-specific mechanisms in the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intrastrand cross-links. Mutat. Res. **424:**23–36.
31. **Macomber, L., C. Rensing, and J. A. Imlay.** 2007. Intracellular copper does not catalyze the formation of oxidative DNA damage in *Escherichia coli*. J. Bacteriol. **189:**1616–1626.

32. **Mehtar, S., I. Wiid, and S. D. Todorov.** 2008. The antimicrobial activity of copper and copper alloys against nosocomial pathogens and *Mycobacterium tuberculosis* isolated from healthcare studies in the Western Cape: an *in vitro* study. J. Hosp. Infect. **68:**45–51.
33. **Michels, H. T., J. O. Noyce, and C. W. Keevil.** 2009. Effects of temperature and humidity on the efficacy of methicillin-resistant *Staphylococcus aureus* challenged antimicrobial materials containing silver and copper. Lett. Appl. Microbiol. **49:**191–195.
34. **Murray, B. E.** 1998. Diversity of multidrug-resistant enterococci. Emerg. Infect. Dis. **4:**37–47.
35. **National Nosocomial Infections Surveillance System.** 2004. National Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1992 through June 2004, issued October 2004. Am. J. Infect. Control **32:**470–485.
36. **Neely, A., and M. P. Maley.** 2000. Survival of enterococci and staphylococci on hospital fabrics and plastic. J. Clin. Microbiol. **38:**724–726.
37. **Noyce, J. O., H. Michels, and C. W. Keevil.** 2006. Potential use of copper surfaces to reduce survival of epidemic methicillin-resistant *Staphylococcus aureus* in the healthcare environment. J. Hosp. Infect. **63:**289–297.
38. **Noyce, J. O., H. Michels, and C. W. Keevil.** 2006. Use of copper cast alloys to control *Escherichia coli* O157 cross-contamination during food processing. Appl. Environ. Microbiol. **72:**4239–4244.
39. **Noyce, J. O., H. Michels, and C. W. Keevil.** 2007. Inactivation of influenza A virus on copper versus stainless steel surfaces. Appl. Environ. Microbiol. **73:**2748–2750.
40. **Ott, M., and H. Wirick.** 2008. Vancomycin-resistant enterococci (VRE) and the role of the healthcare worker. Can. Oper. Room Nurs. J. **26:**21–24, 26–29, 32.
41. **Paulsen, I. T., L. Banerjei, G. S. A. Myers, K. E. Nelson, R. Seshadri, T. D. Read, D. E. Fouts, J. A. Eisen, S. R. Gill, J. F. Heidelberg, H. Tettelin, R. J. Dodson, L. Umayam, L. Brinkac, M. Beanan, S. Daugherty, R. T. DeBoy, S. Durkin, J. Kolonay, R. Madupu, W. Nelson, J. Varmathevan, B. Tran, J. Upton, T. Hansen, J. Shetty, H. Khouri, T. Utterback, D. Radune, K. A. Ketchum, B. A. Dougherty, and C. M. Fraser.** 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science **299:** 2071–2074.
42. **Salgado, C. D., K. A. Sepkowitz, T. Plaskett, J. F. John, J. R. Cantey, H. H. Attaway, L. L. Steed, H. T. Michels, and M. G. Schmidt.** 2008. Microbial burden of objects in ICU rooms, poster K-4117, p. 202. 48th ICAAC. American Society for Microbiology, Washington, DC.
43. **Smith, K., and I. S. Hunter.** 2008. Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. J. Med. Microbiol. **57:**966–973.
44. **Suller, M. T., and A. D. Russell.** 2000. Triclosan and antibiotic resistance in *Staphylococcus aureus*. J. Antimicrob. Chemother. **46:**11–18.
45. **Tacconelli, E., and M. A. Cataldo.** 2008. Vancomycin-resistant enterococci (VRE): transmission and control. Int. J. Antimicrob. Agents **31:**99–106.
46. **Tolba, O., A. Loughrey, C. E. Goldsmith, B. C. Millar, P. J. Rooney, and J. E. Moore.** 2007. Survival of epidemic strains of nosocomial- and community-acquired methicillin-resistant *Staphylococcus aureus* on coins. Am. J. Infect. Control **35:**342–346.
47. **Top, J., R. Willems, and M. Bonten.** 2008. Emergence of CC17 *Enterococcus faecium*: from commensal to hospital adapted pathogen. FEMS Immunol. Med. Microbiol. **52:**297–308.
48. **Weaver, L., H. T. Michels, and C. W. Keevil.** 2008. Survival of *Clostridium difficile* on copper and steel. J. Hosp. Infect. **68:**145–151.
49. **Weaver, L., H. T. Michels, and C. W. Keevil.** 2010. Potential for preventing spread of fungi in air conditioning systems constructed using copper instead of aluminium. Lett. Appl. Microbiol. **50:**18–23.
50. **Werner, G., T. M. Coque, A. M. Hammerum, R. Hope, W. Hryniewicz, A. Johnson, I. Klare, K. G. Kristinsson, R. Leclerq, C. H. Lester, M. Lillie, C. Novais, B. Olsson-Liljuquist, L. V. Peixe, E. Sadowy, G. S. Simonsen, J. Top, J. Vuopio-Varkila, R. J. Willems, W. Witte, and N. Woodford.** 2008. Emergence and spread of vancomycin resistance among enterococci in Europe. Euro Surveill. **13:**1–11.
51. **Wheeldon, L. J., T. Worthington, P. A. Lambert, A. C. Hilton, C. J. Lowden, and T. S. Elliot.** 2008. Antimicrobial efficacy of copper surfaces against spores and vegetative cells of *Clostridium difficile*: the germination theory. J. Antimicrob. Chemother. **62:**522–525.
52. **Wilks, S. A., H. Michels, and C. W. Keevil.** 2005. The survival of *Escherichia coli* O157 on a range of metal surfaces. Int. J. Food Microbiol. **105:**445–454.
53. **Wilks, S. A., H. T. Michels, and C. W. Keevil.** 2006. Survival of *Listeria monocytogenes* Scott A on metal surfaces: implications for cross-contamination. Int. J. Food Microbiol. **111:**93–98.
54. **Witte, W.** 2004. Glycopeptide resistant Staphylococcus. J. Vet. Med. B Infect. Dis. Vet. Public Health **51:**370–373.

Appendix 2

Warnes, S.L. and Keevil, C.W. (2011). Mechanism of copper surface toxicity in vancomycin-resistant enterococci following wet or dry surface contact. *Applied and Environmental Microbiology* 77: 6049-6059



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

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

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

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

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

RESULTS

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

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

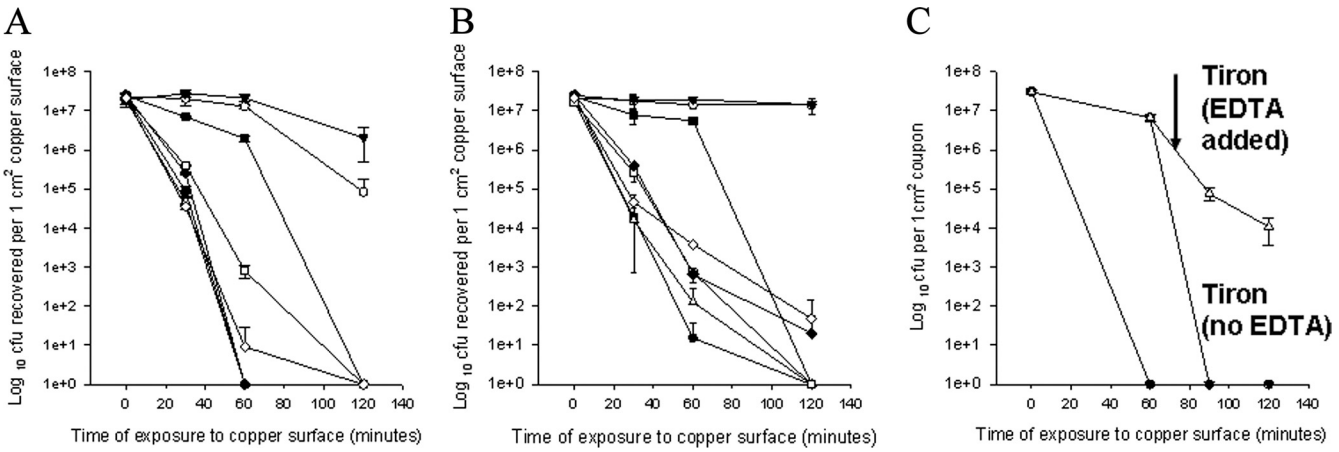

FIG. 1. Survival of vancomycin-resistant *E. faecalis* ATCC 51299 (A) and *E. faecium* NCTC 12202 (B) on copper surfaces with the wet fomite inoculum, with the addition of EDTA prolonging the protective effect of Tiron in *E. faecalis* ATCC 51299 bacteria exposed to copper surfaces (C). (A and B) Approximately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

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

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

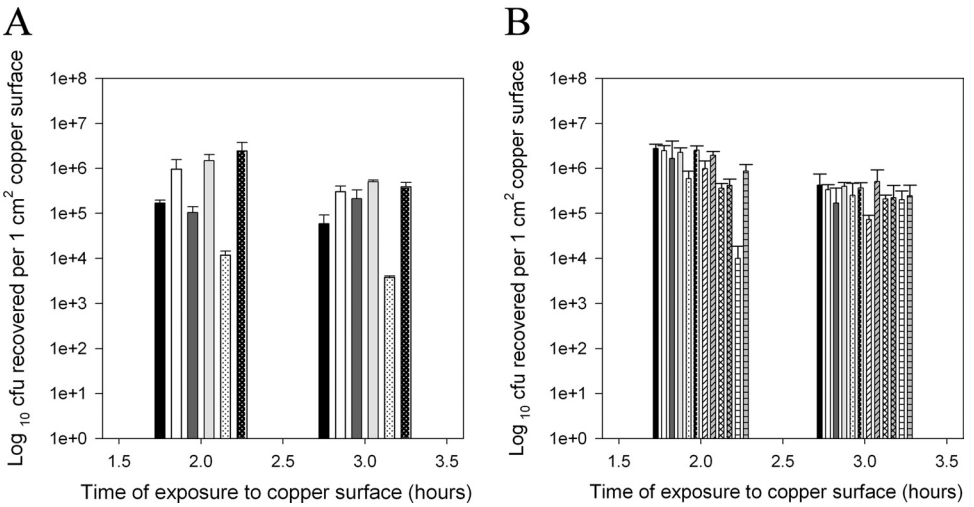

FIG. 2. Survival of enterococcal clinical isolates on copper surfaces in the presence of chelators of Cu(I) (BCS) and Cu(II) ions (EDTA) with the wet fomite inoculum. Approximately 10^7 CFU of *E. faecalis* (A) or *E. faecium* (B), resuspended in 20 μl of 20 mM EDTA or 20 mM BCS, was inoculated onto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

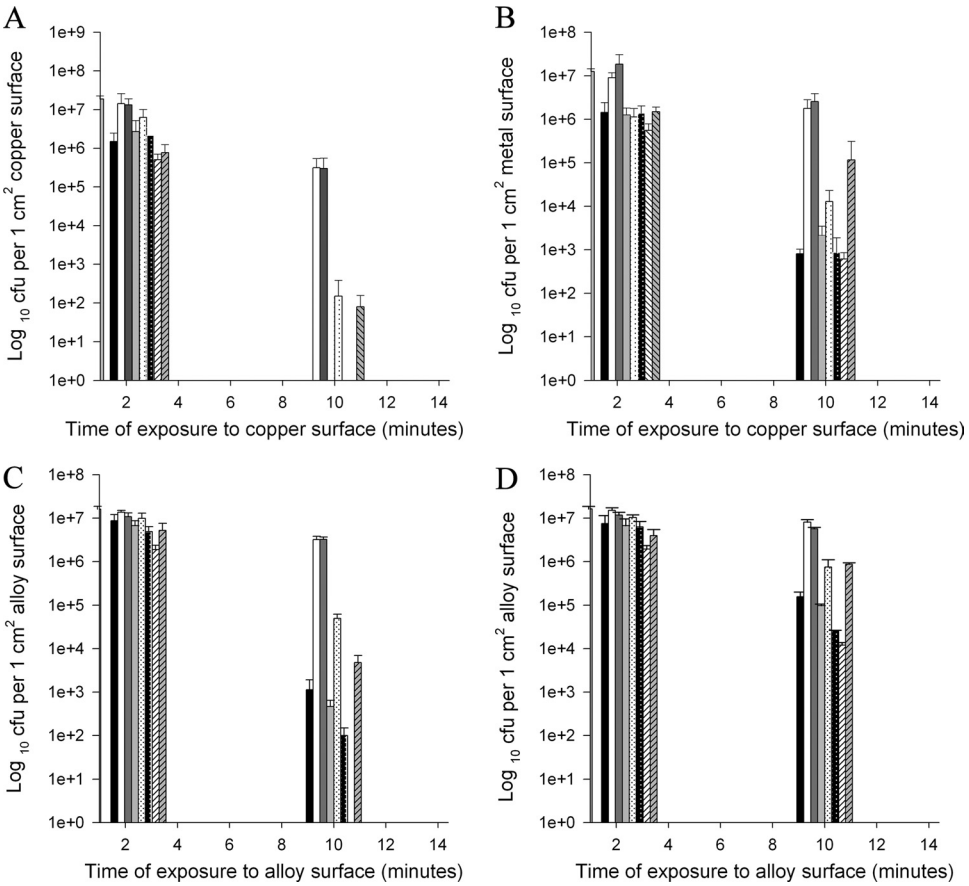

FIG. 3. Survival of vancomycin-resistant *E. faecalis* ATCC 51299 (A and C) and *E. faecium* NCTC 12202 (B and D) on copper (A and B) and copper alloy C26000 (C and D) surfaces with the dry inoculum. Approximately 10^7 CFU in 1 μl was inoculated onto 1-cm^2 coupons in PBS (black bars) or PBS supplemented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

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

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

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

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

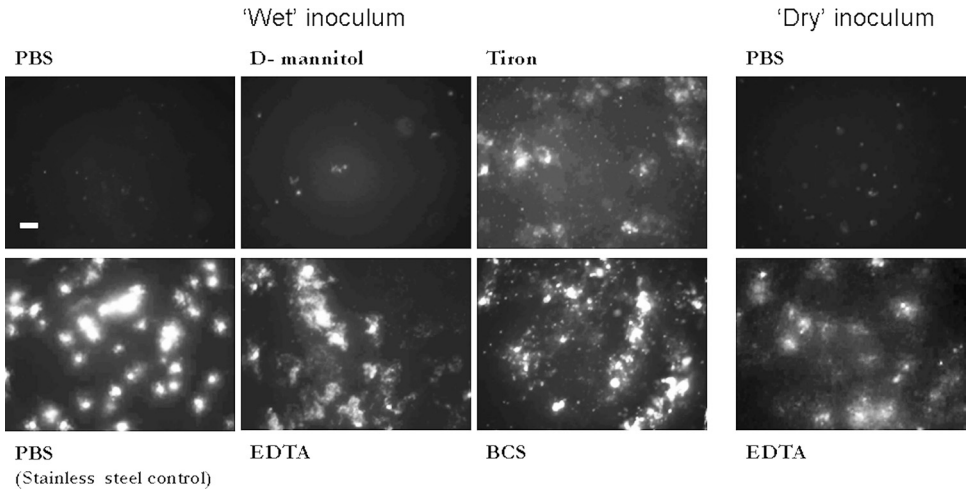

FIG. 4. Protection of bacterial genomic DNA by chelators and ROS quenchers determined by use of a DNA fragmentation assay with the wet and dry inocula. Approximately 10^7 bacterial cells (in 20 μl [wet] or 1 μl [dry]) were inoculated onto copper coupons in PBS (control) or PBS supplemented with chelators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

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

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

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

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

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

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

To determine if the DNA of dead cells also degrades on

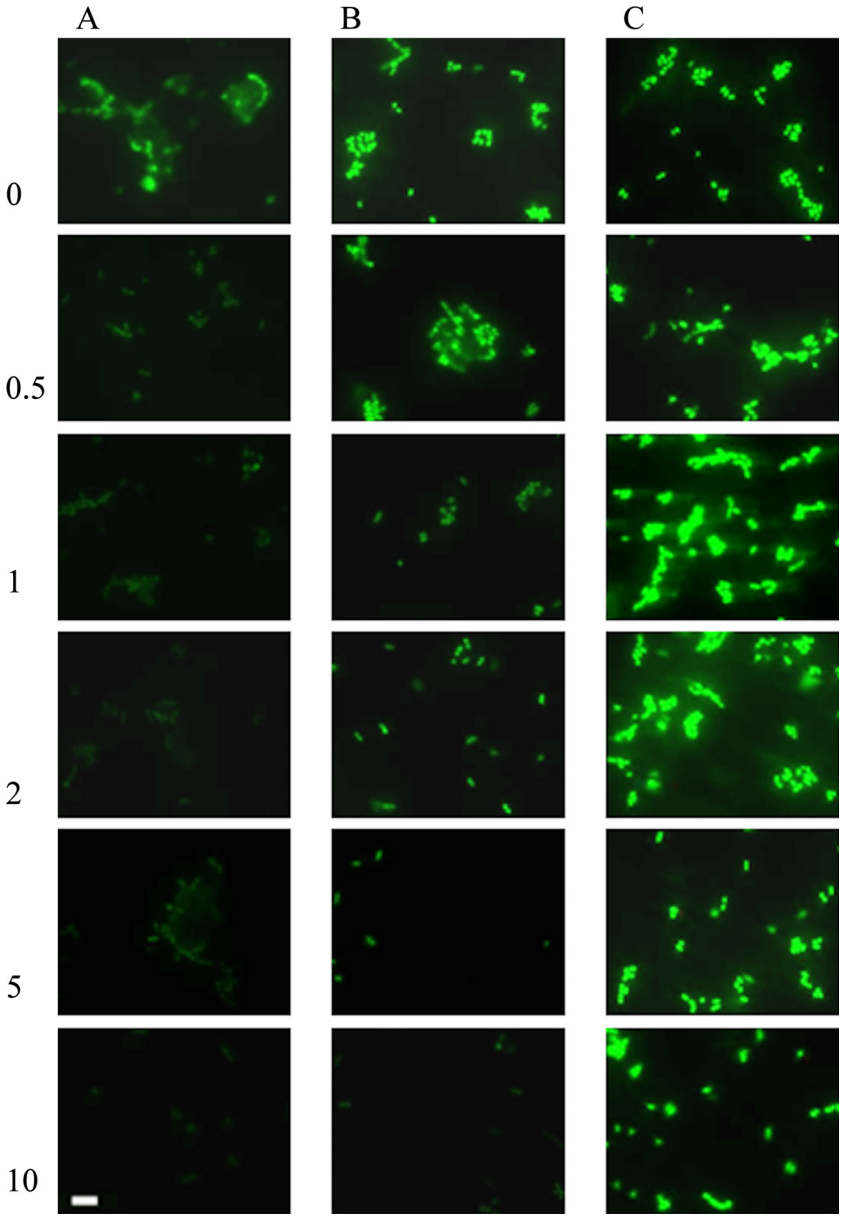

FIG. 5. Rapid breakdown of bacterial DNA on copper (A) and alloy (C26000) (B) surfaces compared to stainless steel (C) occurs as part of the killing mechanism in enterococci. Approximately 10^7 CFU in 1 μl was inoculated onto 1-cm^2 coupons, and cells were removed at the time points indicated (minutes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

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

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

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

Positive staining by the nucleic acid stain SYTO 9 in the

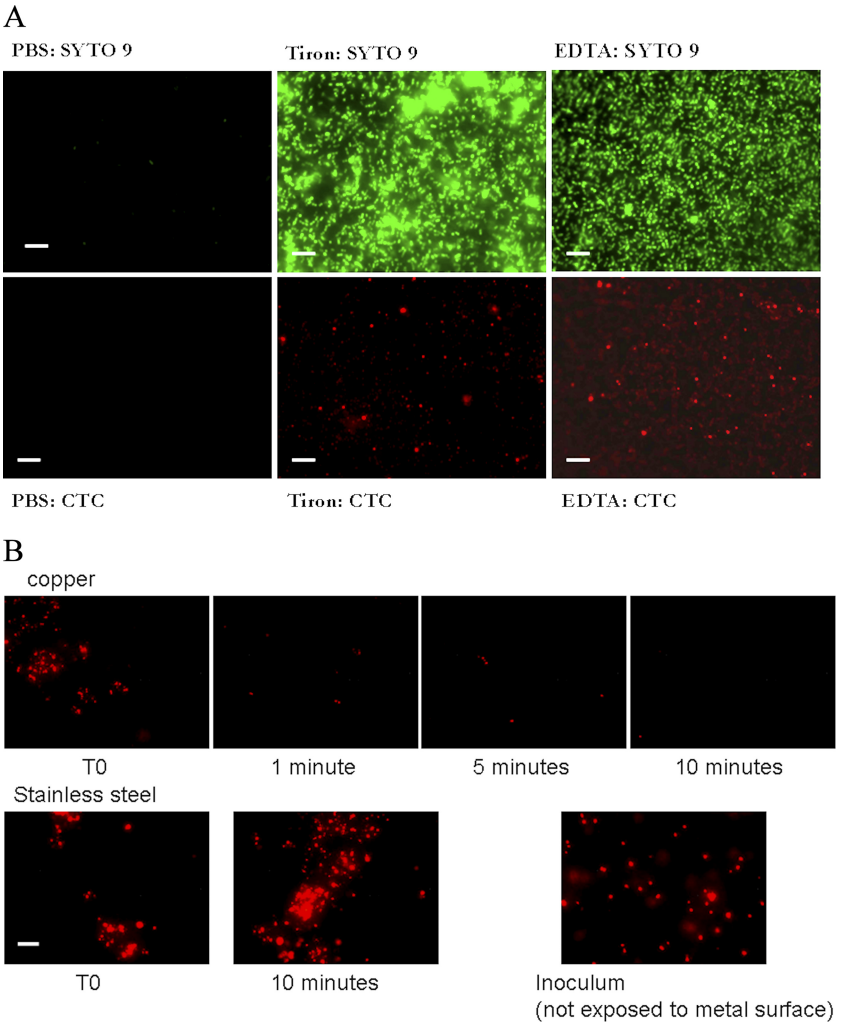

FIG. 6. Protection of bacterial respiratory pathways by chelators and ROS quenchers on copper surfaces with the wet (A) or dry (B) inoculum. Approximately 10^7 bacterial cells (in 20 μl) were inoculated onto copper coupons in PBS or PBS supplemented with chelators or ROS quenchers and incubated at 22°C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

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

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

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

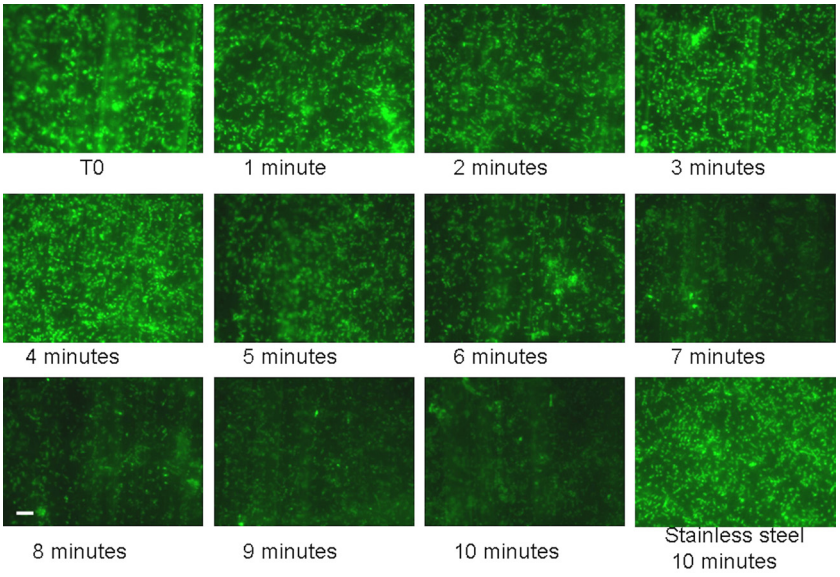

FIG. 7. Detection of changes in membrane potential changes *in situ* in *E. faecalis* ATCC 51299 cells exposed to copper (for the times indicated) and stainless steel (only the 10-min time point is illustrated) surfaces. Approximately 10^7 bacterial cells (in 1 μl) that had been stained with rhodamine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

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

DISCUSSION

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

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

In vivo, enterococci exist primarily under the anoxic condi-

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

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

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

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

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

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

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

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

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

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

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

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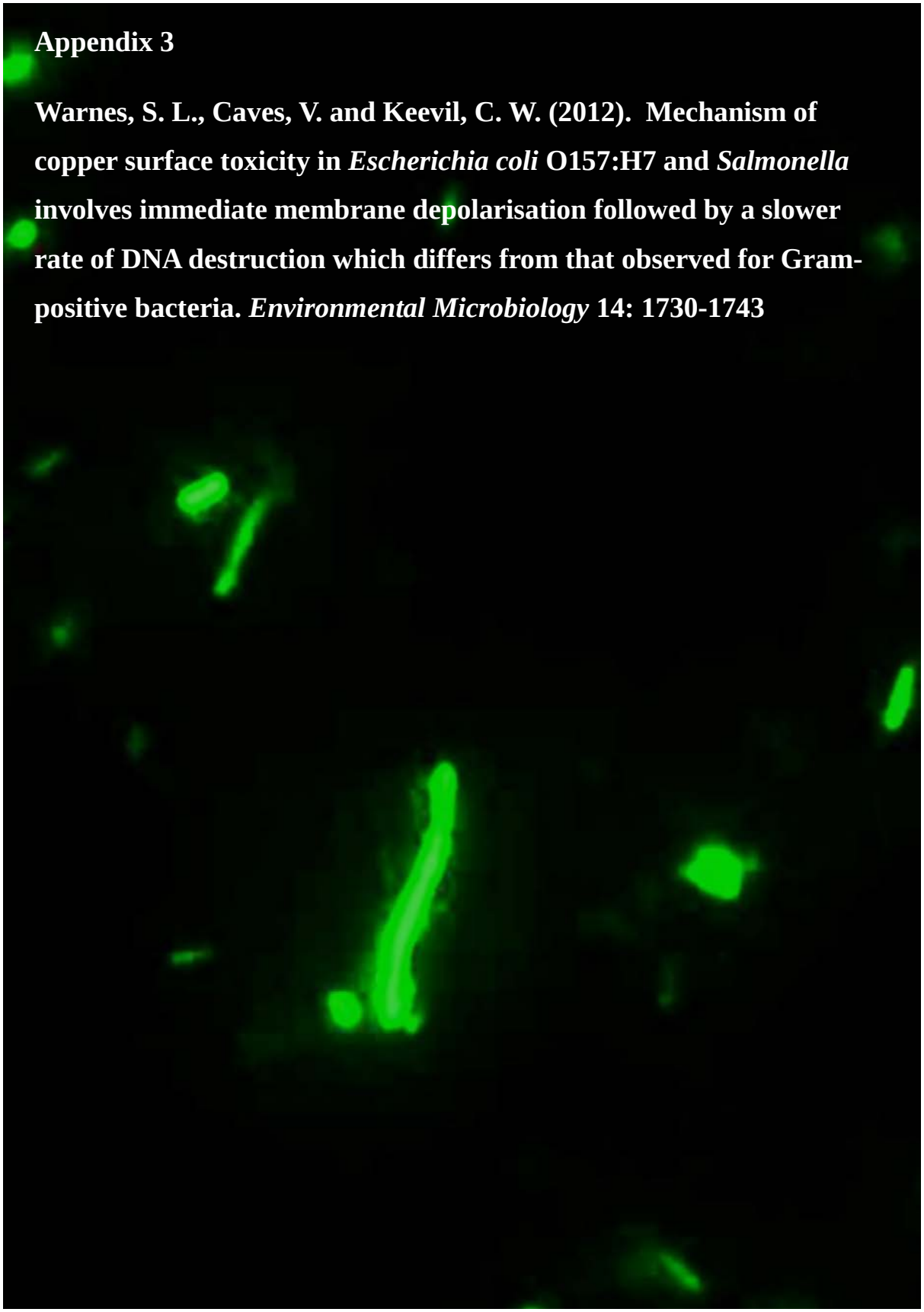
REFERENCES

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

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

Appendix 3

Warnes, S. L., Caves, V. and Keevil, C. W. (2012). Mechanism of copper surface toxicity in *Escherichia coli* O157:H7 and *Salmonella* involves immediate membrane depolarisation followed by a slower rate of DNA destruction which differs from that observed for Gram-positive bacteria. *Environmental Microbiology* 14: 1730-1743



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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

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Summary

We have reported previously that copper I and II ionic species, and superoxide but not Fenton reaction generated hydroxyl radicals, are important in the killing mechanism of pathogenic enterococci on copper surfaces. In this new work we determined if the mechanism was the same in non-pathogenic ancestral (K12) and laboratory (DH5α) strains, and a pathogenic strain (O157), of *Escherichia coli*. The pathogenic strain exhibited prolonged survival on stainless steel surfaces compared with the other *E. coli* strains but all died within 10 min on copper surfaces using a 'dry' inoculum protocol (with approximately 10^7 cfu cm^{-2}) to mimic dry touch contamination. We observed immediate cytoplasmic membrane depolarization, not seen with enterococci or methicillin resistant *Staphylococcus aureus*, and loss of outer membrane integrity, inhibition of respiration and *in situ* generation of reactive oxygen species on copper and copper alloy surfaces that did not occur on stainless steel. Chelation of copper (I) and (II) ionic species still had the most significant impact on bacterial survival but protection by D-mannitol suggests hydroxyl radicals are involved in the killing mechanism. We also observed a much slower rate of DNA destruction on copper surfaces compared with previous results for enterococci. This may be due to protection of the nucleic acid by the periplasm and the extensive cell aggregation that we observed on copper surfaces. Similar results were obtained for *Salmonella* species but partial quenching by D-mannitol suggests radicals other than hydroxyl may be involved. The results indicate that copper biocidal surfaces are effective for Gram-positive and Gram-negative bacteria but bacterial morphology affects the mechanism of toxicity. These surfaces could not only help to prevent infection spread but also prevent horizontal gene transmission which is responsible for the evolution of virulent toxin producing and antibiotic resistant bacteria.

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Introduction

Horizontal gene transfer between bacteria has lead to a global crisis in infection control of hospital and community acquired infections (Peleg and Hooper, 2010). Although warnings were given 10 years ago on the overuse and mis-use of antibiotics ('a gift to be used sparingly') horizontal gene transfer is responsible for the dissemination of antibiotic resistance genes into many bacterial species. Infections caused by resultant multi-drug resistant (MDR) microorganisms is a growing concern and coupled with the lack of new effective antimicrobials means that options for effective antimicrobial therapy are limited. Gene transfer involving stress response genes and virulence factors, and phage transduction has resulted in infectious agents that are highly virulent and able to persist in the environment and on surfaces which continues the spread of infection (Boyce, 2007). The use of biocidal surfaces is one step in this fight and copper and copper alloys have been shown to be effective as constant killing surfaces in the laboratory for bacteria, fungi and viruses (Wilks *et al.*, 2005; Noyce *et al.*, 2006a,b; 2007; Wilks *et al.*, 2006; Mehtar *et al.*, 2008; Weaver *et al.*, 2008; 2009; Wheeldon *et al.*, 2008; Warnes *et al.*, 2010), and also in current clinical trials (Casey *et al.*, 2010; Grass *et al.*, 2011) to a range of bacterial, fungal and viral pathogens. A recent study has observed not only reduced microbiological burden but lower infection rates if copper surfaces are employed in a clinical environment (Schmidt, 2011). In addition, efficacy is retained in varying environmental humidity, unlike silver (Michels *et al.*, 2009). In enterococci, which have a propensity for gene transfer and prolonged environmental survival, we have previously shown that copper surface

toxicity kills the cells by affecting respiration and DNA degradation which is an advantage in potentially reducing the rate of gene transfer (Warnes *et al.*, 2010; Warnes and Keevil, 2011). However, there is an increasing problem with infections caused by Gram-negative cells because of the difficulty in designing antibacterial agents that can cross the double membrane structure and periplasmic space, and overcome the efficient gene regulation, acquisition and multiple resistance mechanisms that can develop in these cells (Rice, 2007; Kunz and Brook, 2010).

Escherichia coli is an inhabitant of the intestinal flora with many strains being commensal in nature and therefore protecting the gastric mucosa from damage. However, there are pathogenic serotypes that have evolved via horizontal gene transfer, of which *E. coli* O157 is one of the most notable. It is often contracted by the ingestion of contaminated food (ground beef, salad crops), water, through person-to-person contact or from animal reservoirs (for reviews see Lim *et al.*, 2010; Pennington, 2010). Outbreaks are often influenced by geographical and weather conditions and in particular have a damaging effect on children. Although the incidence is far less than methicillin-resistant *Staphylococcus aureus* (MRSA) the severity of disease is far greater with symptoms of bloody diarrhoea that can develop into haemorrhagic colitis and haemolytic uraemic syndrome (HUS) which can be fatal. This is due to the acquisition of pathogenicity islands and plasmid pO157 encoding virulence factors responsible for attachment, colonization and production of verocytotoxins, originating from phage transduction, which inflict damage to the mucosa and access the bloodstream which can lead to kidney failure (Vanaja *et al.*, 2010). *Escherichia coli* O157 can survive for long periods in the environment (e.g. food processing plants, chopping boards, farm surfaces and animal hides while retaining acid resistance prolonging survival in acid foods and the stomach; Kruse and Sørum, 1994; Williams *et al.*, 2005; Stephens *et al.*, 2007; Skandamis *et al.*, 2009; King *et al.*, 2010), produce sub-populations of dormant cells (Lewis, 2010) and persist in mixed species biofilms (Habimana *et al.*, 2010; Uhlich *et al.*, 2010; Van Houdt and Michiels, 2010).

This, along with low infectious dose and harbouring genes for virulence, toxins and antibiotic-resistance, increases the risk of perpetuation of infection and possible gene transfer to other bacteria. Research has therefore focussed on the potential of biocidal surfaces to reduce this incidence and the use of copper is documented here. It is essential to understand the modes of action prior to replacing conventional surfaces within healthcare or manufacturing facilities. Many studies describe the mechanism of toxicity of copper ions in solution which is thought to arise from the generation of toxic radicals via Fenton and Haber–Weiss type reactions, which inflict damage on target molecules including nucleic acids, structural and functional proteins (Halliwell and Gutteridge, 1989; Stohs and Bagghi, 1995; Lloyd and Phillips, 1999; Imlay, 2003). Reports have also suggested this happens in prokaryotes (Espirito Santo *et al.*, 2008; Macomber and Imlay, 2009).

Recent studies in our laboratory identified copper (I), (II) and superoxide moieties responsible for toxicity of copper and copper alloy surfaces in enterococci (Warnes and Keevil, 2011). The nucleic acid and respiratory metabolism was affected but not membrane integrity. However, the DNA damage observed was not due to Fenton reaction generated hydroxyl radicals. Published reports on *E. coli* O157 in our laboratory have described the efficacy of copper and alloy surfaces, even in the presence of matrices present in food processing plants for a simulated wet fomite surface contamination (Wilks *et al.*, 2005; Noyce *et al.*, 2006a). In this new work we investigated kill times for dry 'touch' contamination in environmental and pathogenic Gram-negative *E. coli* and if the mechanism of toxicity was the same as in Gram-positive bacteria. We also include data for Gram-negative *Salmonella* species which are one of the most common causes of food poisoning worldwide and persist for extended periods on contact surfaces (Van Houdt and Michiels, 2010).

Results

Survival of ancestral, laboratory and pathogenic strains of E. coli *on metal surfaces in the presence and absence of chelators and ROS quenchers*

Previous work in our laboratory observed that high numbers of *E. coli* O157, in a 'wet' inoculum to mimic fomite surface contamination, die on copper and copper alloy surfaces in several hours compared with over 28 days on stainless steel (Wilks *et al.*, 2005).

In this new work we have observed that a rapidly drying inoculum of *E. coli* pathogenic and non-pathogenic strains, to mimic 'dry' touch contamination of surfaces, dies within minutes on copper (Fig. 1). All strains died within 10 min on copper and alloy surfaces (except the pathogenic strain which survived a little longer on the alloy). However, *E. coli* O157 (C) survived longer on stainless steel surfaces compared with non-pathogenic *E. coli*: approximately 10^4 cfu cm^{-2} were present after 30 min on stainless steel. Another Gram-negative foodborne pathogen, *Salmonella enterica* serovar Typhimurium died more rapidly on copper (all dead at 5 min) but also survived much longer on stainless steel with approximately 10^4 cfu cm^{-2} remaining (D).

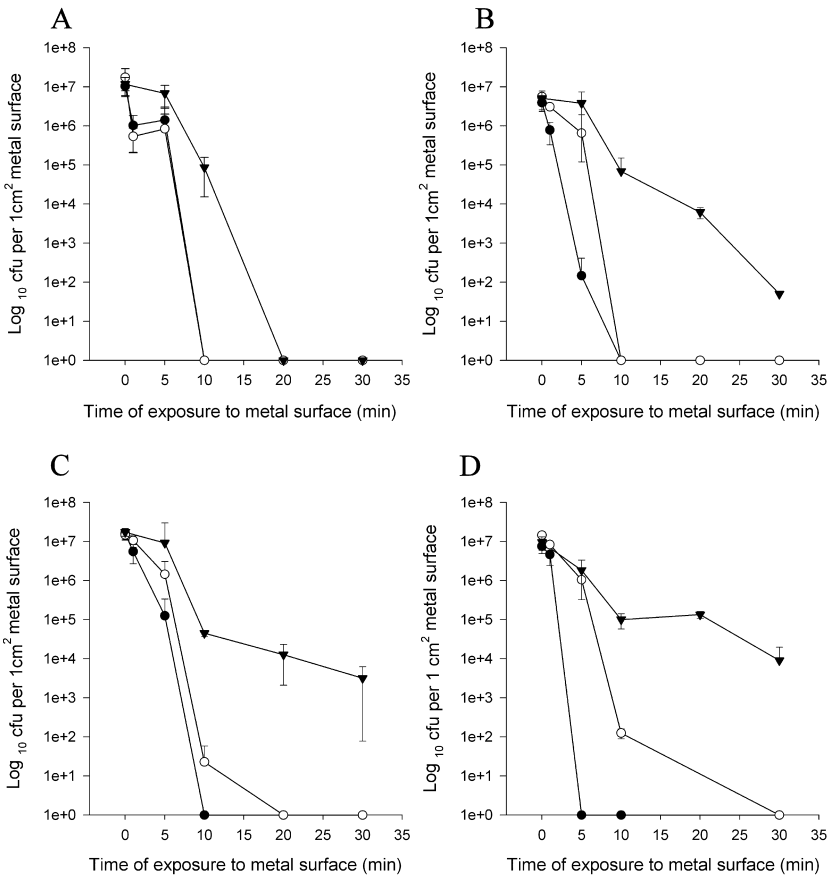

Fig. 1. Survival of *E. coli* ancestral K12 (NCIMB 10214) (A), laboratory (DH5α) (B), pathogenic (NCTC12900, non-toxigenic) (C) strain and *Salmonella* Typhimurium (ATCC 14028 on copper (C11000), cartridge brass (C26000) and stainless steel (S30400) surfaces at 22°C. Approximately 10^7 cfu in 1 μl PBS were inoculated onto 1 cm^2 metal coupons: C11000 (●), C26000 (○), S30400 (▼). Cells were removed from coupons and assessed for culturability as described in the text. Prolonged survival of pathogens was observed on stainless steel but all strains died rapidly on copper and copper alloy.

If EDTA or BCS are present in the inoculum to chelate copper (II) and (I), respectively, a protective effect is seen (Fig. 2) (although it is significantly lower than results on stainless steel at 10 min; $P < 0.001$). A protective effect is also present for D-mannitol but not Tiron suggesting hydroxyl radical generation, but not superoxide, is involved in copper surface toxicity in *E. coli* (results shown for *E. coli* DH5α).

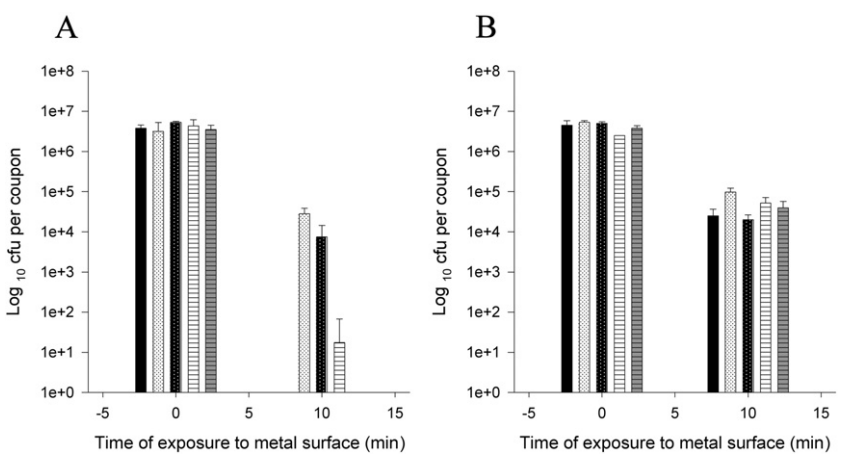

Fig. 2. Protection of bacterial cell death with chelators and D- mannitol on copper (A) or stainless steel (B) surfaces (results shown for *E. coli* DH5α). Approximately 10^7 cfu in 1 μl PBS (control) (black bars) or PBS supplemented with EDTA (white spotted bars), BCS (black spotted bars), D-mannitol (white horizontally striped bars) and Tiron (grey horizontally striped bars) were inoculated onto 1 cm^2 copper coupons: cells were removed from coupons and assessed for culturability as described in the text. In PBS only no viable cells could be detected at 10 min. A protective effect was seen if both chelators and D-mannitol were present suggesting a role for copper (I), (II) and hydroxyl radicals in toxicity. On stainless steel there was a 1–2 log reduction in viable cells recovered following 10 min contact and no significant difference between PBS or supplements ($P < 0.05$).

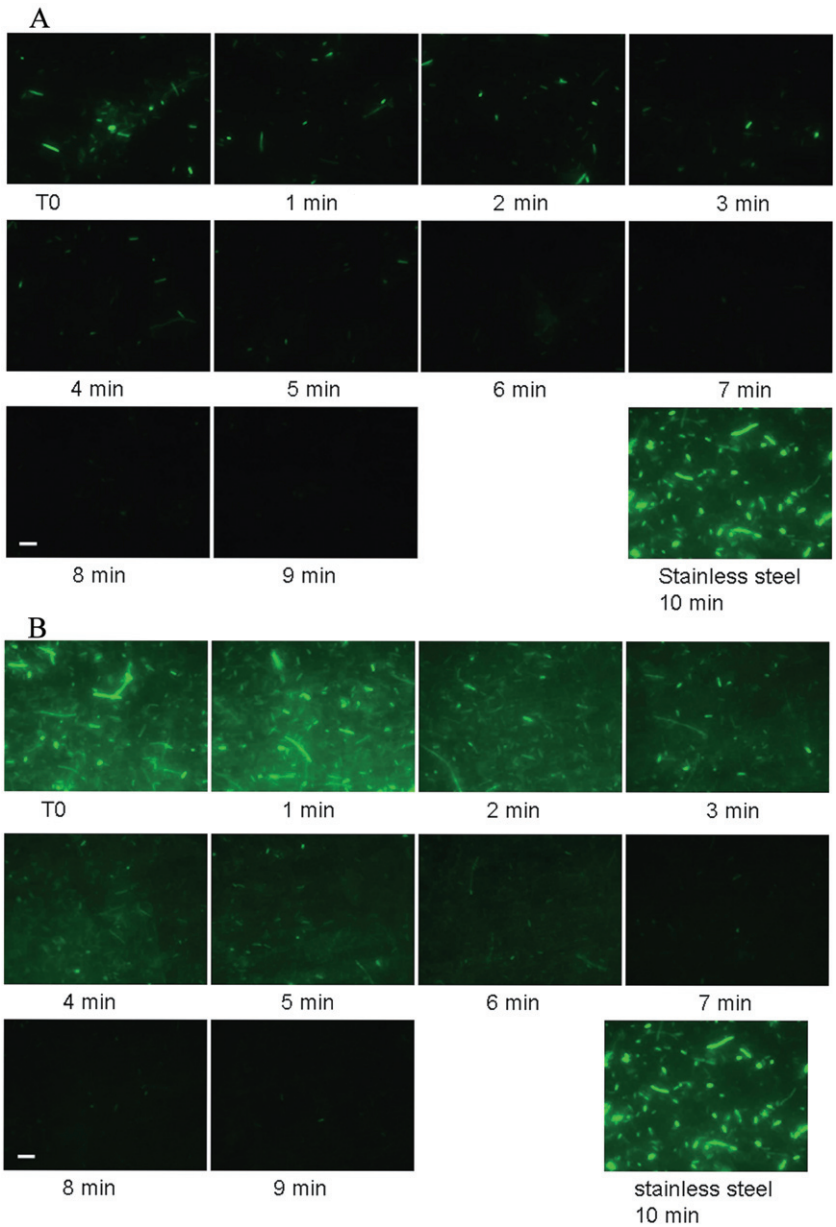

Fig. 3. The effect on bacterial membrane potential *in situ* in *E. coli* (laboratory strain) exposed to copper, brass and stainless steel using lipidophilic stain Rhodamine 123 (Rh123). Bacterial cells were pre-stained with Rh123 (as described in the text) and spread over 1 cm^2 metal coupons (approximately 10^7 cfu in 1 μl). Cells with intact membranes stain brightly due to accumulation of Rh123, however if the membrane is compromised the potential is lost and staining dissipates. On copper surface membrane depolarization occurs almost immediately (A). On alloy, depolarization occurs more slowly but significant staining reduction is observed at 6 min (B). Following 10 min contact with stainless steel membranes are still intact. As a control, to demonstrate that depolarized cells do not retain Rh123 stain, bacterial cells were exposed to the ionophore carbonylcyanide m-chlorophenylhydrazone (CCCP), which increases proton permeability and dissipates membrane potential and proton gradient (proton motive force). In the absence of CCCP bright staining with Rh123 is evident which dissipates if CCCP is added (not shown). Size bar is 10 μm.

Detection of changes in membrane potential in E. coli *on copper surfaces*

Metabolically active bacteria with intact membranes demonstrate an electrical potential difference ($\Delta\Psi$) between the inside and the outside of the cell. This varies between species but is approximately 100–200 mV with the interior of the cells negative. Damage to the membrane, e.g. depolarization by cations, dissipates the potential to 0. Several fluorescent dyes are used to assess $\Delta\Psi$ including the cationic, lipidophilic dye rhodamine 123 (Rh123), which accumulates on the inner surface of intact membranes. If the membrane becomes leaky or ruptures fluorescence diminishes (Breeuwer and Abee, 2004). *Escherichia coli* cells were pre-stained with Rh123 and inoculated onto metal surfaces and fluorescence changes monitored over 10 min contact (Fig. 3). The cells on copper (A) were depolarized immediately on contact. A slower depolarization occurred on the copper alloy surface (B) which was complete after 3–4 min. On stainless steel no depolarization occurred for the duration of the experiment (10 min time point shown) and cells retained bright fluorescence that was also observed in the untreated inoculum (not shown). To ensure these results *in situ* were not due to interaction between copper and the dye bacteria were removed from coupons at specified time points and stained with Rh123 *ex situ*. Figure S1 shows images of *E. coli* O157 removed from copper (A),

brass (B) or stainless steel (C) following 5 min contact. Cells on stainless steel stain brightly with characteristic polar accumulation of Rh123 observed in intact cells. This is reduced on the alloy and eliminated on copper suggesting membrane depolarization has occurred.

Several other bacterial species were stained with Rh123 and inoculated onto metal surfaces. Images were recorded at specific time points and Fig. S2 shows the results following 1 min contact. Once again *E. coli* O157 completely depolarized on copper with a few cells maintaining $\Delta\Psi$ on brass. A similar result was seen for *S.* Typhimurium but depolarization did not occur as rapidly as for *E. coli*. In complete contrast, none of the Gram-positive bacteria tested here showed depolarization on any of the metal surfaces as observed previously (Warnes and Keevil, 2011). Dye uptake was more efficient in Gram-positive bacteria because of the single membrane, hence the brighter staining for these organisms.

Bacterial membrane potential changes were also assessed in *E. coli* using the *Bac*Light Membrane Potential Kit (Invitrogen) (Fig. S3). Cells stained with the carbocyanine dye, DiOC2(3) (3,3′-diethyloxacarbocyanine iodide), emit a green fluorescence but the dye experiences a red shift in cells with intact $\Delta\Psi$ when dye molecules aggregate in the membrane. Dissipation of membrane potential can be visualized as a loss of red fluorescence. *Escherichia coli* stained with DiOC2(3) exhibited red and green fluorescence on stainless steel surfaces (A) but red fluorescence diminished on copper suggesting depolarization had occurred on this surface. However, this method is more suited to flow cytometry and staining intensity was very low. Increasing the dye concentration to aid microscopy observation may affect viability because this dye is known to affect the respiration of bacterial cells, therefore no further studies were performed with this dye but the results do support those obtained with Rh123 that the membrane of *E. coli* is depolarized immediately.

To ensure intact $\Delta\Psi$ was related to dye uptake in all these methods and cell types enforced depolarization with the ionophore CCCP resulted in abolition of staining (not shown).

Lysozyme is a naturally occurring antibacterial which breaks the β (1–4) linkages between *N*-acetyl glucosamine (NAG) and *N*-acetyl muramic acid (NAM) in the peptidoglycan (PG) bacterial cell wall. In Gram-negative bacteria lysozyme is too big to pass through an intact outer membrane (OM). However, if the OM is damaged, lysozyme can access the cell wall and disrupt PG sheets (Peng *et al.*, 2007). We modified this method to visualize the exposed PG using conventional Gram-staining and lysozyme on untreated, detergent-treated (0.1% Triton X-100 for 5 min at room temperature), or cells exposed to copper or stainless steel surfaces. Exposure to copper followed by lysozyme treatment to loosen the cell wall resulted in cells that retained the crystal violet-iodine complex (Fig. S4A). This effect did not occur immediately on contact, suggesting cytoplasmic membrane depolarization occurs first. The cells were also aggregated into large clumps, also observed by Peng and colleagues (2007); Figure S4 shows the results after 5 min exposure to metals. The same result also occurred if cells were exposed to mild detergent to permeabilize the outer membrane followed by lysozyme treatment (not shown). This did not occur on cells recovered from stainless steel (Fig. S4B) or untreated cells. The results suggest exposure to copper also damages the outer membrane of Gram-negative bacteria.

Inhibition of respiration of E. coli *on copper surfaces*

All three *E. coli* strains demonstrated inhibition of respiration on copper surfaces that was not evident on stainless steel using the redox stain 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC), which accumulates red fluorescent formazan in actively respiring cells (Fig. 4). Although the kill time was the same for all strains on copper (10 min, Fig. 1) pathogenic *E. coli* O157 (A) appeared to retain respiring cells for longer than the non-pathogenic strains (B, C). There were less respiring cells of *E. coli* K12 on stainless steel (3) at 10 min which supports the culture results that this strain is more susceptible to desiccation and dies on this surface by 20 min at the inoculum concentration used.

Generation of reactive oxygen species by E. coli *on copper surfaces*

The carboxylated, fluorinated derivative of fluorescein, H_2DFFDA (5-(and-6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate) accumulates in the bacterial cells where it is de-acetylated by intracellular esterases. If ROS are present the dye is oxidized to a fluorescent product, which can be visualized microscopically. This dye is more photostable than chlorinated fluorescein derivatives but care has to be taken in its use to prevent photooxidation (Gomes *et al.*, 2005). *Escherichia coli* cells stained with H_2DFFDA were inoculated onto copper coupons and observed over an 11 min period (Fig. 5A). Positive fluorescence was detected throughout the experiment suggesting ROS generation occurs. This dye is not specific for radicals but because the effect could be almost entirely quenched if D-mannitol was present (Fig. 5B) it suggest a large proportion of the ROS generated are hydroxyl radicals. In preliminary experiments ROS were also generated in *E. coli* strains exposed to cartridge brass (not shown).

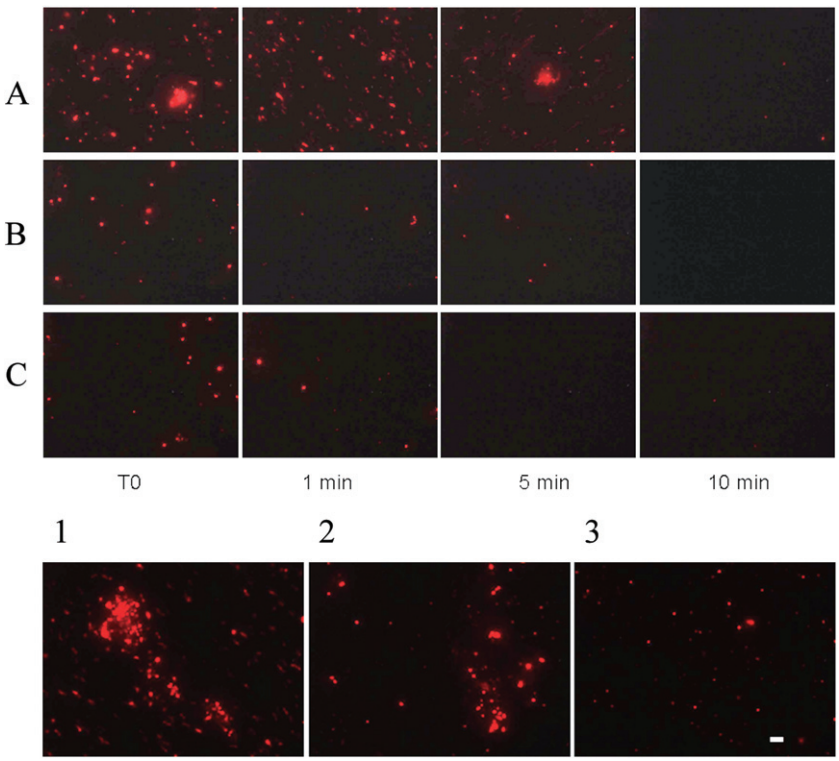

Fig. 4. Inhibition of respiration in *E. coli* O157 (A), DH5α (B) and K12 (C) on copper surfaces using the redox dye CTC (5-cyano-2, 3-ditolyl tetrazolium chloride). This had to be performed *ex situ* because a minimum incubation of 90 min is required for this redox stain. Bacteria were applied to metal coupons for required times, removed and stained with CTC as described in text (same as for culture). No respiring cells are detected following 10 min contact with copper for all strains although inhibition of respiration occurs earlier in non-pathogenic strains. In contrast, actively respiring cells are still evident following 10 min contact with stainless steel in *E. coli* O157 (1), DH5α (2) and K12 (3). Size bar is 10 μm.

Similar results were seen with other Gram-negative species. Results are shown for *S. arizonae* (Fig. S5): no fluorescence was observed on stainless steel with or without D-mannitol (C and D respectively). On copper bright staining was visible (A) which was only partially quenched by D-mannitol (B). Perhaps ROS other than hydroxyl radicals are important in *Salmonella* spp. exposed to copper surfaces.

Degradation of the DNA of E. coli *O157 and* S. *Typhimurium exposed to copper surfaces continues after the death of the bacterial cells*

Escherichia coli O157 (Fig. S6A and B) and *S.* Typhimurium (Fig. S6C and D) cells were stained *in situ* on copper (A and C) or stainless steel surfaces (B and D) with the membrane permeant, non-toxic nucleic acid stain, SYTO 9, which is thought to bind to the minor groove of double-stranded DNA. On copper there was a reduction in staining intensity of individual cells suggesting DNA breakdown but some bright staining was observed at 10 min when cells are dead but the DNA continued to degrade and was undetectable at 60 min. In addition, some aggregation of cells was evident in *E. coli* O157 immediately on contact with copper. On stainless steel bright staining was retained throughout the experiment indicating the DNA is intact. Cells were also stained *ex situ* after exposure to metal surfaces with the same results (not shown). When stained cells were inoculated in the presence of D-mannitol the DNA was protected, indicating that removal of hydroxyl radicals by D-mannitol protects the bacterial DNA from copper toxicity (not shown).

Degradation of the plasmid DNA of E. coli *O157 exposed to copper surfaces*

Although the toxin genes of *E. coli* O157 are located on the chromosome many virulence determinants are on the plasmid (Lim *et al*., 2010). Plasmid DNA was purified from *E. coli* O157 and the fragments separated by agarose gel electrophoresis (Fig. 6). The plasmid DNA from untreated cells (lane 2) or those exposed to stainless steel for 10 min (lane 4) or 1 h (lane 5) is in large fragments > 10 kbp that remain at the loading site. In contrast, cells exposed to copper for 10 min produce a 'smearing' characteristic of disintegration into multi-sized fragments (lane 6), which continue to degrade to smaller fragments observed after 1 h (lane 7). The plasmid DNA of the heat killed cells has also degraded but the fragments are still very large (8–10 kbp).

Discussion

Copper is an essential element but is toxic at elevated concentrations reflected in the complex mechanisms to maintain homeostasis (for review see Grass *et al*., 2011). In *E. coli* chromosomal and plasmid-borne transport chaperones and sequestering proteins are responsible for

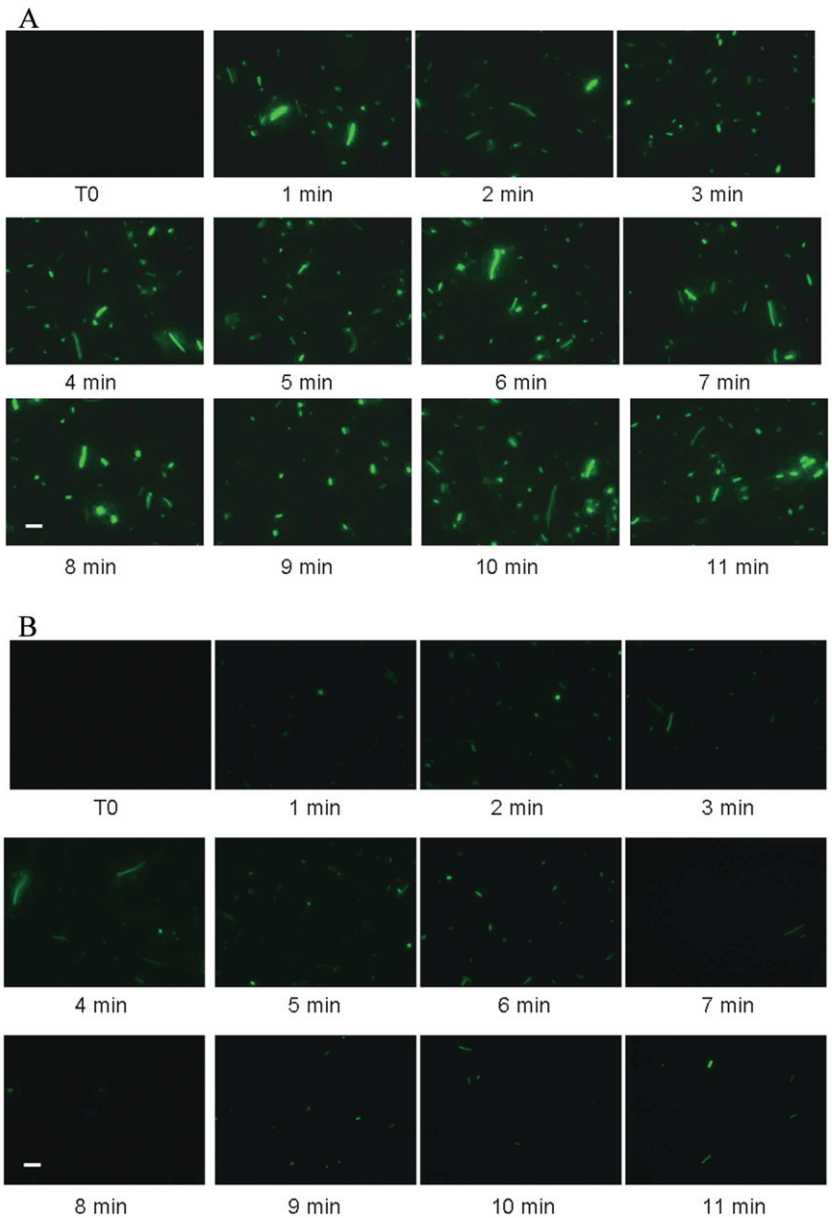

Fig. 5. Generation of reactive oxygen species by *E. coli* on copper surfaces that can be quenched by addition of D-mannitol. Bacterial cells were pre-stained with H_2DFFDA (mixed isomers) (Invitrogen, UK), as described in the text, and spread over 1 cm^2 metal coupons (approximately 10^7 cfu in 1 µl PBS). Images were recorded every minute for 11 min to identify any ROS generation which can be seen as developing fluorescence. ROS are generated by *E. coli* on copper surfaces (A) which can be quenched if D-mannitol is present (B). No fluorescence was observed on stainless steel (not shown). Size bar is 10 µm.

the efflux of excess copper. It has been suggested that the periplasm provides a 'holding area', which can shield the interior of the cell from copper damage (Rice, 2007; Espirito Santo *et al.*, 2008).

Escherichia coli O157 has evolved into an effective pathogen by acquisition of stress resistance and toxin production genes and it is estimated that genes have originated from 53 different species (Lim *et al.*, 2010). *Escherichia coli* O157 is more resistant to complex acids than K12 (King *et al.*, 2010) and differential expression of stress fitness genes even occurs within *E. coli* O157 isolates. Those originating from bovine samples were more resistant to adverse environmental conditions, than clinical isolates which had increased expression of virulence factors (Vanaja *et al.*, 2010).

We have discovered that in dry touch contamination copper and alloy surfaces are very effective in killing all strains of *E. coli* within 10 min for an inoculum of 10^7 cfu cm^{-2} metal surface. K12 did not survive beyond 20 min on stainless steel but the pathogenic strain of *E. coli* and *S.* Typhimurium can survive much longer with approximately 10^4 cfu cm^{-2} present at 30 min. Stainless steel, particularly S30400, is commonly used in food processing plants and it is known that cleaning agents liberate chlorine that can cause pitting of the surface and affect the passivation layer; this can increase the formation of biofilms (Van Houdt and Michiels, 2010). Williams and colleagues (2005) observed that viable *E. coli* O157 persisted on other farm surfaces, including wood, for even longer than stainless steel. Transfer of antibiotic resis-

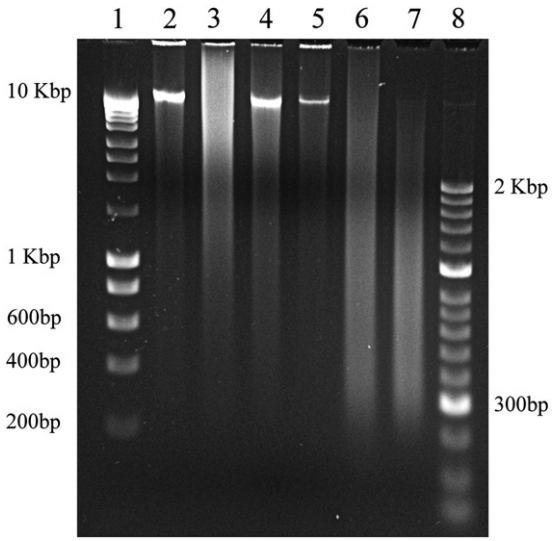

Fig. 6. Degradation of *E. coli* O157 plasmid DNA occurs on copper surfaces. The plasmid DNA of untreated (lane 1), heat killed (lane 2) or cells exposed to metal surfaces [copper for 10 min (lane 6) or 1 h (lane 7); stainless steel for 10 min (lane 4) or 1 h (lane 5)] were purified using Qiaprep Spin miniprep kit (Qiagen, UK) and fragments were separated on a 1% agarose gel using the GelRed DNA stain and buffers (Biotium, UK). The DNA of untreated cells and those exposed to stainless steel demonstrate little effect on DNA, which is in fragments too large to enter the gel (> 10 kbp). However, exposure to copper produces 'smearing' of the DNA characteristic of small fragments of many sizes. After 1 h contact only smaller fragments remain. Control lanes are Bioline Hyperladder I (lane 1) and hyperladder II (lane 8).

tance plasmids has also been observed on surfaces including hand towels and chopping boards (Kruse and Sørum, 1994). Also the low infectious dose means that even persistence of a few viable cells pose an infection risk. The use of copper and copper alloy surfaces which provide a permanently effective biocidal surface could be an effective means of reducing this risk.

We have identified in survival tests that in the various pathogenic and non-pathogenic *E. coli* on copper surfaces release of copper (I), (II) and generation of ROS including hydroxyl radicals are an important part of the killing mechanism. We also used a fluorescence dye to detect ROS generation *in situ* on copper surfaces and observed that the generation occurred throughout contact rather than just on initial inoculation. In our *E. coli* strains D-mannitol, which scavenges hydroxyl radicals, fully quenched the fluorescence. Hydroxyl radicals are powerful non-selective oxidants that can directly damage biomolecules by carbonylation of proteins, membrane peroxidation and DNA damage by base modification, degradation of the ribose ring leading to strand scission (Lloyd and Phillips, 1999; Hossain and Huq, 2002; Imlay, 2003). It has been suggested that copper toxicity results indirectly from the generation of toxic hydroxyl radicals via Fenton reactions with the reduced copper ion (Lloyd and Phillips, 1999; Espirito Santo *et al*., 2008). Our results also suggest that Fenton chemistry is occurring in the various *E. coli* strains exposed to copper surfaces. Of note, although the Fenton reaction is classically associated with iron redox reactions, the reaction is 60 times faster for oxidation of copper (I) than iron (II) (rate constant 4700 M^{-1} s^{-1} compared with 76 M^{-1} s^{-1} respectively (Halliwell and Gutteridge, 1989). In *Salmonella* spp. only partial quenching by D-mannitol was observed, suggesting the possibility that radicals other than hydroxyl radicals are important in this organism. Initial experiments also indicated that chelators increased ROS generation, suggesting hydroxyl radical generation through Haber–Weiss reactions between superoxide and hydrogen peroxide rather than Fenton reactions (not shown), but this must be interpreted with caution because BCS has been observed to oxidize the similar ROS detection dye dichlorofluorescein (Laggner *et al*., 2006).

Recent work has suggested that although hydroxyl radicals are generated on copper surfaces the DNA is not the primary target in bacteria and yeasts and that copper decreases the rate of hydrogen peroxide induced damage in *E. coli* (Macomber *et al*., 2006; Espirito Santo *et al*., 2008; 2010; Quaranta *et al*., 2011). Any DNA damage observed is due to the breakdown of dead cells in common oxidative lethality pathways (Grass *et al*., 2011). The methods used to determine this were designed to detect small changes in the DNA which are then PCR amplified and also using mutagenicity assays (Espirito Santo *et al*., 2011). We have observed, however, that the bulk of DNA in enterococci is rapidly degraded upon exposure to copper by analysis of purified DNA and DNA fragmentation of intact cells in a wet fomite inoculum assay (Warnes *et al*., 2010). In this new study we observed slower but extensive DNA degradation also occurs for the dry touch inoculum assay of *E. coli* on copper surfaces by *in situ* assessment of the DNA of whole cells and purified plasmid DNA. In the former, after 10 min contact with copper surfaces however, some cells are still staining for DNA, which may be due to protection of the nucleic acids by the periplasm and also the aggregation of cells into large clumps, which may prevent copper ion access to cells within the clump. However, the DNA degradation continues after the death of the bacteria and is not detectable after 30 min. Analysis of purified plasmid DNA of cells exposed to copper also revealed degradation was occurring over time although small fragments were still visible at 60 min. No clumping or DNA degradation occurred on stainless steel for both methods. D-mannitol protected the DNA of *E. coli* suggesting hydroxyl radical involvement but as in the ROS generation studies *Salmonella* DNA was only partially protected by D-mannitol. Hydroxyl radicals are known to have a high affinity for and inflict damage on purified *Salmonella* DNA

(Keyhani *et al*., 2006) but perhaps other radicals are important. The DNA damage may also be caused by the direct action of cations including free copper ions, primarily Cu (II) which is the most prevalent ionic species in aerobic aqueous environments. However, copper induced damage is also thought to occur by singlet oxygen (Li and Trush, 1993) and copper peroxide (Tkeshelashvili *et al*., 1990) or copper complexes with other biomolecules (Hossain and Huq, 2002; Selvaraj *et al*., 2009). Cu (II) also increases mis-incorporation of bases by DNA polymerases (Que *et al*., 1979) and Nandakumar and colleagues (2011) noted an upregulation of DnaX, which codes for DNA polymerase III in sublethally and lethally copper stressed cells.

A logical target for copper surface toxicity in Gram-negative bacteria is the outer membrane and associated structures because these are the first parts of the cell to come into contact with the metal surface. Studies have shown that cations have affinity for negatively charged lipopolysaccharide (LPS) in the outer membrane of *E. coli* (Ferris and Beveridge, 1986) and that lead ions disrupt the outer membrane creating unstable patches (Peng *et al*., 2007). We observed that OM breakdown occurred on copper that allowed access of the large enzyme, lysozyme, to the PG layer. We visualized this with conventional Gram-staining and preliminary experiments using the fluorescent wheat germ agglutinin that binds to *N*-acetyl glucosamine suggest copper ions may be affecting the PG itself. However, this effect was not evident immediately on contact with copper but after a few minutes had elapsed.

A conventional way to investigate the status of the inner cytoplasmic membrane is the use of nucleic acid fluorescent stains, e.g. propidium iodide (PI), which can only access DNA of cells with compromised membranes. However, we have had problems using this because the DNA degradation we have observed on copper surfaces prevents binding of the stain to the nucleic acid. Also others have observed that PI staining may be evident but that membrane potential may be unaffected i.e. if there are transient changes in membranes ability to allow entry of PI into the cell (Novo *et al*., 2000). Our results using lipophilic cyanine dyes suggest there is immediate depolarization of *E. coli* and *Salmonella* on copper surfaces, which is slightly delayed on copper alloy presumably due to the lower concentration of copper. This does not occur on stainless steel. Studies have shown that cations have affinity for negatively charged lipopolysaccharide (LPS) in the outer membrane of *E. coli* (Ferris and Beveridge, 1986) and that copper (II) uptake did not affect microviscosity of the bacterial membrane but increased the number of free -SH groups due to disruption of disulfide bonds in membrane-bound ATPases responsible for $K+$ transport possibly by Cu(I) (Lebedev *et al*., 2005; Kirakosyan and Trchounian, 2007; Kirakosyan *et al*., 2008). We have observed inhibition of respiration under dry inoculum conditions, which had previously been observed in the wet (Wilks *et al*., 2005). Volentini and colleagues (2011) used intact cells to show for the first time the role of cytoplasmic membrane-bound respiratory chain components (dehydrogenases and quinones) in copper homeostasis, which may be overwhelmed by the barrage of ion release from dry copper surfaces.

To summarize, copper toxicity in *E. coli* involves copper ionic species and generation of reactive oxygen species, which result in immediate cytoplasmic membrane depolarization followed by inhibition of respiration, DNA degradation and death. The semi-permeable outer membrane is also compromised presumably as copper creates patches in LPS and increased permittivity in the presence of copper (Ferris and Beveridge, 1986; Bai *et al*., 2007) but this is not an immediate effect. Espirito Santo and colleagues (2011) observed that *E. coli* cells exposed to dry copper surfaces accumulated copper ions immediately on contact (much slower accumulation was observed in the wet), which did not increase further. This massive uptake may be enough to diminish the membrane potential but respiratory proteins are able to retain activity for longer. This is in contrast to our previous results for Gram-positive enterococci which although they have only a single membrane it is not depolarized immediately on copper. Why this occurs is unknown because although it is the thick PG cell wall that contacts the metal surface and copper ions can still access the cell interior.

We have observed slow DNA degradation and a few cells retain detectable DNA, especially those in clumps which may also be due to restricted access of copper ions to the nucleic acid until the cytoplasmic membrane depolarizes and weakens. This may explain why other reports have detected bacterial DNA in their amplification methods and surmised that DNA degradation does not occur. Nandakumar and colleagues (2011) have recently undertaken a detailed proteomic study that described the upregulation of genes encoding amino acid metabolism, energy production, envelope biogenesis and DNA replication, recombination and repair in *E. coli* exposed to copper surfaces which supports our results.

By contrast, we observed very rapid DNA destruction in enterococci (much more so than in Gram-negative cells) that was not due to hydroxyl radicals. This was due directly or indirectly to superoxide and copper ions. Therefore, copper surface toxicity in bacteria is significantly affected by their structure and physiology, and cannot be assumed to be due to a single universal mechanism.

The use of copper and alloy surfaces in areas of food handling, processing and preparation may be a useful tool in prevention of food-borne pathogens such as *E. coli* O157 retaining viability on surfaces which is an infection

risk. In addition, the degradation of genomic as well as extrachromosomal DNA, e.g. plasmid, that we have observed on copper but not stainless steel surfaces may also help to prevent transfer of toxin, virulence and antibiotic resistance genes. We are exposed on a daily basis to copper via coin surfaces or through water supplies from copper pipes and there are strict guidelines to maintain safe daily intake of copper ions. Copper cooking utensils have been employed for centuries although non-corrosive linings are recommended for acid foods. We and others have observed that continuous copper ion release is essential for efficacy of copper surfaces (Espirito Santo *et al*., 2008; 2010; Molteni *et al*., 2010; Warnes and Keevil, 2011) and cleaning protocols must be able to remove any substances that may chelate released ions. Some cleaning products have been shown to enhance killing potential (Harrison *et al*., 2008). However, before implementation of copper and new generation copper alloy surfaces in food processing areas can proceed studies have to determine if the copper ion release necessary for efficacy could affect the foodstuffs themselves. Serious consideration has to be made on which surfaces in food manufacture and preparation would be best employed as copper biocidal surfaces.

Experimental procedures

Bacterial strains

Escherichia coli O157 (NCTC 12900), *E. coli* K12 wild-type ATCC 23716 [K (lambda) EMG2], *E. coli* K12-derived laboratory strain DH5α (NCTC 13450), *Salmonella enterica* ssp. *enterica* serovar Typhimurium (referred to as *S.* Typhimurium in this report) (ATCC 14028) and methicillin-resistant *Staphylococcus aureus* NCTC 13143 (EMRSA-16) were supplied by Health Protection Agency, UK and *Salmonella arizonae* (strain 54049/99 (sheep isolate) Central Veterinary Laboratory, UK). Vancomycin-resistant enterococci [*Enterococcus faecalis* (ATCC51299) and *Enterococcus faecium* (NCTC 12202)] were supplied by Oxoid, UK.

Culture preparation

Bacteria were maintained on Glycerol Protect beads (Fisher Scientific, UK) at –80°C. For each experiment, one bead was inoculated into 15 ml sterile Brain Heart Infusion Broth (BHIB) (Oxoid, UK), and incubated aerobically at 37°C for 18 ± 2 h.

Coupon preparation

Metal coupons (1 × 1 × 0.1 cm) were prepared, degreased in acetone and used as described previously (Warnes *et al*., 2010). The metals tested were stainless steel (S30400), copper (C11000) and cartridge brass C26000 (70% copper), supplied by Copper Development Association, New York, USA.

Inoculation and assessment of viable cells by culture in the presence and absence of chelators and reactive oxygen species quenchers

For each exposure time, duplicate coupons were analysed using either culture methods or staining methods; bacteria were washed in phosphate-buffered saline (PBS) to remove any traces of medium and resuspended in either PBS alone (control) or PBS supplemented with chelators or ROS quenchers (supplied by Sigma UK). Supplements and final concentrations were: ethylenediaminetetraacetic acid (EDTA) 20 mM; bathocuproine disulfonic acid (BCS) 20 mM; D-mannitol 20 mM; 4, 5-dihydroxy-1,3-benzene disulfonic acid (Tiron); 20 mM. Approximately 10^7 cfu in 1 µl of bacterial cell suspension was spread evenly over the surface of each coupon and incubated at room temperature (21 ± 2°C) for up to 30 min. Coupons were aseptically transferred to 5 ml PBS containing 20 mM EDTA (to chelate-free copper ions) and 2 mm diameter glass beads, and vortexed for 30 s. Serial dilutions were prepared in PBS and 10 or 100 µl from each dilution was spread over 45 or 90 mm diameter agar plates in triplicate: Tryptone Soy Agar (TSA) and nutrient agar (Oxoid, UK) for *E. coli* strains and for *Salmonella* species respectively. Plates were allowed to dry before inverting and incubating aerobically at 37°C for 24 and 48 h. Colonies on plates were counted by eye and the concentration per coupon was calculated and recorded as colony-forming units (cfu) per coupon (1 cm^2).

Detection of changes in membrane integrity in E. coli *and* Salmonella *spp. exposed to copper and copper alloy surfaces using Rhodamine 123 and BacLight Bacterial Membrane Potential Kit (Invitrogen, UK)* in situ *and* ex situ

To assess changes in membrane potential *in situ* using rhodamine 123 (Rh123) on metal surfaces approximately 5×10^8 cfu (1 ml exponentially growing culture) were washed in PBS to remove traces of medium, resuspended in 50 µl 25 µM Rh123 (Sigma, UK) and incubated at room temperature for 10 min in the dark. Excess stain that had not been internalized was washed away and the cells were resuspended in 50 µl fresh PBS. One microlitre (approximately 10^7 cfu) was spread over each 1 cm^2 metal coupon and any developing fluorescence was photographed using epifluorescence microscope with long working distance objective fitted (Keevil, 2003). Images were recorded every minute for 10–15 min (viability may be affected by the dye for longer periods).

To ascertain if the same results could be obtained *ex situ* coupons were inoculated with unstained cells for required time points and cells removed (as for culture). Cells were stained with Rh123 as described, washed and 5 µl applied to a glass microscope slide with a coverslip and observed using an epifluorescence microscope and an oil immersion objective.

Assessment of changes in membrane potential in *E. coli* exposed to metal surfaces was also done using *Bac*Light Bacterial Membrane Potential Kit (Invitrogen, UK). This contains an oxa-(DiO) carbocyanine dye [$DiOC_2(3)$], which has a

short alkyl tail and accumulates on hyperpolarized membranes before being translocated into the lipid bilayer. This dye has a potential dependant red shift: green (500–575 nm) fluorescence is due to monomers but in the presence of a membrane potential the dye aggregates and fluoresces red (> 600 nm) (Novo *et al*., 2000). This effect is reversed if the $\Delta\Psi$ is dissipated. Approximately 5×10^8 cfu (1 ml exponentially growing culture) were washed in PBS to remove traces of medium, and resuspended in 100 μl fresh PBS. DiOC2(3) was added to a final concentration of 3 μM and incubated for 15 min in the dark at room temperature. Stained cells were observed untreated (applied to microscope slide) or 1 μl was applied to a metal surface and fluorescence recorded over time in the red and green channels.

To confirm that depolarization could be visualized by a reduction in fluorescence the ionophore, carbonylcyanide m-chlorophenylhydrazone (CCCP), which increases proton permeability and dissipates membrane potential and proton gradient (together produce the proton motive force) was added to Rh123 or DiOC2(3) stained cells (10 μM for 10 min at room temperature).

Detection of changes in the integrity of the outer membrane of bacteria exposed to copper and stainless steel surfaces

Bacteria were exposed to metal surfaces and removed as described for culture experiments. Cells were washed in PBS and resuspended in 50 μl (2000 U) lysozyme solution (2.5 mg ml^{-1}) for 4 min at room temperature. Untreated cells from actively growing culture and cells treated with mild detergent to disrupt the outer membrane [0.1% (v/v) Triton X-100 for 5 min at room temperature] were used as controls. Cells were washed in PBS, spread onto the surface of a microscope slide, heat fixed when dry and Gram-stained. A coverslip was applied and observed with bright field microscopy and oil immersion objective.

Detection of respiring cells ex situ *on metal surfaces using the redox dye CTC (5-cyano-2, 3-ditolyl tetrazolium chloride)*

Respiring bacterial cells produce electrons which reduce the redox dye, CTC, to insoluble formazan, which can be visualized using epifluorescence microscopy. Metal coupons were inoculated as described for culture assessment and cells were removed at required time points. Cells were pooled and stained with 5 mM CTC for 60 min at 37°C. Experiments had to be performed *ex situ* because of the long staining times required for this stain; 5 μl stained cells were transferred to a microscope slide and a coverslip applied.

Detection of reactive oxygen species in situ *on copper surfaces*

Harrison and colleagues (2009) detected reactive oxygen species (ROS) generated by *E. coli* in response to soluble metal ions using carboxylated and fluorinated derivatives of fluorescein [H_2DFFDA (mixed isomers); Invitrogen], which fluoresce upon oxidation (acetate groups are removed by intracellular esterases allowing oxidation by ROS to occur). This group quantified their results using a fluorimeter. This method was adapted in our studies to allow episcopic observation of ROS generated at the copper surface. Briefly, approximately 5×10^8 cfu were washed in PBS and resuspended in 2 mM H_2DFFDA in a total volume of 50 μl at room temperature for 20 min. The cells were washed in PBS to remove any dye not internalized and resuspended immediately in 50 μl fresh PBS; 1 μl was spread over the surfaces of copper or stainless steel coupons and observed with epifluorescence microscopy, immediately and up to 30 min duration to record any developing fluorescence.

Detection of changes in the integrity of bacterial DNA in cells exposed to copper and stainless steel surfaces

SYTO 9 (Invitrogen, UK) is a cell permeant, low toxicity, green fluorescent stain that exhibits brighter fluorescence when bound to double-stranded DNA of live and dead cells. If the DNA is compromised this can be visualized as a reduction in fluorescence. Cells were either exposed to metal surfaces for designated times, removed and stained *ex situ* or cells were pre-stained and applied to metal surfaces and observed *in situ*. Staining *ex situ*: bacteria were applied to metal coupons, removed and pooled as described for culture (at least 5 coupons per time point). Cells were stained with 50 μl 5 μM SYTO 9 for 10 min at room temperature, in the dark. Cells were pelleted and resuspended in 50 μl fresh PBS; 5 μl was applied to a microscope slide with coverslip and observed using an oil immersion objective. *In situ* staining protocol: approximately 5×10^8 cfu (1 ml exponentially growing culture) were washed in PBS to remove traces of medium, resuspended in 50 μl of 5 μM SYTO 9 and incubated for 10 min in the dark at room temperature. Unbound stain was removed by washing the cells in PBS and resuspending the final pellet in 50 μl fresh PBS. One microlitre (approximately 10^7 cfu) was spread over 1 cm^2 metal coupons and any developing fluorescence was photographed using an epifluorescence microscope with long working distance objective fitted (Keevil, 2003). Images were recorded every minute for 10–15 min and periodically up to 60 min contact. Experiments were also conducted by inoculating the cells onto metal surfaces in the presence of 20 mM D-mannitol which negates the effect of any hydroxyl radicals produced.

To determine if the plasmid DNA of Gram-negative bacteria was also degraded on copper surfaces cells were inoculated onto copper or stainless steel coupons (approximately 10^7 cfu cm^{-2}), incubated at room temperature for 10 min or 1 h then the cells were removed and pooled as described previously (30 coupons per time point). Plasmid DNA was purified from metal exposed, untreated and heat killed cells (equivalent number of cells as used for metal exposure) using the Qiaprep Spin miniprep kit (Qiagen, UK) according to the manufacturer's instruction and fragments were separated on a 1% agarose gel using GelRed Nucleic Acid Prestaining Kit (Biotium, UK). DNA ladders were supplied by Bioline as described in the Fig. 6 legend. Gels were observed and photographed using GeneSnap software and a Syngene UV light box. Results shown for *E. coli* O157 in Fig. 6.

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 11.

Acknowledgements

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References

Bai, W., Zhao, K., and Asami, K. (2007) Effects of copper on dielectric properties of *E. coli* cells. *Colloids Surf B Biointerfaces* **58:** 105–115.

Boyce, J.M. (2007) Environmental contamination makes an important contribution to hospital infection. *J Hosp Infect* **65** (Suppl. 2)**:** 50–54.

Breeuwer, P., and Abee, T. (2004) Assessment of membrane potential, intracellular pH and respiration of bacteria employing fluorescent techniques. *Mol Microb Ecol Man* **8:** 1563–1580.

Casey, A.L., Adams, D., Karpanen, T.J., Lambert, P.A., Cookson, B.D., Nightingale, P., *et al*. (2010) The role of copper in the reduction of contamination of the hospital environment. *J Hosp Infect* **74:** 72–77.

Espirito Santo, C., Morais, P.V., and Grass, G. (2010) Isolation and characterisation of bacteria resistant to metallic copper surfaces. *Appl Environ Microbiol* **76:** 1341–1348.

Espirito Santo, C., Lam, E.W., Elowsky, C.G., Quarant, D., Domaille, D.W., Chang, C.J., and Grass, G. (2011) Bacterial killing by dry metallic copper surfaces. *Appl Environ Microbiol* **77:** 794–802.

Espirito Santo, C.E., Taudte, N., Nies, D.H., and Grass, G. (2008) Contribution of copper ion resistance to survival of *E. coli* on metallic copper surfaces. *Appl Environ Microbiol* **74:** 977–986.

Ferris, F.G., and Beveridge, T.J. (1986) Site specificity of metallic ion binding in *Escherichia coli* K12 lipopolysaccharide. *Can J Microbiol* **32:** 52–55.

Gomes, A., Fernandes, E., and Lima, J.L.F.C. (2005) Fluorescence probes used for the detection of reactive oxygen species. *J Biochem Biophys Methods* **65:** 45–80.

Grass, G., Rensing, C., and Solioz, M. (2011) Metallic copper as an antimicrobial surface. *Appl Environ Microbiol* **77:** 1541–1547.

Habimana, O., Heir, E., Langsrud, S., Åsli, A.W., and Møretrø, T. (2010) Enhanced surface colonisation by *Escherichia coli* O157:H7 in biofilms formed by an *Acinetobacter calcoaceticus* isolate from meat processing environments. *Appl Environ Microbiol* **76:** 4557–4559.

Halliwell, B., and Gutteridge, J.M.C. (1989) *Free Radicals in Biology and Medicine*, 2nd edn. Oxford, UK: Clarendon Press.

Harrison, J.J., Turner, R.J., Joo, D.A., Stan, M.A., Chan, C.S., Allan, N.D., *et al*. (2008) Copper and quaternary ammonium cations exert synergistic bactericidal and antibiofilm activity against *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **52:** 2870–2881.

Harrison, J.J., Tremaroli, V., Stan, M.A., Chan, C.S., Vacchi-Suzzi, C., Heyne, B.J., *et al*. (2009) Chromosomal antioxidant genes have metal ion-specific roles as determinants of bacterial metal tolerance. *Environ Microbiol* **11:** 2491–2509.

Hossain, Z., and Huq, F. (2002) Studies on the interaction between Cd2+ ions and DNA. *J Inorg Biochem* **90:** 85–96.

Imlay, J.A. (2003) Pathways of oxidative damage. *Annu Rev Microbiol* **57:** 395–418.

Keevil, C.W. (2003) Rapid detection of biofilms and adherent pathogens using scanning confocal laser microscopy and episcopic differential interference contrast microscopy. *Water Sci Technol* **47:** 105–116.

Keyhani, E., Abdi-Oskouei, F., Attar, F., and Keyhani, J. (2006) DNA strand breaks by metal-induced oxygen radicals in purified *Salmonella typhimurium* DNA. *Ann N Y Acad Sci* **1091:** 52–64.

King, T., Luccini, S., Hinton, J.C.D., and Gobius, K. (2010) Transciptomic analysis of *Escherichia coli* O157:H7 and K12 cultures exposed to inorganic and organic acids in stationary phase reveals acidulant- and strain specific acid tolerance responses. *Appl Environ Microbiol* **76:** 6514–6528.

Kirakosyan, G., and Trchounian, A. (2007) Redox sensing by *Escherichia coli*: effect of copper ions as oxidisers on proton-coupled membrane transport. *Bioelectrochemistry* **70:** 58–63.

Kirakosyan, G., Trchounian, K., Vardanyan, Z., and Trchounian, A. (2008) Copper (II) ions affect *Escherichia coli* membrane vesicles' SH-groups and a disulphide-dithiol interchange between membrane proteins. *Cell Biochem Biophys* **51:** 45–50.

Kruse, H., and Sørum, H. (1994) Transfer of drug resistance plasmids between bacteria of diverse origins in natural microenvironments. *Appl Environ Microbiol* **60:** 4015–4021.

Kunz, A.N., and Brook, I. (2010) Emerging resistant Gram-negative aerobic bacilli in hospital-acquired infections. *Chemotherapy* **56:** 492–500.

Laggner, H., Hermann, M., Gmeiner, M.K., and Kapiotis, S. (2006) Cu2+ and Cu+ bathocuproine disulfonate complexes promote the oxidation of the ROS detecting compound dichlorofluorescin (DCFH). *Anal Bioanal Chem* **385:** 959–961.

Lebedev, V.S., Volodina, L.A., and Deĭnega, E. (2005) Structural modifications of the surface of *Escherichia coli* bacteria and copper-induced permeability of plasma membrane. *Biofizika* **50:** 107–113.

Lewis, K. (2010) Persister cells. *Ann Rev Microbiol* **64:** 357–372.

Li, Y., and Trush, M.A. (1993) DNA damage resulting from the oxidation of hydroquinone by copper: role for a Cu(II)/Cu(I) redox cycle and reactive oxygen generation. *Carcinogenesis* **14:** 1303–1311.

Lim, J.-Y., Yoon, J.W., and Hovde, C.J. (2010) A brief overview of *Escherichia coli* O157:H7 and its plasmid O157. *J Microbiol Biotechnol* **20:** 1–10.

Lloyd, D.R., and Phillips, D.H. (1999) Oxidative DNA damage mediated by copper (II), iron (II) and nickel (II) Fenton reactions: evidence for site-specific mechanisms in the formation of double-strand breaks, 8-hydroxy deoxyguanosine and putative intrastrand cross-links. *Mutat Res* **424:** 23–36.

Macomber, L., and Imlay, J.A. (2009) The iron-sulfur clusters of dehydratases are primary targets of copper toxicity. *Proc Natl Acad Sci USA* **106:** 8344–8349.

Macomber, L., Rensing, C., and Imlay, J.A. (2006) Intracellular copper does not catalyse the formation of oxidative DNA damage in *E. coli*. *J Bacteriol* **189:** 1616–1626.

Mehtar, S., Wiid, I., and Todorov, S.D. (2008) The antimicrobial activity of copper and copper alloys against nosocomial pathogens and *Mycobacterium tuberculosis* isolated from healthcare studies in the Western Cape: an *in vitro* study. *J Hosp Infect* **68:** 45–51.

Michels, H.T., Noyce, J.O., and Keevil, C.W. (2009) Effects of temperature and humidity on the efficacy of methicillin-resistant *Staphylococcus aureus* challenged antimicrobial materials containing silver and copper. *Lett Appl Microbiol* **49:** 191–195.

Molteni, C., Abicht, H.K., and Solioz, M. (2010) Killing of bacteria by copper surfaces involves dissolved copper. *Appl Environ Microbiol* **76:** 4099–4101.

Nandakumar, R., Espirito Santo, C., Madayiputhiya, N., and Grass, G. (2011) Quantitative profiling of the *Escherichia coli* response to metal stress. *Biometals* **24:** 429–444.

Novo, D.J., Perlmutter, N.G., Hunt, R.H., and Shapiro, H.M. (2000) Multiparameter flow cytometric analysis of antibiotic effects on membrane potential, permeability and bacterial counts of *Staphylococcus aureus* and *Micrococcus aureus*. *Antimicrob Agents Chemother* **44:** 827–834.

Noyce, J.O., Michels, H., and Keevil, C.W. (2006a) Use of copper cast alloys to control *Escherichia coli* O157 cross-contamination during food processing. *Appl Environ Microbiol* **72:** 4239–4244.

Noyce, J.O., Michels, H., and Keevil, C.W. (2006b) Potential use of copper surfaces to reduce survival of epidemic methicillin-resistant *Staphylococcus aureus* in the healthcare environment. *J Hosp Infect* **63:** 289–297.

Noyce, J.O., Michels, H., and Keevil, C.W. (2007) Inactivation of influenza A virus on copper versus stainless steel surfaces. *Appl Environ Microbiol* **73:** 2748–2750.

Peleg, A.Y., and Hooper, D.C. (2010) Hospital-acquired infections due to Gram-negative bacteria. *N Engl J Med* **362:** 1084–1813.

Peng, L., Lifang, R., Hongyu, X., Xi, L., and Chaocan, Z. (2007) Study on the toxic effect of lead (II) ion on *Escherichia coli*. *Biol Trace Elem Res* **115:** 195–202.

Pennington, H. (2010) *Escherichia coli* O157. *Lancet* **376:** 1428–1435.

Quaranta, D., Krans, T., Espirito Santo, C., Elowsky, C.G., Domaille, D.W., Chang, C.J., and Grass, G. (2011) Mechanisms of contact killing of yeast cells on dry metallic copper surfaces. *Appl Environ Microbiol* **77:** 416–426.

Que, B.G., Downey, K.M., and So, A.G. (1979) Selective inhibition of the polymerase activity of DNA polymerase I: Further evidence of separate active sites for polymerase and 3′ to 5′ exonuclease activities. *Biochemistry* **18:** 2064–2068.

Rice, L.B. (2007) Emerging issues in the management of infections caused by multidrug-resistant Gram-negative bacteria. *Cleve Clin J Med* **74:** S12–S20.

Schmidt, M.G. (2011) Copper surfaces in the ICU reduced the relative risk of acquiring an infection while hospitalised. *BMC Proc* **5** (Suppl. 6)**:** O53.

Selvaraj, S., Saha, K.C., Chakraborty, A., Bhattacharyya, S.N., and Saha, A. (2009) Toxicity of free and various aminocarboxylic ligands sequestered copper(II) ions to *Escherichia coli*. *J Hazard Mater* **166:** 1403–1409.

Skandamis, P.N., Stopforth, J.D., Ashton, L.V., Geornaras, I., and Kendall, P.A. (2009) *E. coli* O157:H7 survival, biofilm formation and acid tolerance under simulated slaughter plant moist and dry conditions. *Food Microbiol* **26:** 112–119.

Stephens, T.P., Loneragan, G.H., Thompson, T.W., Sridhara, A., Branham, L.A., Pitchiah, S., and Brashears, M.M. (2007) Distribution of *Escherichia coli* on hide surfaces, the oral cavity, and in feces of feedlot cattle. *J Food Prot* **70:** 1346–1349.

Stohs, S.J., and Bagghi, D. (1995) Oxidative mechanisms in the toxicity of metal ions. *Free Radic Biol Med* **18:** 321–336.

Tkeshelashvili, L.K., McBride, T., Spence, K., and Loeb, L.A. (1990) Mutation spectrum of copper-induced DNA damage. *J Biol Chem* **266:** 6401–6406.

Uhlich, G.A., Rogers, D.P., and Mosier, D.A. (2010) *Escherichia coli* serotype O157:H7 retention on solid surfaces and peroxide resistance is enhanced by dual-strain biofilm formation. *Foodborne Pathog Dis* **7:** 935–943.

Van Houdt, R., and Michiels, C.W. (2010) Biofilm formation and the food industry, a focus on the bacterial outer surface. *J Appl Microbiol* **109:** 1117–1131.

Vanaja, S.K., Springman, A.C., Besser, T.E., Whittam, T.S., and Manning, S.D. (2010) Differential expression of virulence and stress fitness genes between *Escherichia coli* O157:H7 strains with clinical of bovine-based genotypes. *Appl Environ Microbiol* **76:** 60–68.

Volentini, S.I., Farías, R.N., Rodríguez-Montelongo, L., and Rapisarda, V.A. (2011) Cu(II) reduction by *Escherichia coli* cells is dependant on respiratory chain components. *Biometals.* **24:** 827–835. doi: 10.1007/s10534-011-9436-3.

Warnes, S.L., and Keevil, C.W. (2011) Mechanism of copper surface toxicity in vancomycin-resistant enterococci following ‘wet’ or ‘dry’ contact. *Appl Environ Microbiol* **77:** 6049–6059. AEM Accepts AEM00597-11.

Warnes, S.L., Green, S.M., Michels, H.T., and Keevil, C.W. (2010) Biocidal efficacy of copper alloys against pathogenic enterococci involves degradation of genomic and plasmid DNA. *Appl Environ Microbiol* **76:** 5390–5401.

Weaver, L., Michels, H.T., and Keevil, C.W. (2008) Survival of *Clostridium difficile* on copper and steel. *J Hosp Infect* **68:** 145–151.

Weaver, L., Michels, H.T., and Keevil, C.W. (2009) Potential for preventing spread of fungi in air conditioning systems constructed using copper instead of aluminium. *Lett Appl Microbiol* **50:** 18–23.

Wheeldon, L.J., Worthington, T., Lambert, P.A., Hilton, A.C., Lowden, C.J., and Elliot, T.S. (2008) Antimicrobial efficacy

of copper surfaces against spores and vegetative cells of *Clostridium difficile*: the germination theory. *J Antimicrob Chemother* **62:** 522–525.

Wilks, S.A., Michels, H., and Keevil, C.W. (2005) The survival of *Escherichia coli* O157 on a range of metal surfaces. *Int J Food Microbiol* **105:** 445–454.

Wilks, S.A., Michels, H.T., and Keevil, C.W. (2006) Survival of *Listeria monocytogenes* Scott A on metal surfaces: Implications for cross-contamination. *Int J Food Microbiol* **111:** 93–98.

Williams, A.P., Avery, L.M., Killham, K., and Jones, D.L. (2005) Persistence of *Escherichia coli* O157 on farm surfaces under different environmental conditions. *J Appl Microbiol* **98:** 1075–1083.

Supporting information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. The effect on bacterial membrane potential in *E. coli* O157 exposed to copper, brass and stainless steel using lipidophilic stain rhodamine 123 (*ex situ*). Approximately 10^7 cfu in 1 μl PBS were spread over the surface of a 1 cm^2 metal coupon. After 5 min at 22°C cells were removed as described in the text, and stained with Rh123. On stainless steel (C) bright staining (characteristically brightest at cell poles) is visible. The degree of staining is reduced for brass (B) and eliminated on copper (A) suggesting depolarization has taken place. Size bar is 10 μm.

Fig. S2. Destruction of membrane potential in *E. coli* O157 and *Salmonella* Typhimurium *in situ* on copper and brass that is not evident in Gram-positive species. Bacterial cells were pre-stained with Rh123 (as described in the text) and spread over 1 cm^2 metal coupons (approximately 10^7 cfu in 1 μl) and observed following 1 min contact (it must be noted that equilibrium fluorescence is greater for Gram-positive species due to more efficient dye uptake and only a single membrane). Rapid depolarization has already occurred in Gram-negative species (especially *E. coli* O157) on copper and brass. Membranes are intact in *E. faecalis*, *E. faecium* and EMRSA on all surfaces tested. Size bar is 10 μm.

Fig. S3. Detection of changes in bacterial membrane potential in *E. coli* DH5α using *Bac*Light Bacterial Membrane Potential Kit (Invitrogen, UK). This kit employs a single oxa-(DiO) carbocyanine dye that accumulates in bacterial membrane in monomers (stain green) and in aggregates (red). Cells with intact membranes and membrane potential stain red and green. If the potential dissipates the degree of red staining diminishes (intensity of red stain is proportional to the membrane potential). On stainless steel bright red and green staining can be seen (A). However, on copper all the red staining has diminished suggesting loss of membrane potential (B). Size bar is 10 μm.

Fig. S4. Exposure to copper surfaces also results in damage to outer membrane of Gram-negative bacteria. *E. coli* O157 exposed to copper (A) or stainless steel (B) surfaces for 5 min were removed from coupons, treated with 5000U lysozyme (which can only access the cell wall if the outer membrane is compromised) for 4 min and Gram-stained. Extensive cell clumping occurs in *E. coli* O157 on copper surfaces and staining pattern is similar to detergent and lysozyme controls i.e. breakdown of peptidoglycan which retains crystal-violet-iodine complex (controls not shown).

Fig. S5. Generation of reactive oxygen species by *S. arizonae* on copper surfaces that can be quenched by addition of D-mannitol. Bacterial cells were pre-stained with H_2DFFDA (mixed isomers) (Invitrogen, UK), as described in the text and for Fig. 5). ROS are generated by on copper surfaces (A) which can be partially quenched if D-mannitol is present (B). No fluorescence was observed on stainless steel in PBS (C) or D-mannitol (D). Size bar is 10 μm.

Fig. S6. Slow degradation of DNA observed *in situ* of *E. coli* O157 and *Salmonella* Typhimurium. Bacterial cells were pre-stained with double-stranded (ds) DNA stain, SYTO 9 (as described in the text) and spread over 1 cm^2 metal coupons (Approximately 10^7 cfu in 1 μl PBS) and observed over 60 min. The DNA degrades on copper surfaces as observed by a reduction in SYTO 9 staining (this stain binds to intact ds DNA). Although no cells are viable after 10 min a small minority of cells retain DNA for longer suggesting possible presence of persister cells. Cells appear to clump as soon as they contact the copper surface (particularly in *E. coli* O157 contact times 0 and 10 min). However, there has been no DNA degradation on stainless steel for both species. Size bar is 10 μm.

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environmental microbiology

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

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Supporting information

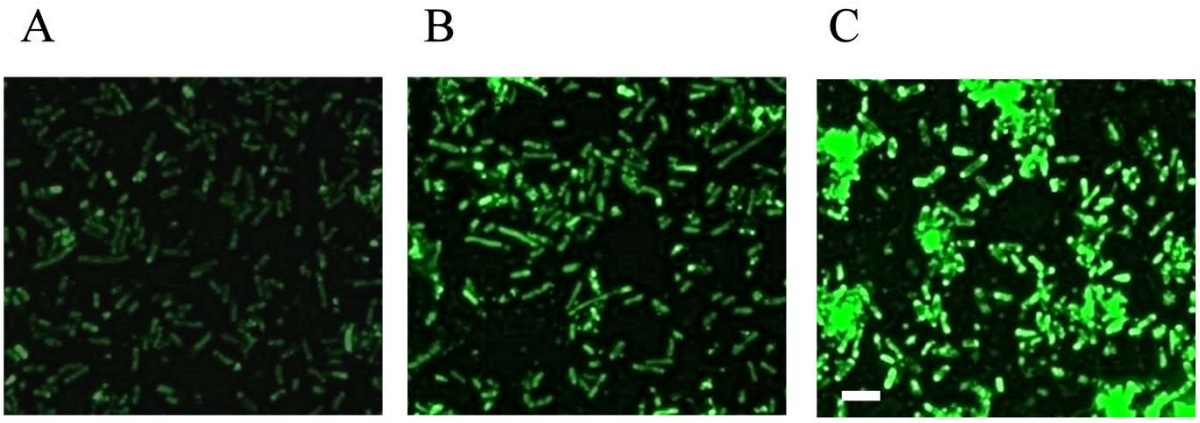


Fig. S1. The effect on bacterial membrane potential in *E. coli* O157 exposed to copper, brass and stainless steel using lipidophilic stain rhodamine 123 (*ex situ*). Approximately 10^7 cfu in 1 µl PBS were spread over the surface of a 1 cm^2 metal coupon. After 5 min at 22°C cells were removed as described in the text, and stained with Rh123. On stainless steel (C) bright staining (characteristically brightest at cell poles) is visible. The degree of staining is reduced for brass (B) and eliminated on copper (A) suggesting depolarization has taken place. Size bar is 10 µm.

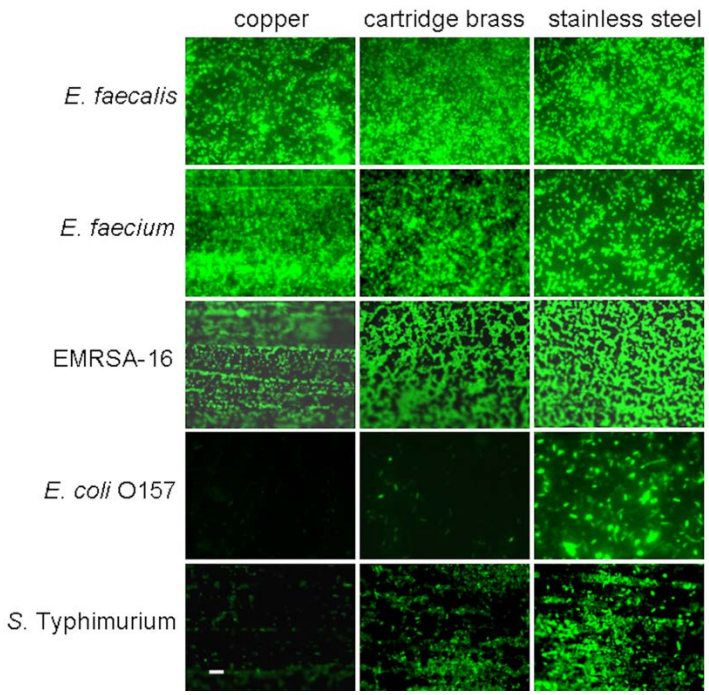


Fig. S2. Destruction of membrane potential in *E. coli* O157 and *Salmonella* Typhimurium *in situ* on copper and brass that is not evident in Gram-positive species. Bacterial cells were pre-stained with Rh123 (as described in the text) and spread over 1 cm^2 metal coupons (approximately 10^7 cfu in 1 µl) and observed following 1 min contact (it must be noted that equilibrium fluorescence is greater for Gram-positive species due to more efficient dye uptake and only a single membrane). Rapid depolarization has already occurred in Gram-negative species (especially *E. coli* O157) on copper and brass. Membranes are intact in *E. faecalis*, *E. faecium* and EMRSA on all surfaces tested. Size bar is 10 µm.

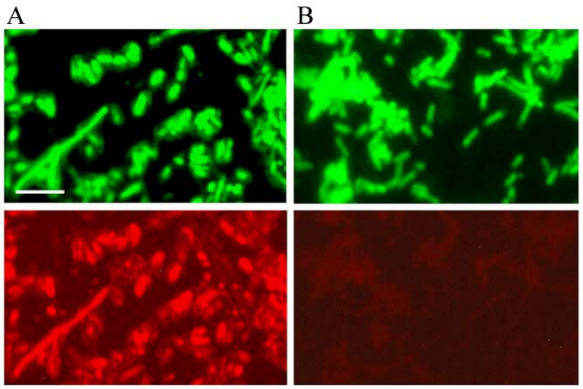


Fig. S3. Detection of changes in bacterial membrane potential in *E. coli* DH5α using *Bac*Light Bacterial Membrane Potential Kit (Invitrogen, UK). This kit employs a single oxa-(DiO) carbocyanine dye that accumulates in bacterial membrane in monomers (stain green) and in aggregates (red). Cells with intact membranes and membrane potential stain red and green. If the potential dissipates the degree of red staining diminishes (intensity of red stain is proportional to the membrane potential). On stainless steel bright red and green staining can be seen (A). However, on copper all the red staining has diminished suggesting loss of membrane potential (B). Size bar is 10 µm.

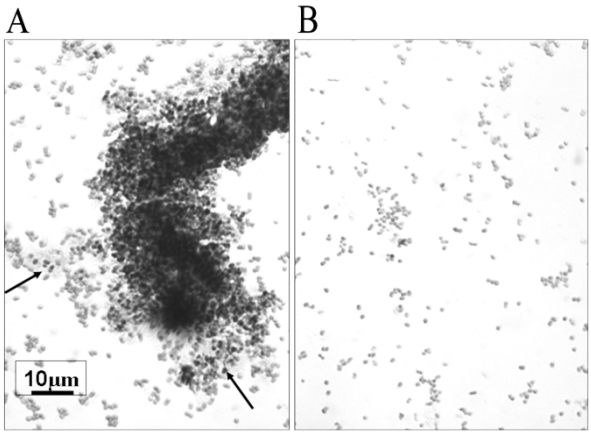


Fig. S4. Exposure to copper surfaces also results in damage to outer membrane of Gram-negative bacteria. *E. coli* O157 exposed to copper (A) or stainless steel (B) surfaces for 5 min were removed from coupons, treated with 5000U lysozyme (which can only access the cell wall if the outer membrane is compromised) for 4 min and Gram-stained. Extensive cell clumping occurs in *E. coli* O157 on copper surfaces and staining pattern is similar to detergent and lysozyme controls i.e. breakdown of peptidoglycan which retains crystal-violet-iodine complex (controls not shown).

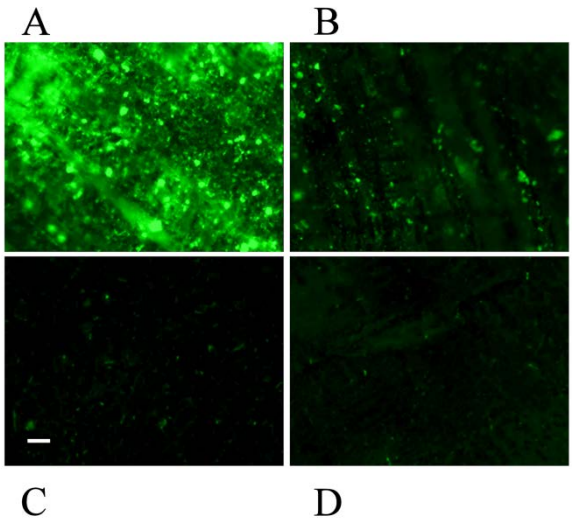


Fig. S5. Generation of reactive oxygen species by *S. arizonae* on copper surfaces that can be quenched by addition of D-mannitol. Bacterial cells were pre-stained with H_2DFFDA (mixed isomers) (Invitrogen, UK), as described in the text and for Fig. 5). ROS are generated by on copper surfaces (A) which can be partially quenched if D-mannitol is present (B). No fluorescence was observed on stainless steel in PBS (C) or D-mannitol (D). Size bar is 10 µm.

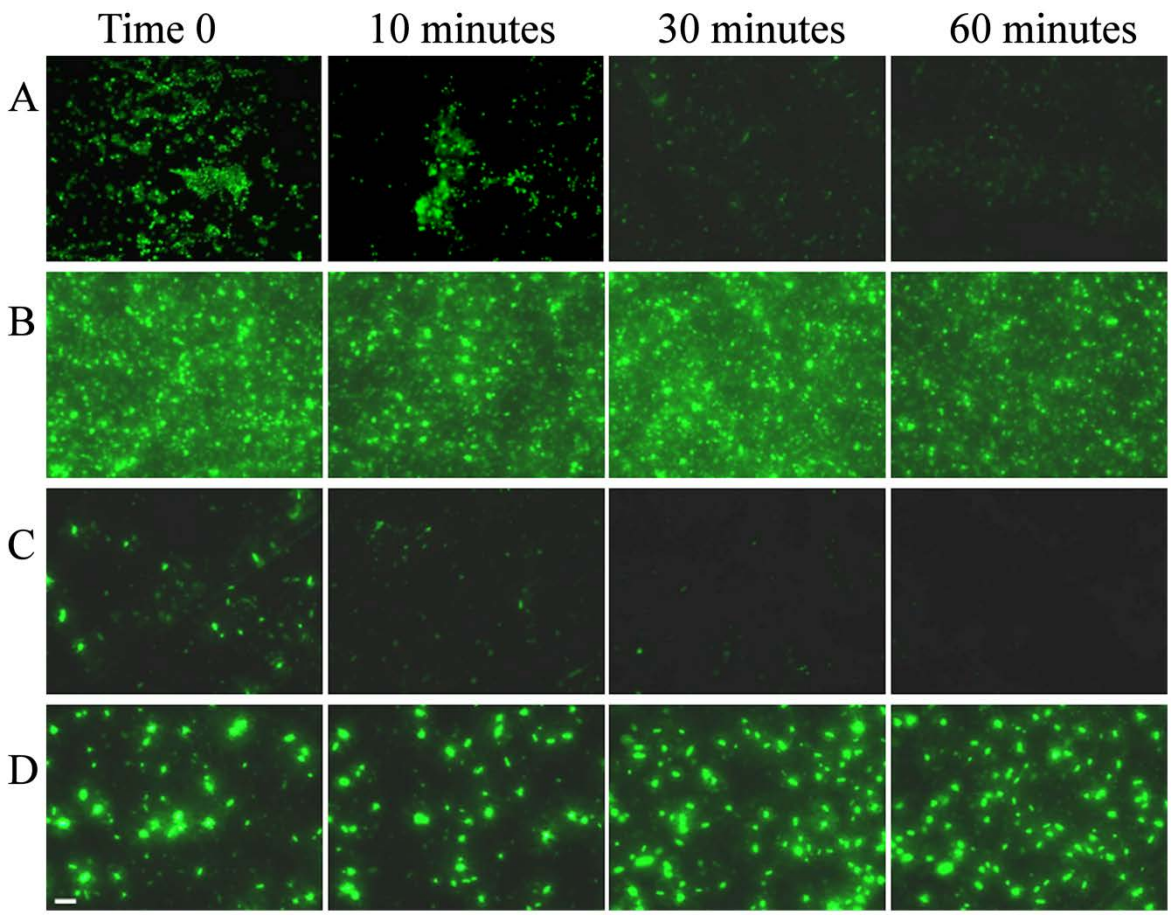
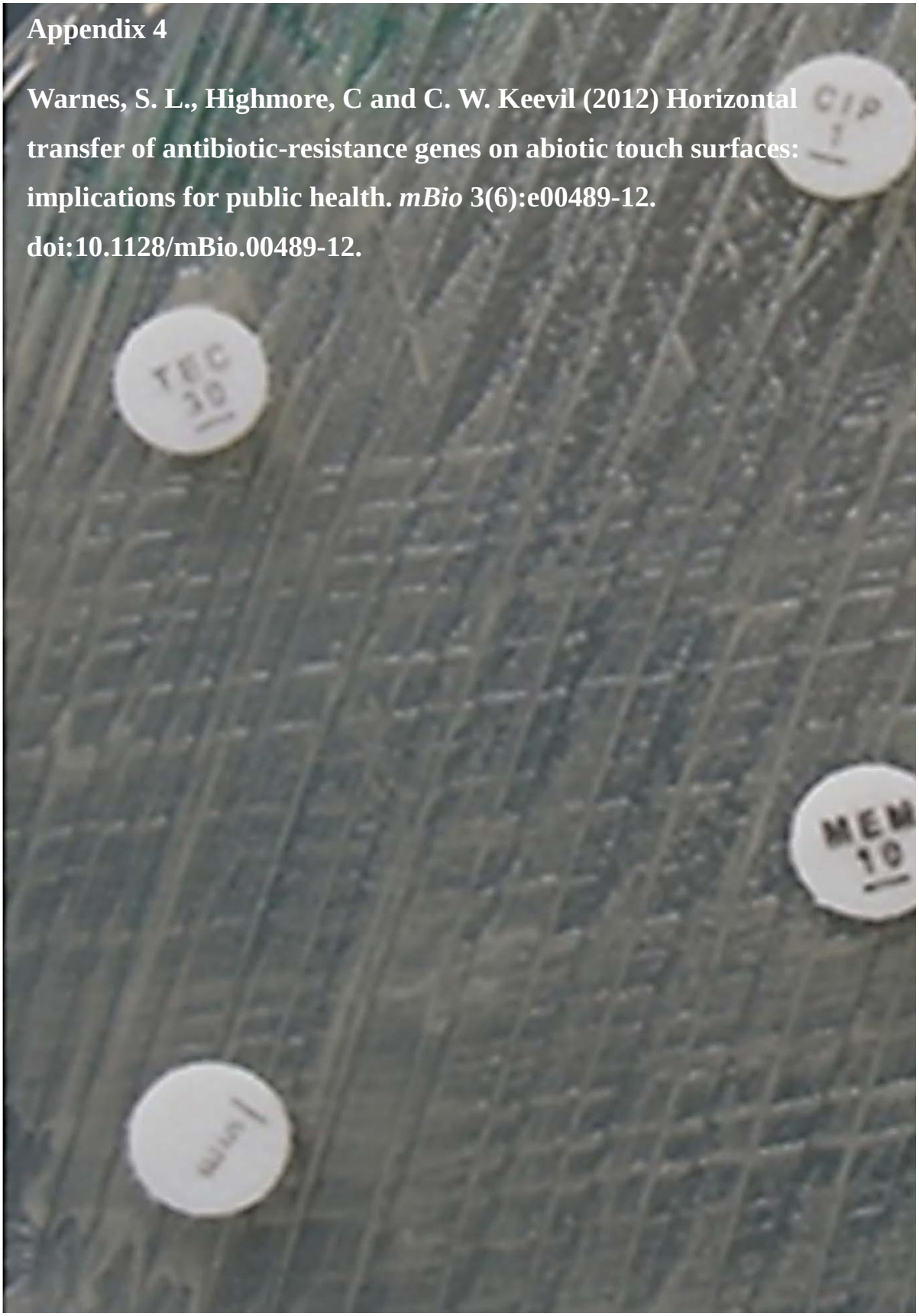


Fig. S6. Slow degradation of DNA observed *in situ* of *E. coli* O157 and *Salmonella* Typhimurium. Bacterial cells were pre-stained with double-stranded (ds) DNA stain, SYTO 9 (as described in the text) and spread over 1 cm^2 metal coupons (Approximately 10^7 cfu in 1 µl PBS) and observed over 60 min. The DNA degrades on copper surfaces as observed by a reduction in SYTO 9 staining (this stain binds to intact ds DNA). Although no cells are viable after 10 min a small minority of cells retain DNA for longer suggesting possible presence of persister cells. Cells appear to clump as soon as they contact the copper surface (particularly in *E. coli* O157 contact times 0 and 10 min). However, there has been no DNA degradation on stainless steel for both species. Size bar is 10 µm.

Appendix 4



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Horizontal Transfer of Antibiotic Resistance Genes on Abiotic Touch Surfaces: Implications for Public Health

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ABSTRACT **Horizontal gene transfer (HGT) is largely responsible for increasing the incidence of antibiotic-resistant infections worldwide. While studies have focused on HGT *in vivo*, this work investigates whether the ability of pathogens to persist in the environment, particularly on touch surfaces, may also play an important role. *Escherichia coli*, virulent clone ST131, and *Klebsiella pneumoniae* harboring extended-spectrum-β-lactamase (ESBL) $bla_{CTX\text{-}M\text{-}15}$ and metallo-β-lactamase $bla_{NDM\text{-}1}$, respectively, exhibited prolonged survival on stainless steel, with approximately 10^4 viable cells remaining from an inoculum of 10^7 CFU per cm^2 after 1 month at 21°C. HGT of *bla* to an antibiotic-sensitive but azide-resistant recipient *E. coli* strain occurred on stainless steel dry touch surfaces and in suspension but not on dry copper. The conjugation frequency was approximately 10 to 50 times greater and occurred immediately, and resulting transconjugants were more stable with ESBL *E. coli* as the donor cell than with *K. pneumoniae*, but $bla_{NDM\text{-}1}$ transfer increased with time. Transconjugants also exhibited the same resistance profile as the donor, suggesting multiple gene transfer. Rapid death, inhibition of respiration, and destruction of genomic and plasmid DNA of both pathogens occurred on copper alloys accompanied by a reduction in *bla* copy number. Naked *E. coli* DNA degraded on copper at 21°C and 37°C but slowly at 4°C, suggesting a direct role for the metal. Persistence of viable pathogenic bacteria on touch surfaces may not only increase the risk of infection transmission but may also contribute to the spread of antibiotic resistance by HGT. The use of copper alloys as antimicrobial touch surfaces may help reduce infection and HGT.**

IMPORTANCE **Horizontal gene transfer (HGT) conferring resistance to many classes of antimicrobials has resulted in a worldwide epidemic of nosocomial and community infections caused by multidrug-resistant microorganisms, leading to suggestions that we are in effect returning to the preantibiotic era. While studies have focused on HGT *in vivo*, this work investigates whether the ability of pathogens to persist in the environment, particularly on touch surfaces, may also play an important role. Here we show prolonged (several-week) survival of multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae* on stainless steel surfaces. Plasmid-mediated HGT of β-lactamase genes to an azide-resistant recipient *E. coli* strain occurred when the donor and recipient cells were mixed together on stainless steel and in suspension but not on copper surfaces. In addition, rapid death of both antibiotic-resistant strains and destruction of plasmid and genomic DNA were observed on copper and copper alloy surfaces, which could be useful in the prevention of infection spread and gene transfer.**

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Editor Bonnie Bassler, Princeton University

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The discovery in 1928 of an antimicrobial compound containing a β-lactam ring that exerted its effect by disrupting bacterial cell wall synthesis and construction revolutionized the treatment of infectious diseases. Continuous use, overuse, and misuse of many antimicrobials in human and animal health applications and food production and the lack of effective regimens to prevent the spread of infection over the last 50 years has led to the evolution of many classes of β-lactamase enzymes and the subsequent development of many generations of β-lactams to try to overcome this (reviewed in reference 1). The most worrying trend is the horizontal transmission of antibiotic resistance genes via mobile elements such as plasmids and transposons, especially in Gram-negative pathogens (1–3).

Over the last 15 years, β-lactamase enzymes that have an extended spectrum of activity (ESBL) against the majority of β-lactams, including cephalosporins but not carbapenemases, have evolved. One of these, CTX-M-15, initially found in *Escherichia coli* but now found in other members of *Enterobacteriaceae* and frequently associated with a specific lineage, uropathogenic clone ST131 (1, 2), has spread worldwide. It is often located on highly mobile IncFII plasmids (4, 5) and associated with mobile genetic element IS26 (1). The risk of infection is particularly high in individuals in association with prolonged hospitalization, catheterization, nursing home residency, previous antibiotic treatment, underlying renal or liver pathology, and travel to high-risk areas (4). This has generated a high selection pressure, particularly because the use of carbapenemases has been necessary to treat ESBL infections and now β-lactamases have evolved that are effective against carbapenems as well. There has been no choice for treatment, because delay in the use of carbapenems increases mor-

tality in ESBL patients (6). *Klebsiella pneumoniae* carbapenemases (KPC) have now spread worldwide, and it has been estimated that up to 40% of intensive care unit (ICU) patients in New York harbor carbapenem-resistant *Klebsiella* strains, with a 14-day mortality rate of 47% (reviewed in reference 4). There are several metallo-β-lactamases, including VIM and NDM-1, with 1 or 2 zinc ions in the active site, and these are more prevalent in Europe and Asia (1, 4, 7).

NDM-1 was isolated in 2008 from a patient who had visited India and contracted a urinary tract infection (UTI) (8). One significance of this important discovery was that the gene was not only present in different species in the same patient but was also located on a plasmid associated with genes conferring resistance not only to all β-lactams but also to other antibiotic classes and those affecting virulence and pathogenesis. The subsequent increase in nosocomial infections caused by bacteria harboring NDM-1 has been linked to travel to India, Pakistan, and the Balkans (1, 7, 9), especially for surgical procedures, including transplantation and cosmetic surgery often described as "medical tourism" (1, 7, 9). A worrying development is the spread to the community, resulting in infections primarily from orofecal transmission via direct contact with contaminated humans, animals, or the environment as well as via the ingestion of contaminated food and water (4, 10–12). The discovery that in certain areas there is widespread environmental contamination with NDM-1, lack of access to adequate sanitation, and extensive environmental contamination with fecal matter (4) and that the gene is increasingly found in *E. coli*, a major cause of community diarrhea, is a huge cause for concern; in Pakistan, approximately 20% of the population carry the gene (4, 7). In addition, initial isolates were not associated with clonal spread; however, the recent discovery of $bla_{\text{NDM-1}}$ in the ubiquitous *E. coli* ST131 lineage and the recent discovery of the gene on the IncFII plasmid which has been primarily responsible for the rapid dissemination of $bla_{\text{CTX-M15}}$ is adding to concerns of a similar pandemic spread (4, 7, 13). In addition, as for methicillin-resistant *Staphylococcus aureus* (MRSA), asymptomatic colonization, which may precede infection, may mask the size of the reservoir (4). NDM enzymes, such as NDM-4, are now evolving that are more catalytically efficient (14). A clonal outbreak observed in the United Kingdom, when 9 patients were contaminated, was traced to a failure of disinfection of a single endoscope camera. $bla_{\text{NDM-1}}$ is now found in a large number of *Enterobacteriaceae* and opportunistic environmental species, although carriage is not as stable *in vitro* in some nonfermenters, including *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, although NDM-1 has been isolated from these species (4, 7, 15).

This is such a serious problem it has been likened to the development of acquired immunodeficiency syndrome in the 1980s and the current epidemic of drug-resistant tuberculosis (16). The main concern is that the lack of effective antimicrobials and the delay in the development of new ones may return us to the preantibiotic era such that minor infections may escalate into serious, systemic life-threatening pathologies (1, 4, 16).

Within clinical and domestic environments, touch surfaces such as stainless steel are employed primarily for their resilience, cost, and ability to be cleaned regularly. However, several pathogens have been shown to exhibit prolonged survival on stainless steel and other dry surfaces, and microscopic striations on apparently smooth surfaces can harbor viable microorganisms that evade cleaning regimens (17–19). Guet-Revillet et al. (20) observed contamination by fecal carriage of ESBL-producing *Enterobacteriaceae*, especially *Klebsiella*, in clinical environments frequented by patients and also saw no difference in levels of environmental contamination before and after routine cleaning procedures.

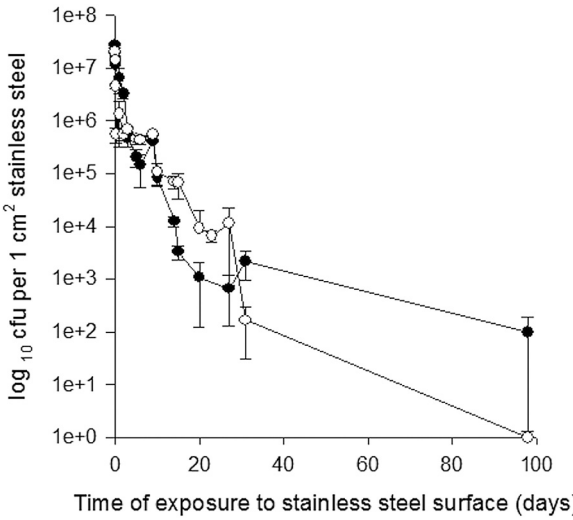

FIG 1 Survival of *E. coli* (NCTC 13441) (●) and *K. pneumoniae* (NCTC 13443) (○) containing $bla_{\text{CTX-M-15}}$ and $bla_{\text{NDM-1}}$, respectively, on stainless steel at 22°C. Approximately 10^7 CFU in 20 μl were inoculated onto 1-cm^2 metal coupons in bacteriological medium. Cells were removed and assessed for culturability as described in the text. For both species, a 2-log reduction in viable cell numbers was observed over the first 10 days, gradually declining to a 3- to 4-log reduction over the following month. Viable *E. coli* bacteria were detected at 100 days. Error bars represent ± SD (standard deviations), and data are from multiple independent experiments.

Consequently, we investigated the survival of *E. coli* (clone ST131) and *K. pneumoniae* containing $bla_{\text{CTX-M-15}}$ and $bla_{\text{NDM-1}}$, respectively, on abiotic surfaces. It has been shown that these genes are transferable in suspension *in vitro* and even *in situ* in the gut to other species (8, 15, 21). We investigated if horizontal gene transfer could occur on stainless steel surfaces, which may have implications for the rapid spread of these genes to potentially hardier and more virulent bacteria. We also investigated the efficacy of antimicrobial copper alloy surfaces which could be employed in high-risk areas in clinical and community settings.

RESULTS AND DISCUSSION

ESBL-producing *E. coli* and NDM-1-producing *K. pneumoniae* exhibit prolonged survival on stainless steel surfaces. Previous work in our laboratory had shown that both Gram-positive and Gram-negative pathogens survive for long periods on stainless steel surfaces (18, 22). In this new work, we observed the same for ESBL-producing *E. coli* and NDM-1-producing *K. pneumoniae*: with a starting inoculum of approximately 10^7 CFU per cm^2 of metal surface, there was a 2-log reduction in viable cell numbers for both species over the first 10 days, gradually declining to a 3- to 4-log reduction at 4 weeks. Viable *E. coli* bacteria were detected at 100 days (Fig. 1). In addition, there was no significant reduction in viability when cells were exposed to other common surfaces, including glass, ceramic tiles, and acrylic, for 5 h (not shown). The results suggest that the presence of antibiotic resistance genes that

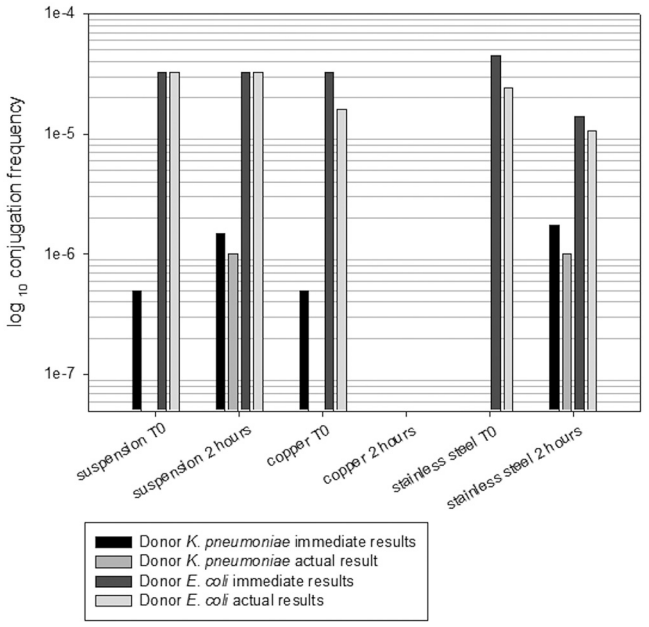

FIG 2 Comparison of the frequencies of conjugation of donor *E. coli* (NCTC 13441) and *K. pneumoniae* (NCTC 13443) containing $bla_{\text{CTX-M-15}}$ and $bla_{\text{NDM-1}}$, respectively, versus the recipient *E. coli* strain (Az$^{\text{r}}$ [F^{-} *met pro*], sensitive to β-lactam antibiotics) in suspension and on surfaces. Conjugation frequency is expressed as the number of transconjugants per donor cell as described in the text and is shown as the immediate result (initial isolation) or as the actual result following subculture (i.e., stable transconjugants that retain the gene). Transfer of $bla_{\text{CTX-M15}}$ occurred immediately in the suspension at a frequency of approximately 3×10^{-5} and did not increase after 2 h. Similar results were observed on stainless steel, but no transconjugants were recovered from copper after 2 h of contact. Transfer of $bla_{\text{NDM-1}}$ from *K. pneumoniae* occurred at a lower frequency of approximately 3×10^{-7}, and although transfer did occur immediately when the cells were mixed in suspension and on copper, the transconjugants were not stable and died on subculturing. However, after 2 h, stable transconjugants were produced on stainless steel and in suspension.

confer an advantage to the organism *in vivo* does not affect fitness in the environment. In addition, the prolonged environmental survival can increase the risk of infection spread (23).

Horizontal transfer of β-lactamase genes occurs in suspension and on stainless steel surfaces. Clinical isolates are often screened for the ability to transfer genes to recipient strains in suspension. Using an azide-resistant recipient strain of *E. coli*, we selected for transconjugants produced in suspension or on surfaces at room temperature (21°C ± 2°C) using culture medium containing antibiotic and sodium azide concentrations that together would kill donor and recipient cells. The frequency of conjugation was over 10 times greater and more stable with *E. coli* containing $bla_{\text{CTX-M-15}}$ as the donor than with *K. pneumoniae* containing $bla_{\text{NDM-1}}$ (Fig. 2).

Gene transfer from ESBL *E. coli* to strain J53 in suspension occurred in the few minutes required to process t_0 samples, i.e., as soon as the cells were mixed together. After 2 h, there was no change in the conjugation frequency (approximately 3×10^{-5}), and all transconjugants were stable upon subculture. On stainless steel, at t_0, the conjugation frequency was twice that of cells in suspension, but the actual number of transconjugants which were stable was less than in suspension. After 2 h of contact, there was no increase in gene transfer, suggesting that the significant gene transfer occurred as soon as cells were mixed at t_0.

The transfer of carbapenemase genes occurred at a significantly lower frequency (~4×10^{-7}). In suspension, unstable transconjugants were produced at t_0; however, over 2 h, the number of transconjugants increased (~2×10^{-6}) and the majority of these were then stable.

Horizontal transmission of carbapenemase genes is often accompanied by transmission of flanking genes, including those conferring resistance to other antimicrobials and affecting expression, including multiple promoters and novel replicase genes (reviewed in reference 4). In our work, transconjugants generated from both species had the same multidrug resistance profile as the donor strain, demonstrating multiple gene transfer and the potential for rapid dissemination of multiple antibiotic resistance genes to the same and other species. *E. coli* serotype O157: H7 has evolved as a significant pathogen by the acquisition of genes from 53 different species, including pathogenicity islands and virulence factors responsible for attachment, colonization, and production of toxins, and gene transfer on towels and chopping boards has been observed (24). The advent of ESBL- and metallo-β-lactamase-producing organisms has rendered virtually all β-lactams obsolete and has severely limited treatment options, and a huge concern is that these pathogens may also acquire other genes, increasing virulence. Increased mortality caused by severe infections with *Pseudomonas* metallo-β-lactamase producers has already been observed, but there is a wide variation in the results of infections by *Enterobacteriaceae* (1). The lack of development of new antimicrobials (3) and the emergence of resistance to resurrected "savage" older treatments (25) to counteract this exacerbates the problem. A few potential treatments, including new applications for existing drugs such as the angiotensin converting enzyme (ACE) inhibitor D-captopril, and new treatments, including a non-β-lactam inhibitor of metallo-β-lactamases, NXL104 (1).

Walsh et al. (7) observed that the highest frequency of conjugation between cells in suspension occurred at 30°C compared to 25°C and 37°C and suggested that gene transfer may be more frequent in areas of the world with a higher ambient temperature. This may be extrapolated to the gene transfer on surfaces that we have observed. The combination of the ability to survive for long periods in the environment and the propensity to acquire genes increasing virulence has resulted in the development of significant pathogens. Guet-Revillet et al. (20) identified contamination of surfaces in clinical facilities that had been occupied by ESBL-colonized or -infected children and observed that the contamination was more common with *K. pneumoniae* than *E. coli*. Our results suggest that transfer of $bla_{\text{CTX-M-15}}$ to recipient cells can occur immediately and that transfer of $bla_{\text{NDM-1}}$ from *Klebsiella* can occur on surfaces with increasing numbers over time at room temperature. This suggests that immediate effective decontamination of surfaces exposed to patients is required; otherwise, gene transfer to other pathogens in the vicinity could occur. Prolonged pathogen survival and horizontal gene transfer (HGT) pose a significance risk not only for exacerbations of multiple antibiotic-resistant health care-associated infections but also for widespread dissemination in public transportation systems where hand contact is unavoidable, particularly in societies where personal hygiene compliance is poor.

TABLE 1 Composition of metal coupons

Metal type	UNS[a] no.	% composition					
		Cu	Zn	Sn	Ni	Fe	Cr
Copper	C11000	100					
Phosphor bronze (contains ~0.26% P)	C51000	95		5			
Copper nickel	C70600	89			10	1	
Cartridge brass	C26000	70	30				
Nickel silver	C75200	65	17		18		
Muntz metal	C28000	60	40				
Stainless steel	S30400				8	74	18

[a] UNS, unified numbering system.

Horizontal transfer of β-lactamase genes does not occur on antimicrobial copper surfaces. A reduction in the microbial burden and infection rate has previously been observed with dry copper alloy surfaces for other bacterial, viral, and fungal pathogens in laboratory studies and clinical trials incorporating copper alloys into a range of fitments, including door handles and push plates, toilet seats, bed rails, and intravenous poles in high-risk clinical and community environments throughout the world (18, 19, 22, 26–35). Previous work with enterococci has suggested that dry copper surfaces evoke death by release of copper ions and effects on growth and respiration; genomic and plasmid DNA is a primary target (18, 22). DNA is also degraded in Gram-negative cells exposed to copper but not as rapidly; the cell membrane is a primary target because depolarization occurs immediately on contact and peroxidation of membrane lipids occurs (32, 36). After we had observed HGT on stainless steel, we investigated whether it would occur on dry copper surfaces.

On copper surfaces, the transconjugation frequency of $bla_{\text{CTX-M-15}}$ was the same as that seen in cells in suspension at t_0 but a small, significant ($P < 0.001$) number of transconjugants were unstable (Fig. 2). No transconjugants were evident after 2 h of contact, presumably due to the effects of antimicrobial copper. On copper at t_0, no stable $bla_{\text{NDM-1}}$-containing transconjugants were produced (the same frequency as cells in suspension), and none were evident after 2 h of contact. The results suggest that copper surfaces may prevent HGT.

Under conditions of wet fomite or dry touch surface contamination with *E. coli* and *K. pneumoniae* carrying $bla_{\text{CTX-M-15}}$ and $bla_{\text{NDM-1}}$, respectively, the pathogens die rapidly on antimicrobial copper surfaces at room temperature (21°C ± 2°C).

Approximately 10^7 CFU of ESBL-producing *E. coli* in phosphate-buffered saline (PBS) were spread over 1-cm^2 coupons of copper and alloys containing between 95% and 60% copper (Table 1) in 20 μl or 1 μl to mimic wet fomite and dry touch surface contamination, respectively. Results for *E. coli* fomite contamination are shown in Fig. 3. Cells inoculated in PBS (panel A) were dead on copper and copper nickel after 30 min of contact. Death occurred on all other alloys by 90 min, although the rate of killing over the first 30 min was proportional to the percentage of copper, i.e., the rate was higher for phosphor bronze than Muntz metal containing 95% and 60% copper, respectively. When cells were inoculated in complex matrices of carbohydrates, proteins, and lipids to represent naturally occurring soils (bacteriological media tryptone soy broth [TSB] [panel B] and brain heart infusion broth [BHIB] [panel C]), death on copper and copper alloys was delayed.

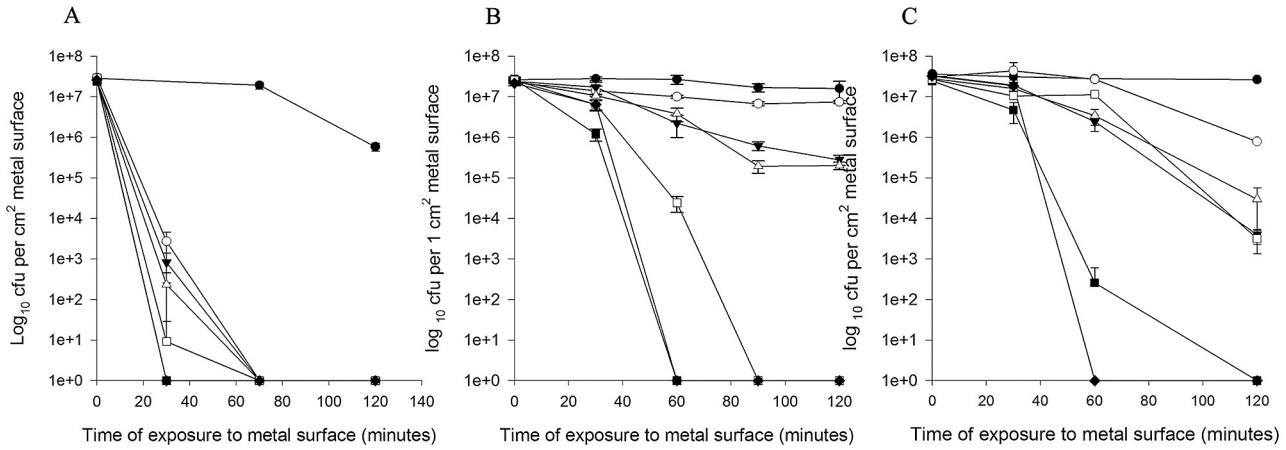

FIG 3 Survival of a wet fomite inoculum of extended-spectrum-β-lactamase-producing *E. coli* (NCTC 13441) containing $bla_{\text{CTX-M-15}}$ on copper and copper alloys at 22°C inoculated in a range of matrices. Approximately 10^7 CFU in 20 μl were inoculated onto the following 1-cm^2 metal coupons in PBS (A), tryptone soy broth (TSB) (B), or brain heart infusion broth (BHIB) (C) (see Table 1 for constituents of coupons): S30400 (●), C28000 (○), C75200 (▼), C26000 (Δ), C70600 (■), C51000 (□), and C11000 (◆). Cells were removed and assessed for culturability as described in the text. Prolonged survival was observed on stainless steel with no significant reduction in cell viability for all matrices. Cells in PBS died very rapidly, but death was delayed in the other two matrices. However, there was a significant reduction in numbers of viable cells at 2 h for all alloys and matrices ($P < 0.05$) except cells inoculated in TSB onto C28000, the alloy with the lowest copper content. Error bars represent ± SD, and data are from multiple independent experiments.

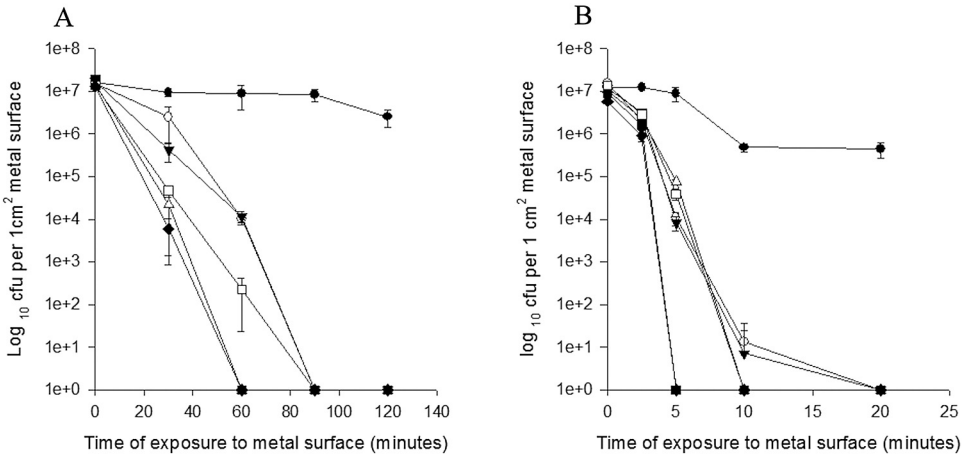

FIG 4 Survival of a wet fomite (A) and dry touch surface (B) inoculum of *K. pneumoniae* (NCTC 13443) containing bla_{NDM-1} on copper and copper alloys at 22°C. Approximately 10^7 CFU in 20 μl (dries in 30 min) or 1 μl (dries in seconds) were inoculated onto the following 1-cm^2 metal coupons in PBS: S30400 (●), C28000 (○), C75200 (▼), C26000 (Δ), C70600 (■), C51000 (□), and C11000 (◆). Cells were removed and assessed for culturability as described in the text. Prolonged survival was observed on stainless steel, but rapid death occurred on copper and copper alloys in proportion to the percentage of copper, especially for the dry touch surface contamination, where all cells were dead on copper and copper nickel after 5 min of contact. Similar results were obtained for extended-spectrum-β-lactamase-producing *E. coli* (not shown). Error bars represent ± SD, and data are from multiple independent experiments.

In TSB, death occurred by 60 min on copper and copper nickel and 90 min on phosphor bronze, and a significant reduction ($P < 0.05$) occurred on cartridge brass and nickel silver following 2 h of contact. On Muntz metal, which has the lowest copper content tested, the reduction in viable cells was not significant at 2 h ($P = 0.243$).

In BHIB, which has high lipid content, death occurred on copper and copper nickel within 2 h and there was a significant reduction in viable cells on all other alloys (a 3- to 4-log reduction on phosphor bronze, cartridge brass, and nickel silver).

This delay may have been due either to constituents in the matrices chelating copper ions and therefore preventing access to the cells or possibly to interactions with the Gram-negative outer membrane. Previous experiments with Gram-positive enterococci indicate the latter, because there was no significant delay between kill times in complex matrices on copper surfaces.

Similar results were observed for *K. pneumoniae*, with slightly longer times to death: all cells had died on copper, copper nickel, and cartridge brass by 60 min or 90 min for the remaining alloys (Fig. 4A). Death occurred considerably more quickly with dry touch surface contamination, a phenomenon we have observed previously (32): death occurred on copper and copper nickel by 5 min and by 20 min for the remaining alloys (Fig. 4B).

Therefore, the rate of killing on copper and copper alloys is determined not only by the aqueous content of the inoculating matrix but also by the constituents of the matrix itself. Death was most rapid in PBS and slightly slower in complex matrices. Similar results were observed by Noyce et al. (28), who observed a delay in killing of *E. coli* O157 on copper surfaces in the presence of blood and fat in meat juices.

As expected, in light of the long-term survival noted previously, no significant reduction in viable cells on stainless steel in all three matrices was observed after 2 h of exposure at room temperature. The high inoculum used for these experiments represents a worst-case scenario, because, in reality, the naturally occurring bioburden of surfaces is much less (31), and previous experiments have shown that death occurs even more rapidly with a reduced inoculum size (18).

The results do highlight the fact that antimicrobial copper and copper alloy can provide a constant killing surface, providing that suitable cleaning protocols are employed that use nonchelating substances to ensure continuous copper ion release.

Exposure to dry copper surfaces inhibits the respiration of β-lactamase producers. Bacteria may exist in a viable-but-nonculturable (VBNC) state under stressful environmental conditions with the potential to cause infection if conditions change to those more optimal for growth. Bacteria in a VBNC state can be identified by the presence of intact, respiring cells that do not grow in culture. CTC (5-cyano-2,3-ditolyl tetrazolium chloride) is a nonfluorescent redox dye that can be used to detect active respiration in bacteria by acting as an alternative electron acceptor. When respiration is occurring, the dye is reduced to a red, fluorescent non-water-soluble formazan product.

Although culture results suggested that no viable cells could be recovered from copper and cartridge brass surfaces following 2 h of contact at room temperature (Fig. 3A and 4A), cells were stained with CTC to determine if any VBNC cells were present (results are shown for ESBL-producing *E. coli* in Fig. S1 in the supplemental material). Actively respiring cells (stained red) were evident on stainless steel (panel B) after 2 h of contact but not on copper (panel F) or brass (panel D) surfaces (see Fig. S1 in the supplemental material), suggesting the absence of VBNC cells. The same preparations were also stained with the cell-permeable, low-toxicity, green fluorescent stain SYTO 9, which exhibits brighter fluorescence when bound to double-stranded DNA of live and dead cells. On stainless steel (panel A), the cells stained brightly, suggesting intact nucleic acid. However, on copper (panel E) and brass (panel C), the staining intensity was diminished, suggesting that DNA breakdown had occurred and that the dye was unable to bind. We have observed similar results in enterococci and other *E. coli* species (22, 32).

Therefore, the use of antimicrobial surfaces in conjunction

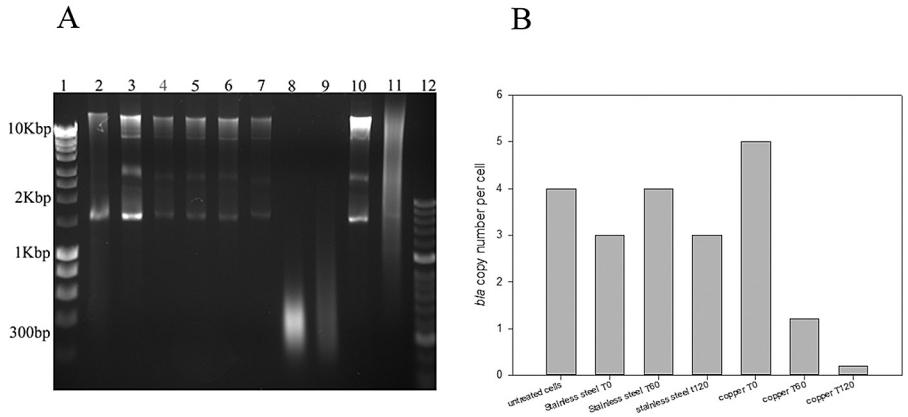

FIG 5 Degradation of plasmid DNA and a reduction of copy numbers of $bla_{CTX\text{-}M\text{-}15}$ occur in extended-spectrum-β-lactamase-producing *E. coli* exposed to copper but not stainless steel surfaces; wet fomite inoculum. (A) The plasmid DNAs of untreated cells (lanes 3 and 10), heat-killed cells (lane 11), or cells exposed to metal surfaces (stainless steel, 0, 60, and 120 min in lanes 4, 5, and 6, respectively; copper, 0, 60, and 120 min in lanes 7, 8, and 9, respectively) were purified and separated by agarose gel electrophoresis as described in the text. The DNAs of untreated cells and those exposed to stainless steel demonstrate the same plasmid bands, indicating that no degradation of DNA had occurred. The reduction of fragment size and smearing of plasmid DNA from cells exposed to copper suggest that extensive degradation was occurring which increased with time. Plasmid DNA from *K. pneumoniae* is represented in lane 2. Control lanes represent Bioline Hyperladder I (lane 1) and Hyperladder II (lane 12). (B) The same plasmid preparations were assessed for concentrations of $bla_{CTX\text{-}M\text{-}15}$ with gene-specific qPCR as described in the text. Approximately 3 to 4 copies of the gene were present in untreated cells and in those exposed to stainless steel. The copy number increased slightly on cells exposed to copper and immediately removed but then diminished to <1 upon longer exposure; i.e., many cells did not contain the gene.

with efficient cleaning and disinfection routines could help in the prevention of infection spread.

Degradation of plasmid DNA occurs in β-lactamase-producing *E. coli* and *K. pneumoniae* exposed to copper surfaces accompanied by reduced copy numbers of *bla* genes. We have observed the prevention of HGT on copper surfaces. We extracted the entire plasmid DNA of β-lactamase producers exposed to copper surfaces to ascertain the extent of DNA damage.

For *E. coli* containing $bla_{CTX\text{-}M\text{-}15}$, the plasmid profiles of untreated cells (Fig. 5A, lanes 3 and 10) and cells exposed to stainless steel for 0, 60, and 120 min (Fig. 5A, lanes 4, 5, and 6) are similar, with several plasmids of multiple sizes greater than 1.5 kbp. However, on copper there was a progressive degradation of the plasmid DNA with time so that after 120 min a smear of small fragments with an average size of 300 bp was present (Fig. 5A, lanes 7, 8, and 9). The concentration of the *bla* gene determined by quantitative PCR (qPCR) in the same plasmid samples indicates that, although the copy number was reduced following contact with copper surfaces (after 60 min, the copy number was less than 1, suggesting that many cells did not contain a plasmid), copies were still detectable. However, the qPCR amplifies only a small fraction of the gene, and we have not yet investigated the expression and functionality of the β-lactamase itself. Interestingly, the cells that had been removed from copper immediately (t_0) had a greater copy number than control cells (Fig. 5B).

The results were similar for the "dry" inoculum (see Fig. S2 in the supplemental material), where there was minimal effect on plasmid DNA from cells exposed to stainless steel for 0, 10, 30, and 60 min (see Fig. S2A, lanes 2 to 5, in the supplemental material) but rapid degradation on copper at the same time points (see Fig. S2A, lanes 6 to 9). It is interesting that some degradation occurred immediately on contact and that DNA fragments had massively degraded to 300-bp fragments by 10 min. qPCR detection of $bla_{CTX\text{-}M\text{-}15}$ (see Fig. S2B in the supplemental material) also showed a reduction in copy numbers in cells exposed to copper, but there was greater variation in copy numbers in all cells.

Naked DNA degrades on copper surfaces and in a temperature-dependent manner. We have previously shown that enterococcal genomic and plasmid DNA is actively degraded on copper and copper alloy surfaces (18, 22) as a result of copper ion release and generation of reactive oxygen species. We have also observed the effect of copper on Gram-negative bacteria and observed an immediate effect on the outer membrane and a much slower degradation of the bacterial DNA (32). There is some dispute with regard to whether the DNA degradation observed is the cause or effect of release of lytic enzymes and compounds post-cell death. Naked plasmid DNA (60 ng) was applied to the surface of 1-cm^2 stainless steel or copper coupons in 1- or 10-μl volumes (dry within few seconds or by 10 min, respectively) for 20 min at room temperature. On stainless steel, the DNA remained intact in large fragments (several bands are visible due to the presence of several plasmid polymeric, relaxed, and supercoiled isoforms) (Fig. 6A, lanes 4 and 5). On copper, the DNA completely degraded in the rapidly dried application but traces remained in the "wet" application (Fig. 6A, lanes 6 and 7).

When the DNA was applied to copper (Fig. 6B, lanes 8 to 10) or cartridge brass (Fig. 6B, lanes 5 to 7) (dry inoculum) at a range of temperatures (4°C, 22°C, and 37°C, respectively), the DNA totally fragmented after 20 min at 37°C and 22°C but not at 4°C. The inoculum was not completely dry at this temperature.

Figure 6C shows the results seen with the DNA applied in 20 μl at 37°C (lanes 3, 6, and 9), 22°C (lanes 4, 7, and 10), or 4°C (lanes 5, 8, and 11): the extent of degradation after 45 min was greater on copper (lanes 9 to 11) than on cartridge brass (lanes 6 to 8), presumably due to reduced copper content in the latter, and became greater as the temperature increased. No change in DNA exposed to stainless steel (lanes 3 to 5) was observed under any of the 3 temperature conditions.

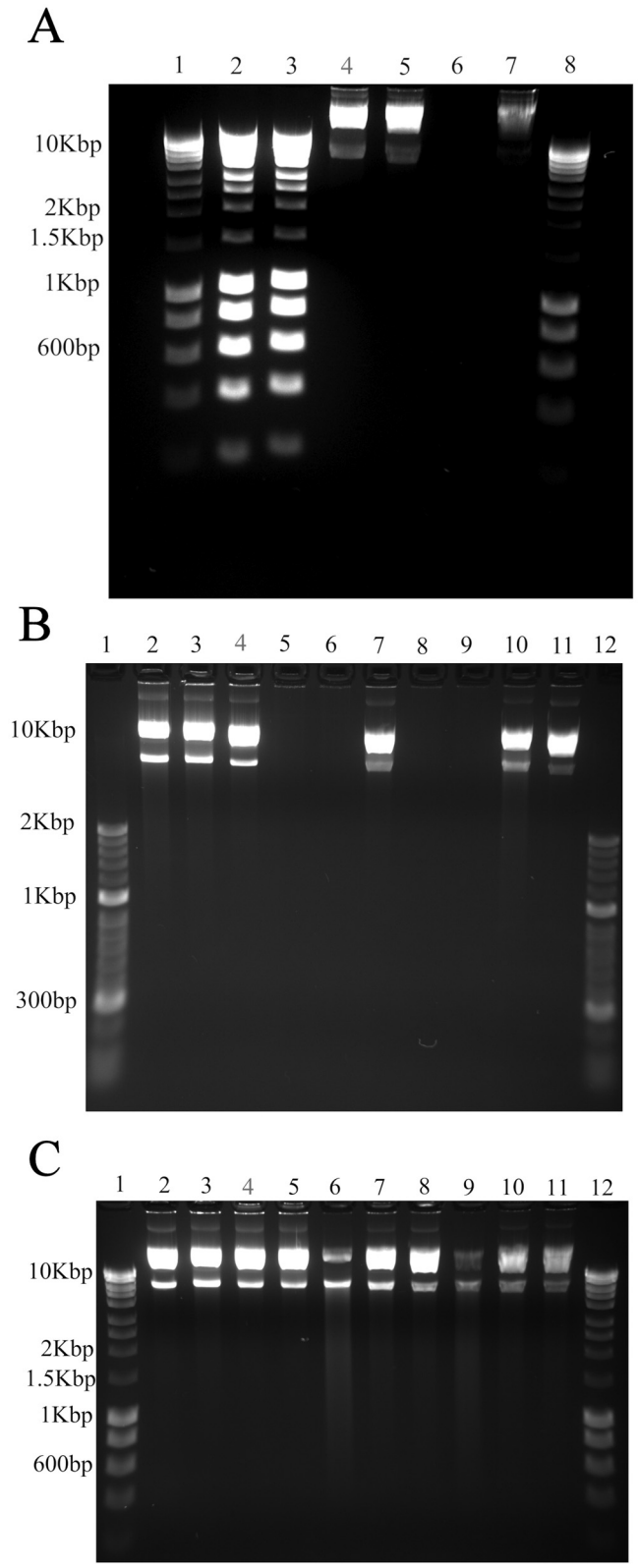

FIG 6 Degradation of naked plasmid DNA (pBR322) on copper surfaces is dependent on temperature and aqueous content. (A) Sixty nanograms of naked plasmid DNA was applied to metal surfaces for 20 min at room temperature in 1 μl, which dried in seconds ("dry"), or in 10 μl ("wet"). The DNA was

(Continued)

No degradation of DNA occurred on stainless steel (wet or dry) at any of the temperatures tested.

There is controversy in the literature, with some researchers believing that the DNA degradation observed is entirely a result of post-cell-death breakdown and toxicity from released products (37). Our experiments performed with naked DNA suggest that active DNA breakdown does occur on copper and brass surfaces that is more extensive in rapidly drying samples at temperatures greater than or equal to room temperature. However, at 4°C, DNA degradation is very slow but, given that the optimal temperature for conjugation is 30°C, the chances of conjugation may be minimal. Perhaps the DNA denaturation we have observed in Gram-negative species here represents a combination of active processes and lysis of dead cells releasing deleterious compounds.

CONCLUSION

It is estimated that up to 10% of patients admitted to modern hospitals acquire one or more infections and that the proportion rises to 25% in some developing countries. In Europe and the United States, this is equivalent to 4 and 2 million cases, leading to approximately 37,000 and 99,000 deaths per year, respectively, with a combined resultant cost of over 13 billion dollars (3). The meteoric rise in ESBL-producing *E. coli* and *Klebsiella* spp., which have been the most common *Enterobacteriaceae* responsible for the nosocomial spread in ESBL, may now be followed by carbapenemase producers. Nordmann et al. (4) warn of an increase in *E. coli* NDM-1 and OXA-48 community-acquired infections and *Klebsiella* species KPC, IMP, NDM, and OXA-48 infections in high-risk hospitalized individuals. It is very difficult to estimate the size of the reservoir of multidrug resistance genes surviving in resilient bacteria over the long term in the environment along with increased colonization of humans and animals. This study demonstrated that HGT readily occurs on dry touch surfaces such as stainless steel, providing a potentially important route for multidrug resistance emergence and dissemination in public buildings

Figure Legend Continued

removed by pipetting and integrity determined by agarose electrophoresis, as described in the text. For the dry inoculum, plasmid DNA recovered from stainless steel (lane 4) produced two multimer bands, the same as plasmid DNA that had not been exposed to metal (B, lane 11), but the DNA completely degraded on copper (lane 6). The wet inoculum was the same on stainless steel (lane 5), and although extensive degradation occurred on copper (lane 7), traces of DNA were still visible. Control lanes (lanes 1 and 8) represent Bioline Hyperladder I. The ladder DNA was applied directly to stainless steel (lane 2) and copper (lane 3) surfaces. No breakdown of the DNA was evident because the chelator, EDTA, and glycerol components used to stabilize DNA for long-term storage protected the DNA from damaging copper ions released from the surface. (B) Plasmid DNA ("dry" inoculum) was applied directly to stainless steel (lanes 2 to 4), cartridge brass (lanes 5 to 7), or copper (lanes 8 to 10) surfaces at 37°C (lanes 2, 5, and 8), 22°C (lanes 3, 6, and 9), or 4°C (lanes 4, 7, and 10) for 20 min. On stainless steel, DNA remained intact at all temperatures. However, on brass and copper, the DNA degraded completely at 37°C and 22°C, but very little degradation occurred at 4°C. (C) Plasmid DNA ("wet" inoculum) was also applied to stainless steel (lanes 3 to 5), cartridge brass (lanes 6 to 8), or copper (lanes 9 to 11) surfaces at 37°C (lanes 3, 6, and 9), 22°C (lanes 4, 7, and 10), or 4°C (lanes 5, 8, and 11) for 45 min. The DNA remained intact on stainless steel the same as it did on the dry inoculum. On brass and copper, results were similar to those seen with dry inoculation, i.e., most DNA broke down at the highest temperature of 37°C, but the effect was not as extensive. The amount of DNA breakdown was proportional to the copper content. Control lanes (lanes 1 and 12) represent Bioline Hyperladder I, and lane 2 is the untreated plasmid.

and transportation systems if surfaces are not regularly and efficiently cleaned. Although previous studies in our laboratory have demonstrated that plasmid and genomic DNA degradation of antibiotic-resistant enterococci and *E. coli* O157 occurs on dry copper surfaces (18, 32), the next step is to investigate whether HGT in these and other pathogens, including the transfer of specific genes responsible for enhanced virulence and toxin production as well as resistance to antimicrobial therapies, is prevented. The effect of various environmental conditions, contact times, and bacterial densities on the rate of HGT also has to be determined. The use of copper alloys in clinical and community settings could help reduce infection spread and also reduce the incidence of horizontal transmission genes conferring drug resistance, virulence, and pathogenesis and expression efficiency. Considerable concern has been expressed and blame has been assigned in describing the nature, origin, and nomenclature of the newly evolving resistance genes, but more accessible global travel has made the world a much smaller place, and the evolution of potentially untreatable infectious diseases will eventually and inevitably affect us all.

MATERIALS AND METHODS

Bacterial strains. *Klebsiella pneumoniae* NCTC 13443 (encodes $bla_{\text{NDM-1}}$ metallo-beta-lactamase) and *Escherichia coli* NCTC 13441 (encodes $bla_{\text{CTX-M-15}}$ on plasmid pEK499) (5) were supplied by Health Protection Agency, Porton Down, United Kingdom. Sodium azide-resistant *E. coli* J53 (J53 Az^r [F$^-$ *met pro*]), used as the recipient strain in conjugation experiments, was kindly supplied by George Jacoby (Lahey Clinic, Burlington, United States) (38) and James Anson (University of Liverpool, United Kingdom).

Preparation of sample surfaces. Metal coupons (10 by 10 by 0.5 mm) were degreased in acetone, stored in absolute ethanol, and flamed prior to use as described previously (18). The constituents of each metal tested are detailed in Table 1, and all were supplied by the Copper Development Association. Other surfaces were glass microscope slides (Fisher; Thermo Scientific), ceramic tiles, and acrylic (polymethylmethacrylate [PMMA]). These surfaces, and stainless steel as a method comparison control, were degreased in mild detergent solution and sterilized by immersion in ethanol for 10 min. Samples were dried under a sterile airflow prior to use (results described in text).

Culture preparation. Bacterial stocks were prepared on Protect Cryo beads (Microbiological Supply Company, United Kingdom) and stored at −80°C. Cultures were prepared by inoculation of 1 bead into 15 ml sterile brain heart infusion broth (BHIB) or TSB (Oxoid, United Kingdom) and incubated aerobically at 37°C for 16 ± 2 h.

Metal coupon inoculation (vortex/bead method) and assessment of viable cells by culture to simulate wet fomite or dry touch surface contamination. The surfaces of coupons were inoculated with approximately 10^7 CFU in either bacteriological medium or PBS. The volume was 20 μl or 1 μl for the same number of cells, with the former representing a wet fomite inoculum that dries in 30 to 40 min at 22°C and the latter drying in seconds in a manner equivalent to a dry touch surface contamination. Drying time was included in the exposure time, and this method has been described previously by us for enterococci (18). Bacteria were removed from the coupons at the required time point using a vortex procedure and 5 ml phosphate-buffered saline (PBS) containing EDTA (20 mM) to chelate and neutralize free copper ions and 2-mm-diameter glass beads and subjected to a vortex procedure for 30 s. A range of dilutions was prepared immediately, and aliquots were plated onto nutrient agar and tryptone soy agar (TSA) for *K. pneumoniae* and *E. coli*, respectively. When dry, the plates were inverted and incubated aerobically at 37°C for up to 48 h to quantify viable cells. Duplicate coupons were analyzed for each time point.

Inoculation of glass, ceramic, acrylic, and stainless steel surfaces and assessment of viable cells by culture (wet fomite contamination only). These surfaces were inoculated as described for metal coupons, but the cells were removed by addition of 100 μl PBS which was vigorously pipetted up and down and the cells transferred to a sterile tube. This was repeated 5 more times; the cells were then pooled, and the volume was increased to 5 ml with the addition of sterile PBS. Dilutions were prepared and aliquots spread over nutrient agar (NA) and TSA plates as described for metal surfaces.

Detection of respiring cells on metal surfaces in situ using the redox dye CTC (5-cyano-2,3-ditolyl tetrazolium chloride): "wet" inoculum only. Actively respiring bacteria can reduce the redox dye, CTC, to red fluorescent formazan which can be visualized by epifluorescence microscopy. Copper, cartridge brass, and stainless steel coupons were inoculated with ESBL-producing *E. coli* in petri dishes as described. When the coupons had dried (45 min), 50 μl of 5 mM CTC was added to each coupon, which was covered and incubated for a further 65 min at 37°C. A further 50 μl of the double-stranded DNA stain SYTO 9 (5 μM) was added to each coupon, which was incubated at room temperature for 10 min. Coupons were observed using epifluorescence microscopy with a metal halide light source and a long-working-distance 100× objective as described previously (18, 22, 39). A positive control of inoculum was stained in suspension at the same time.

Detection of changes in the integrity of plasmid DNA from CTX-M-15- and NDM-1-producing *E. coli* and *K. pneumoniae*, respectively. Cells were inoculated onto 1-cm^2 metal coupons as described for culture assessment (10 coupons per time point). Cells were removed and pooled, and the plasmid DNA was extracted using a Qiaprep Spin miniprep kit (Qiagen, United Kingdom) according to the manufacturer's instructions. Fragments were separated on 0.9% (wt/vol) agarose gel using a GelRed nucleic acid prestaining kit (Biotium, United Kingdom) and Hyperladder I and II size markers (Bioline, United Kingdom). Gels were observed in a Syngene UV light box and photographed using GeneSnap software.

Quantification of $bla_{\text{CTX-M-15}}$ and $bla_{\text{NDM-1}}$ using quantitative PCR of purified plasmid DNA. Primers were designed to amplify 80- and 118-bp fragments of $bla_{\text{NDM-1}}$ and $bla_{\text{CTX-M15}}$ as follows: for accession number FN396876, plasmid pKpANDM-1, sense, CCGCCATCCCTGAC GATC (position 2969), antisense, GTCTGGCAGCACACTTCCT (position 3048); and for accession number *000046, plasmid pEK499_p079, sense, TGAGGCTGGGTGAAGTAAGTG (position 68), antisense, CCTG GGTTGTGGGGGATAAAA (position 185) (PrimerDesign Ltd., Southampton, United Kingdom). Amplification was performed on a Bio-Rad iQ5 cycler, and standard curves were prepared from known copy number standards to determine copy numbers in test samples.

Horizontal transfer of $bla_{\text{CTX-M-15}}$ and $bla_{\text{NDM-1}}$ from donor cells to recipient *E. coli* J53 Az^r (F$^-$ *met pro*) cells on surfaces. Cultures of $bla_{\text{CTX-M-15}}$- and $bla_{\text{NDM-1}}$-containing *E. coli* and *K. pneumoniae*, respectively (donor cells), and recipient *E. coli* J53 Az^r cells were prepared in BHIB. The cells were pelleted and mixed together at a 10:1 ratio of donor to recipient in the same volume of Mueller-Hinton broth (Oxoid, United Kingdom); 20 μl mixed cells (total cell concentration, 5×10^8 CFU/ml) was applied immediately to the surface of copper or stainless steel coupons. Cells were removed by gentle pipetting in 2× 100 μl sterile PBS and spread over medium selecting for transconjugants, i.e., TSA containing 100 μg/ml sodium azide and 2 μg/ml cefotaxime (for the selection of J53 that had received $bla_{\text{CTX-M-15}}$) or 2 μg/ml meropenem (for the selection of J53 that had received $bla_{\text{NDM-1}}$). Prior to the experiment, donor cells were found to be sensitive to sodium azide and recipient cells sensitive to the antibiotic concentrations that were used. This method differs from that described for culture, so efficiency of recovery was determined. All of the donor cells and between 99.71% and 99.94% of the recipient J53 cells were recovered.

Transconjugants were subcultured, plasmids were prepared, and DNA integrity was checked by gel electrophoresis; qPCR confirmed the presence and copy numbers of $bla_{\text{CTX-M-15}}$ and $bla_{\text{NDM-1}}$. Those transcon-

jugants that were isolated initially on selective media but did not survive subculture and/or lost $bla_{CTX\text{-}M\text{-}15}$ and $bla_{NDM\text{-}1}$ when retested by gene-specific qPCR were considered to be unstable.

The conjugation frequency was calculated from the number of transconjugants per coupon/number of donor cells per coupon (and from equivalent numbers of cells in suspension). $bla_{CTX\text{-}M\text{-}15}$ transconjugants were also resistant to the same macrolide, fluoroquinolone, aminoglycoside, and glycopeptide antibiotics as the donor strain, as shown by testing with a disc diffusion assay (Oxoid, United Kingdom). The same was true of $bla_{NDM\text{-}1}$ transconjugants.

Assessment of the integrity of naked plasmid DNA on metal surfaces at a range of temperatures. Sixty nanograms of purified *E. coli* plasmid BR322 DNA (Sigma-Aldrich, United Kingdom) was spread over 1-cm^2 copper, cartridge brass, or stainless steel surfaces in a final volume of 1 or 10 μl. After 20 or 45 minutes of contact at 4, 22, or 37°C, the DNA was removed by addition of 10 μl distilled deionized water to the surface, gentle pipetting, and transfer to a DNase-free tube. The integrity of the DNA samples was investigated by agarose gel electrophoresis as described previously.

It should, however, be noted that this is a commercial plasmid preparation lyophilized in a solution containing 1 mM EDTA to retain stability. The sample was diluted to give a final concentration of <5 μM EDTA, which is significantly less than the 20 mM concentration found to be protective on copper surfaces in our previous work.

Statistical analysis. Data are expressed as means ± standard deviations (SD) and are the result of multiple independent experiments. Differences between duplicate samples were assessed using the Mann-Whitney rank *t* test. Group comparisons were analyzed 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 11.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00489-12/-/DCSupplemental.

Figure S1, TIF file, 3.1 MB.

Figure S2, TIF file, 0.8 MB.

ACKNOWLEDGMENTS

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REFERENCES

1. **Bush K, Fisher JF.** 2011. Epidemiological expansion, structural studies, and clinical challenges of new β-lactamases from gram-negative bacteria. Annu. Rev. Microbiol. **65**:455–478.
2. **Woodford N, Turton JF, Livermore DM.** 2011. Multiresistant gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol. Rev. **35**:736–755.
3. **World Health Organization.** 2011. Report on the burden of endemic health care-associated infection worldwide. World Health Organization, Geneva, Switzerland. ISBN 978 92 4 150150 7.
4. **Nordmann P, Poirel L, Walsh TR, Livermore DM.** 2011. The emerging NDM carbapenemases. Trends Microbiol. **19**:588–595.
5. **Woodford N, et al.** 2009. Complete nucleotide sequences of plasmids pEK204, pEK499, and pEK516, encoding CTX-M enzymes in three major Escherichia coli lineages from the United Kingdom, all belonging to the international O25:H4-ST131 clone. Antimicrob. Agents Chemother. **53**:4472–4482.
6. **Tuon FF, Kruger M, Terreri M, Penteado-Filho SR, Gortz L.** 2011. Klebsiella ESBL bacteremia-mortality and risk factors. Braz. J. Infect. Dis. **15**:594–598.
7. **Walsh TR, Weeks J, Livermore DM, Toleman MA.** 2011. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. Lancet Infect. Dis. **11**:355–362.
8. **Yong D, et al.** 2009. Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrob. Agents Chemother. **53**:5046–5054.
9. **Kumarasamy KK, et al.** 2010. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect. Dis. **10**:597–602.
10. **Cohen Stuart J, et al.** 2012. Comparison of ESBL contamination in organic and conventional retail chicken meat. Int. J. Food Microbiol. **154**:212–214.
11. **O'Keefe A, Hutton TA, Schifferli DM, Rankin SC.** 2010. First detection of CTX-M and SHV extended-spectrum beta-lactamases in *Escherichia coli* urinary tract isolates from dogs and cats in the United States. Antimicrob. Agents Chemother. **54**:3489–3492.
12. **Overdevest I, et al.** 2011. Extended-spectrum β-lactamase genes of *Escherichia coli* in chicken meat and humans, The Netherlands. Emerg. Infect. Dis. **17**:1216–1222.
13. **Bonnin RA, Poirel L, Carattoli A, Nordmann P.** 2012. Characterization of an IncFII plasmid encoding NDM-1 from *Escherichia coli* ST131. PLoS One **7**:e34752.
14. **Nordmann P, Boulanger AE, Poirel L.** 2012. NDM-4 metallo-β-lactamase with increased carbapenemase activity from *Escherichia coli*. Antimicrob. Agents Chemother. **56**:2184–2186.
15. **Potron A, Poirel L, Nordmann P.** 2011. Plasmid-mediated transfer of the bla(NDM-1) gene in Gram-negative rods. FEMS Microbiol. Lett. **324**:111–116.
16. **Bonomo RA.** 2011. New Delhi metallo-β-lactamase and multidrug resistance: a global SOS? Clin. Infect. Dis. **52**:485–487.
17. **Kramer A, Schwebke I, Kampf G.** 2006. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC Infect. Dis. **6**:130.
18. **Warnes SL, Green SM, Michels HT, Keevil CW.** 2010. Biocidal efficacy of copper alloys against pathogenic enterococci involves degradation of genomic and plasmid DNAs. Appl. Environ. Microbiol. **76**:5390–5401.
19. **Wilks SA, Michels H, Keevil CW.** 2005. The survival of Escherichia coli O157 on a range of metal surfaces. Int. J. Food Microbiol. **105**:445–454.
20. **Guet-Revillet H, et al.** 2012. Environmental contamination with extended-spectrum beta-lactamases: is there any difference between Escherichia coli and Klebsiella spp? Am. J. Infect. Control **40**:845–848.
21. **Goren MG, et al.** 2010. Transfer of carbapenem-resistant plasmid from *Klebsiella* pneumoniae ST258 to *Escherichia coli* in patient. Emerg. Infect. Dis. **16**:1014–1017.
22. **Warnes SL, Keevil CW.** 2011. Mechanism of copper surface toxicity in vancomycin-resistant enterococci following wet or dry surface contact. Appl. Environ. Microbiol. **77**:6049–6059.
23. **Boyce JM.** 2007. Environmental contamination makes an important contribution to hospital infection. J. Hosp. Infect. **65**(Suppl 2):50–54.
24. **Kruse H, Sørum H.** 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Appl. Environ. Microbiol. **60**:4015–4021.
25. **McGann P, et al.** 2012. Complete sequence of a novel 178-kilobase plasmid carrying bla(NDM-1) in a Providencia stuartii strain isolated in Afghanistan. Antimicrob. Agents Chemother. **56**:1673–1679.
26. **Mehtar S, Wiid I, Todorov SD.** 2008. The antimicrobial activity of copper and copper alloys against nosocomial pathogens and Mycobacterium tuberculosis isolated from healthcare facilities in the Western Cape: an in-vitro study. J. Hosp. Infect. **68**:45–51.
27. **Noyce JO, Michels H, Keevil CW.** 2006. Potential use of copper surfaces to reduce survival of epidemic meticillin-resistant Staphylococcus aureus in the healthcare environment. J. Hosp. Infect. **63**:289–297.
28. **Noyce JO, Michels H, Keevil CW.** 2006. Use of copper cast alloys to control *Escherichia coli* O157 cross-contamination during food processing. Appl. Environ. Microbiol. **72**:4239–4244.
29. **Noyce JO, Michels H, Keevil CW.** 2007. Inactivation of influenza A virus on copper versus stainless steel surfaces. Appl. Environ. Microbiol. **73**:2748–2750.
30. **Schmidt MG.** 2011. Copper surfaces in the ICU reduced the relative risk of acquiring an infection while hospitalised. BMC Proc. **5**(Suppl 6):O53.
31. **Schmidt MG, et al.** 2012. Sustained reduction of microbial burden on common hospital surfaces through introduction of copper. J. Clin. Microbiol. **50**:2217–2223.
32. **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.
33. **Weaver L, Michels HT, Keevil CW.** 2008. Survival of *Clostridium* difficile on copper and steel: futuristic options for hospital hygiene. J. Hosp. Infect. **68**:145–151.
34. **Weaver L, Michels HT, Keevil CW.** 2010. Potential for preventing spread of fungi in air-conditioning systems constructed using copper instead of aluminium. Lett. Appl. Microbiol. **50**:18–23.
35. **Wilks SA, Michels HT, Keevil CW.** 2006. Survival of Listeria monocytogenes Scott A on metal surfaces: implications for cross-contamination. Int. J. Food Microbiol. **111**:93–98.
36. **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.
37. **Espírito Santo C, et al.** 2011. Bacterial killing by dry metallic copper surfaces. Appl. Environ. Microbiol. **77**:794–802.
38. **Jacoby GA.** 1998. Epidemiology of extended-spectrum beta-lactamases. Clin. Infect. Dis. **27**:81–83.
39. **Keevil CW.** 2003. Rapid detection of biofilms and adherent pathogens using scanning confocal laser microscopy and episcopic differential interference contrast microscopy. Water Sci. Technol. **47**:105–116.

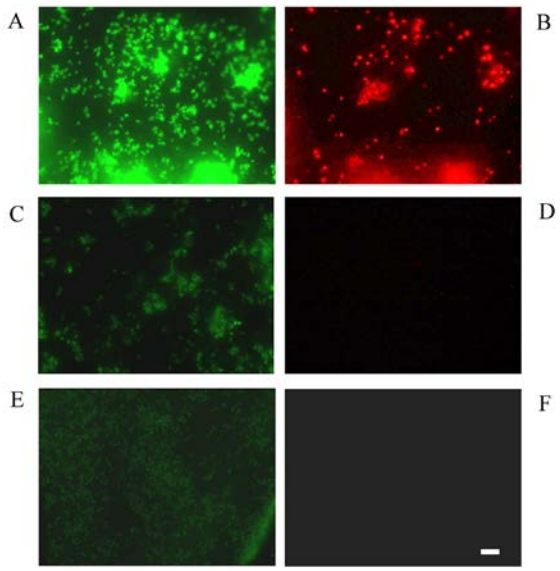
Horizontal Transfer of Antibiotic Resistance Genes on Abiotic Touch Surfaces: Implications for Public Health

Supplementary Data

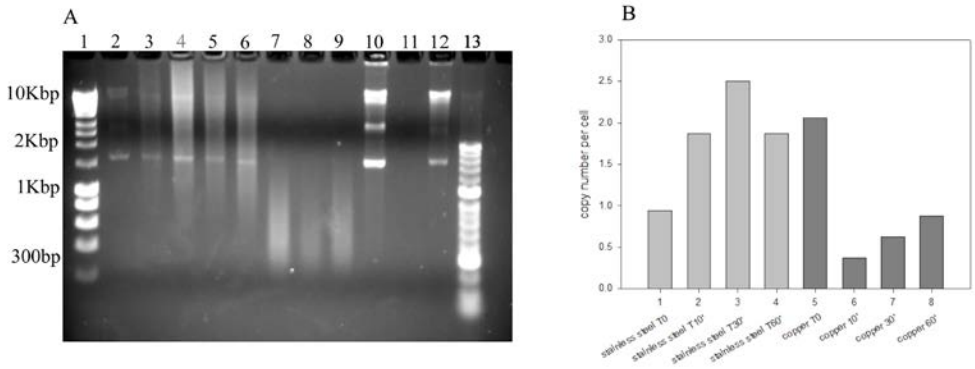
Supplementary Data

Files in this Data Supplement:

- **Figure sf01, TIF** - Figure sf01, TIF
- **Figure sf02, TIF** - Figure sf02, TIF



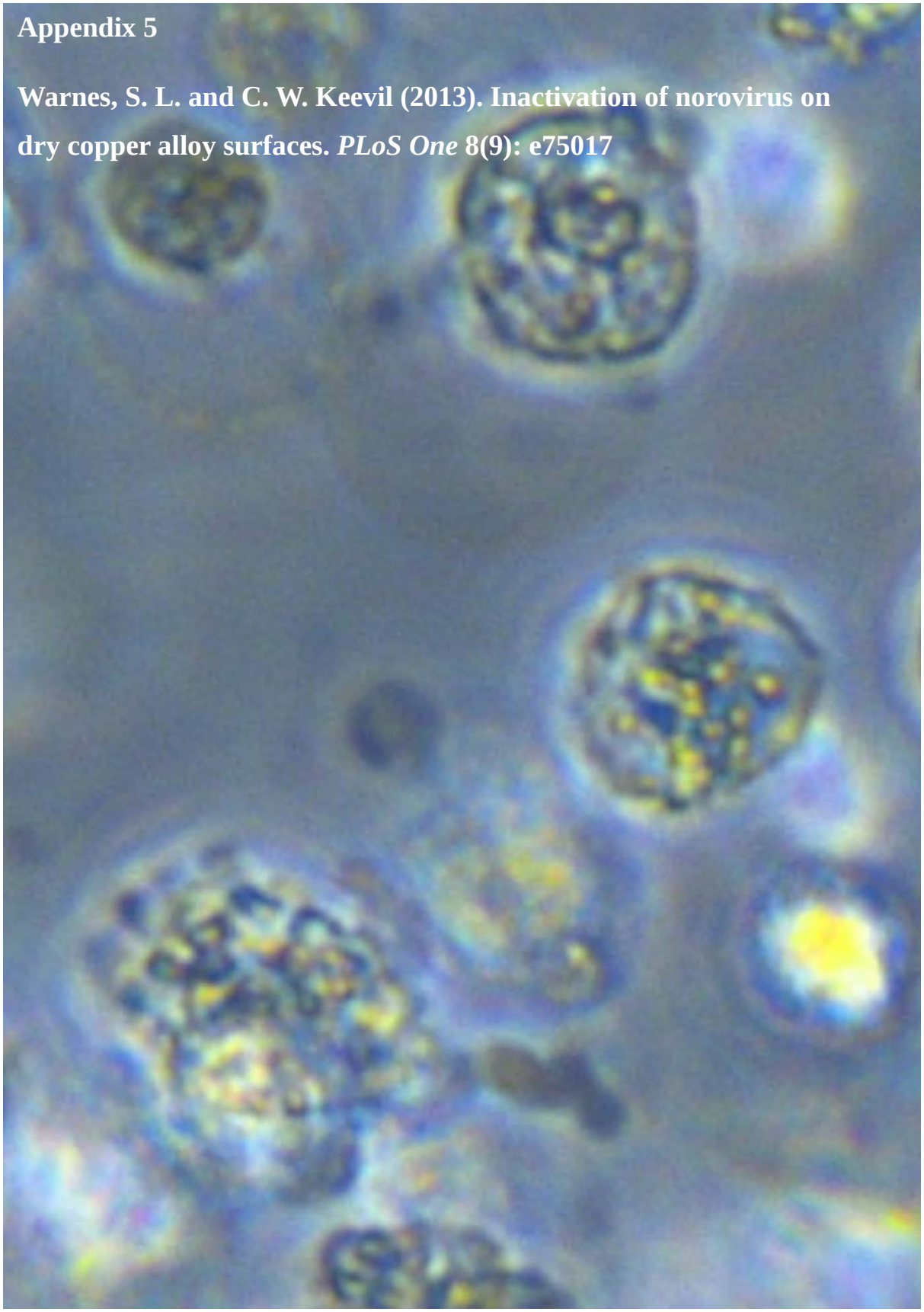
- **FIGURE S1**
Inhibition of respiration of extended-spectrum-β-lactamase-producing *E. coli* occurs on cartridge brass (C and D) and copper (E and F) but not stainless steel (A and B) surfaces following 2 h of contact at 22°C. Approximately 10^7 CFU in 20 µl were inoculated onto 1-cm^2 metal coupons in PBS. Once dry, the cells were stained *in situ* with the redox dye, CTC (which is reduced to a red fluorescent product in actively respiring cells) (B, D, and F), and with the non-vital stain, SYTO 9 (which fluoresces green when intercalated into double-stranded DNA) (A, C, and E), as described in the text. Actively respiring cells were present on stainless steel, and bright SYTO 9 staining suggests that the DNA of live and dead cells was intact. On copper and brass surfaces, no respiring cells were present, which, together with the culture results, suggests the absence of VBNC (viable-but-non-culturable) cells on this surface. In addition, the reduction in SYTO 9 staining suggests that DNA had broken down and that the dye was unable to bind. Bar, 10 µm. Download **Figure S1, TIF file, 3.1 MB**.



- **FIGURE S2**
  Degradation of plasmid DNA and a reduction of copy numbers of $bla_{\text{CTX-M-15}}$ occur in extended-spectrum-β-lactamase-producing *E. coli* exposed to copper but not stainless steel surfaces; dry touch surface contamination (10^7 CFU in 1 µl per cm^2 of metal surface). (A) The plasmid DNAs of untreated cells (lane 12) or cells exposed to metal surfaces (stainless steel, 0, 10, 30, and 60 min in lanes 2, 3, 4, and 5, respectively; copper, 0, 10, 30, and 60 min in lanes 6, 7, 8, and 9, respectively) were purified and separated by agarose gel electrophoresis as described in the text. The DNAs of untreated cells and those exposed to stainless steel demonstrate the same plasmid bands, indicating that no degradation of DNA had occurred. The reduction of fragment size and smearing of plasmid DNA from cells exposed to copper suggest that extensive degradation had occurred by 10 min. Plasmid DNA from *E. coli* is represented in lane 10. Control lanes represent Bioline Hyperladder I (lane 1) and Hyperladder II (lane 13). (B) The same plasmid preparations were assessed for concentrations of $bla_{\text{NDM-1}}$ with gene-specific qPCR as described in the text. Approximately 2 copies of the gene were present in untreated cells and in those exposed to stainless steel. The copy number increased slightly on cells exposed to copper and immediately removed but then diminished to <1 upon longer exposure, i.e., many cells did not contain the gene. Download **Figure S2, TIF file, 0.8 MB**.

Appendix 5

Warnes, S. L. and C. W. Keevil (2013). Inactivation of norovirus on dry copper alloy surfaces. *PLoS One* 8(9): e75017



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Inactivation of Norovirus on Dry Copper Alloy Surfaces

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Abstract

Noroviruses (family *Caliciviridae*) are the primary cause of viral gastroenteritis worldwide. The virus is highly infectious and touching contaminated surfaces can contribute to infection spread. Although the virus was identified over 40 years ago the lack of methods to assess infectivity has hampered the study of the human pathogen. Recently the murine virus, MNV-1, has successfully been used as a close surrogate. Copper alloys have previously been shown to be effective antimicrobial surfaces against a range of bacteria and fungi. We now report rapid inactivation of murine norovirus on alloys, containing over 60% copper, at room temperature but no reduction of infectivity on stainless steel dry surfaces in simulated wet fomite and dry touch contamination. The rate of inactivation was initially very rapid and proportional to copper content of alloy tested. Viral inactivation was not as rapid on brass as previously observed for bacteria but copper-nickel alloy was very effective. The use of chelators and quenchers of reactive oxygen species (ROS) determined that Cu(II) and especially Cu(I) ions are still the primary effectors of toxicity but quenching superoxide and hydroxyl radicals did not confer protection. This suggests Fenton generation of ROS is not important for the inactivation mechanism. One of the targets of copper toxicity was the viral genome and a reduced copy number of the gene for a viral encoded protein, VPg (viral-protein-genome-linked), which is essential for infectivity, was observed following contact with copper and brass dry surfaces. The use of antimicrobial surfaces containing copper in high risk closed environments such as cruise ships and care facilities could help to reduce the spread of this highly infectious and costly pathogen.

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Introduction

Gastroenteritis is a major cause of morbidity and mortality worldwide and is responsible for approximately 5–8 million deaths per year. It is estimated that norovirus (family *Caliciviridae*) gives rise to more than 267 million infections worldwide per year including 23 million in the US alone. This small, single stranded, positive sense RNA virus is responsible for over 90% cases of non-bacterial and approximately half of all cases of gastroenteritis (reviewed in [1,2,3]). Norovirus is now as important as rotavirus as a cause of diarrhoea and vomiting in hospitalised children in some countries [4].

The disease is usually contracted by ingestion of contaminated food, water, person-to-person contact and touching contaminated surfaces [5,6]. Infection is also transmitted by aerosols and prolonged viral shedding of high virus load, including asymptomatic individuals, increases the risk of infection spread [7]. Norovirus gastroenteritis is self-limiting but extremely infectious with a low infectious dose and is responsible for many outbreaks, often seasonal, especially in closed environments e.g. cruise ships and health-care facilities. Most reported cases are in the under 5 years old but the highest economic costs are in the care of elderly patients in residential care [5]. The disease may be life threatening in severely ill and vulnerable patients and has been linked to Crohn's disease and necrotising enterocolitis in neonates [8].

The virus does not have an envelope, conferring resistance to some cleaning detergents, alcohols, food preservation chemicals; it can survive on surfaces (especially if surfaces are contaminated with detritus and food residues [9] in the environment, and resist a wide pH range and temperatures from $-20^{\circ}C$ to $72^{\circ}C$ [10,11,12]. The contribution of contaminated surfaces in the spread of infection has been described previously [3,6]. In human challenge studies Gerhardts et al. [13] demonstrated a chain of bacterial and viral transfer from a single contaminated individual to surfaces and from there to other personnel with the risk of infection greatest in pathogenic strains with a low infectious dose. Thornley et al. [14] suggested that persistently contaminated fomites resulted in transmission of infection in flight attendants over an 8-day period following a single vomiting incident of a passenger with norovirus. Studies have also shown the transfer of noroviruses from cleaning cloths of varying composition and absorbency to surfaces and *vice versa* and also spread of virus from a single fingertip to up to 7 surfaces [15,16] perpetuating the spread of infectious virions.

The use of antimicrobial surfaces in clinical and community environments may help to reduce the spread of infection, especially if combined with rigorous and effective cleaning regimes. Laboratory studies have described the rapid death of bacterial, fungal and viral pathogens on copper alloy surfaces [17–27] and also prevention of antibiotic resistance horizontal gene transfer between pathogens [27]. The results from these studies led to clinical trials worldwide in clinical and children's facilities where a reduction in microbial bioburden was observed in rooms with copper surfaces [28,29]. Of great significance, a recent study of 3 US hospital intensive care units has shown more than a 50% reduction in the infection rate when copper alloys have replaced conventional touch surfaces for 6 highly touched objects (bed rails,

over-bed tables, chair arm, call button, computer accessories and intravenous poles [30]).

Sensitive detection methods for human norovirus are available, primarily PCR amplification of genes encoding viral capsid or viral RNA dependant RNA polymerase (RdRp) from cDNA [31]. However, there is no correlation between these methods and infectivity [32] and there are no available methods to assess viral infectivity, other than human challenge, because of the absence of suitable tissue culture systems [33]. Therefore research has concentrated on feline or murine surrogates. In this study we have investigated the infectivity of murine norovirus (MNV), the closest phylogenetic surrogate to the human virus, exposed to dry touch copper and copper alloy surfaces, containing at least 60% copper, assessed by plaque assay in mouse macrophage monocyte cell line, RAW 264.7 [34,35]. Stainless steel was used as a control surface throughout. We investigated the possible roles of Cu (I) and Cu (II) in viral inactivation and their effect on the integrity of the viral genome following contact of the virus with copper surfaces. The norovirus genome consists of a positive strand RNA of approximately 7.5 kb, and replicates in the host cell cytoplasm. There are 4 open reading frames (ORF); ORF 2 and 3 encode for the capsid proteins and a recently discovered ORF 4 [36] produces a protein that, although not essential for infectivity, affects virulence. The production of sub-genomic strand duplicating ORF 2–4 increases the capacity of the relatively small genome. The ORF1 encodes a polyprotein that is cleaved by viral protease, NS6, into several non-structural proteins. One of these, NS5, encodes for VPg (viral-protein-genome-linked), which is essential for infectivity. It binds to 5′ end of the viral genome acting as a primer initiating translation of viral RNA and also as a protein primer for the viral RdRp [37]. We have observed previously the destruction of bacterial plasmid and genomic nucleic acid on copper and copper alloy dry surfaces. In this study we investigated the effect of norovirus exposure to copper surfaces on the entire genome and as a more sensitive and quantitative assay investigated the effect on a single gene i.e. production of VPg using reverse transcriptase quantitative PCR (RT-qPCR).

Results

Infectivity of murine norovirus (MNV) is destroyed on copper and copper alloy surfaces but not on stainless steel for simulated wet fomite and dry touch contamination

An inoculum of 5×10^4 pfu MNV applied to copper, and high copper content alloys, phosphor bronze and copper nickel, to simulate wet fomite contamination was rapidly inactivated at room temperature using plaque assay. No infectious virus was evident after 30 minutes on copper and 60 minutes on copper nickel (Figure 1A). There was a 2–4 log reduction for phosphor bronze, cartridge brass and nickel silver respectively after 2 hours at room temperature. Increasing the viral load 50×did not affect kill times (data not shown). There was no significant reduction in infectivity following 2 hours contact with stainless steel at room temperature.

Figure 1B demonstrates that virus inactivation is even more rapid if a 'dry' inoculum of virus is used i.e. same size inoculum is applied in very low volume (1 µL) which dries instantly on contact and corresponds to dry touch contamination. All virus is inactivated on copper and copper nickel over the first 5 minutes contact and after 10 and 30 minutes for phosphor bronze and cartridge brass, respectively. Nickel silver, which has the lowest copper content, was inactivated after 2 hours. There was a slight reduction in infectivity on virus exposed to stainless steel, suggesting rapid drying also has an effect.

Calculation of inactivation rate at specific times reveals that the highest rate of MNV inactivation on copper surfaces occurs upon immediate contact (Figure 2). Inactivation was up to 10 times faster in 'dry' touch contamination (Figure 2B); on copper rates were -2.06 and -0.3 for dry and wet contamination, respectively (for the initial timepoints). Inactivation rates were proportional to percentage copper: $R^2 = 0.926$ (Figure S1 Supporting Information) except that copper nickel (89% copper) was slightly more effective than phosphor bronze (95%). This is now being investigated further with a larger range of copper nickels and also investigating if the surface finish affects the virus inactivation rate.

Rate of inactivation of MNV on copper surfaces is affected by temperature

If MNV is inoculated onto surfaces at 4°C inactivation still occurs on copper but at least 4 times more slowly and significant reduction was seen after 2 hours on copper nickel. Little inactivation had occurred on other metals at this time. The inoculum remained wet over the 2 hour testing period (Figure 3A). In contrast at 37°C although for the first 30 minutes of contact there was little reduction of MNV infectivity subsequent inactivation was faster with at least a 3-log reduction on all alloys at 2 hours (Figure 3B). It is unclear if the initial lag is due to any differences between the temperatures of inoculum and the metal. After 2 hours contact with stainless steel at 37°C norovirus was still infectious but there was a considerable reduction in infectivity (2-log) compared to results at room temperature.

Inactivation of MNV on dry copper surfaces involves copper (II) and especially copper (I) ions but not superoxide or hydroxyl radicals ('wet' inoculum)

Addition of D-mannitol or Tiron, at the same time as virus, to quench hydroxyl radicals or superoxide (Figure 4A striped and cross hatch bars respectively) does not protect MNV from inactivation on copper and the virus is inactivated following 60 minutes contact, the same as virus inoculated without quenchers (Figure 4A white bars). Varying the concentration of the quenchers and addition of superoxide dismutase did not affect results (data not shown). There was no significant loss of infectivity for virus inoculated onto stainless steel compared to virus inoculated with or without quenchers with approximately 10^4 pfu recovered after 2 hours contact (Figure 4C).

Addition of EDTA at the same time as the virus to chelate Cu(II) was protective for the initial 60 minutes of virus contact with copper (Figure 4B striped bars) but prolonged protection was observed in the presence of BCS (Figure 4B cross hatch bars). This suggests that Cu (I) is important in the inactivation of MNV on dry copper. Inoculation without chelators present resulted in total inactivation by 60 minutes (Figure 4B white bars). There was no significant loss of infectivity in virus inoculated onto stainless steel compared to virus inoculated with or without chelators (Figure 4D).

Degradation of the entire MNV genome occurs on copper and brass surfaces

PEG concentrated virus was exposed to copper, brass and stainless steel for 2 hours. Virus was removed and total RNA purified and fragments separated by non-denaturing agarose gel electrophoresis (Figure 5). There is a band of native viral RNA from stainless steel that is not visible in samples exposed to

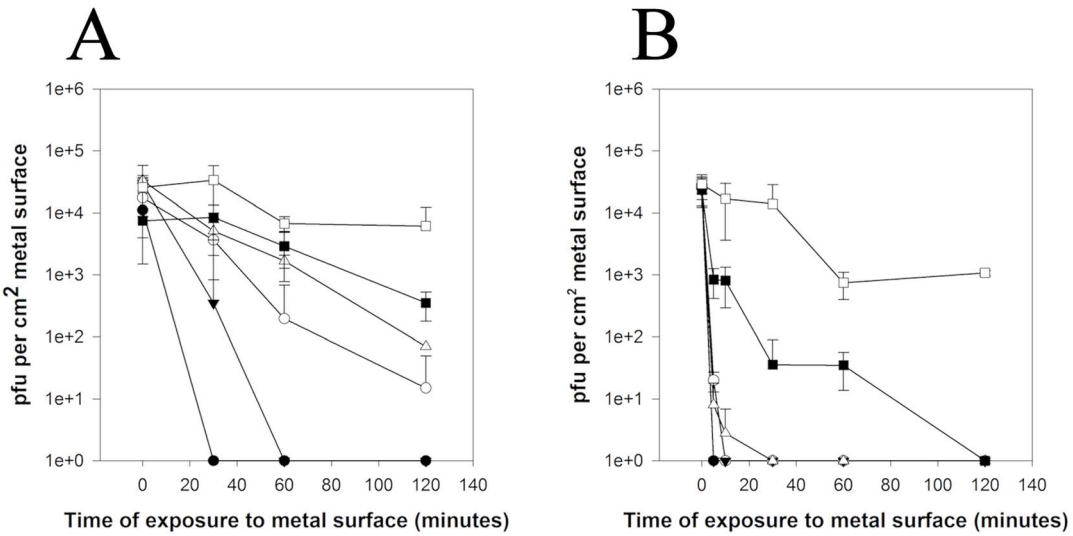

Figure 1. Efficacy of copper alloys to reduce infectivity of wet fomite (A) and dry touch (B) contamination with MNV at room temperature. Plaque assay is described in the text but briefly a dilution series of control and test virus was plated for 60 minutes onto a monolayer of RAW 264.7 cells, then overlaid with agarose and incubated 48–72 hours. Monolayers were stained with vital stain, Neutral Red, and areas of infected and lysed cells can be visualised as plaques and enumerated. Approximately 5×10^4 pfu were applied to test surfaces (copper (●), phosphor bronze (95% copper) (○), copper nickel (89% copper) (▼), cartridge brass (70% copper) (Δ), nickel silver (65% copper) (■), stainless steel (□)) in either 20 μL (dries in 30 minutes) or 1 μL (dries in seconds) to represent wet and dry contamination, respectively. No significant loss of infectivity was observed on stainless steel for both types of inocula. Error bars represent ± SD and data are from multiple experiments.
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copper or cartridge brass. There is some evidence of degraded RNA (less than 300bp) in all three samples. Negative controls were prepared from mock infected cells. MNV used for inoculation but not exposed to metal surfaces was used as a control; purified genomic RNA was analysed by non-denaturing electrophoresis producing band at 2000–2500 bp for native conformation (total size denatured genome is 7382 bp)(Figure S2, Supporting Information)

MNV exposed to copper and brass surfaces has a lower concentration of viral gene, *NS5*, essential for infectivity because of production of VPg (viral-protein-genome-linked)

The genomic RNA of MNV exposed to copper, brass and stainless steel surfaces was purified and cDNA prepared. qPCR amplification of a 70 bp region of VPg demonstrated a reduction

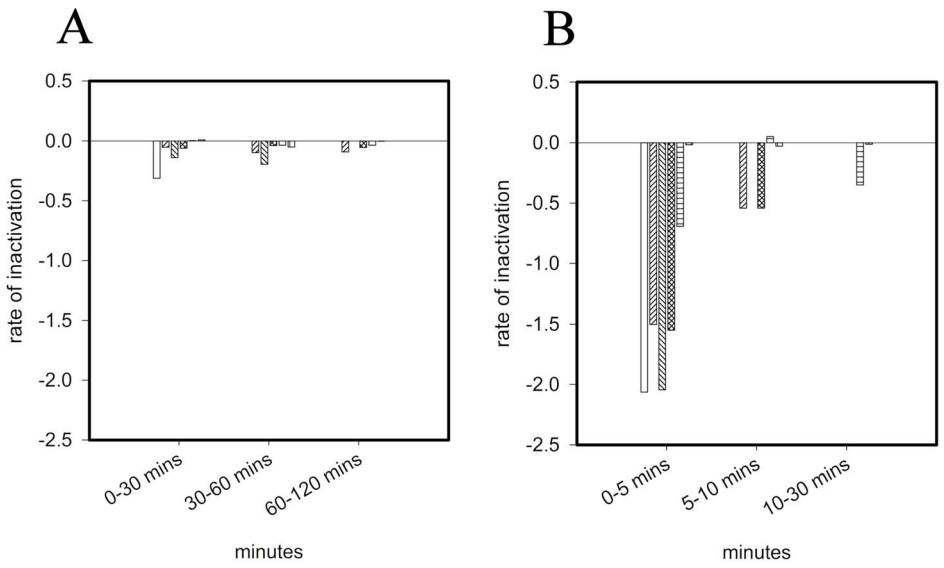

Figure 2. Comparison between inactivation rates of MNV in wet fomite (A) and dry touch (B) contamination on copper surfaces. Inactivation rates were calculated for various contact times of MNV exposed to test surfaces as described in the text (from the results generated in Figure 1). (copper (white bars), phosphor bronze (95% copper) (forward diagonal striped bars), copper nickel (89% copper) (backward diagonal striped bars), cartridge brass (70% copper) (cross hatch bars), nickel silver (65% copper) (horizontal striped bars) and stainless steel (vertical striped bars)). Error bars represent ± SD and data are from multiple experiments.
doi:10.1371/journal.pone.0075017.g002

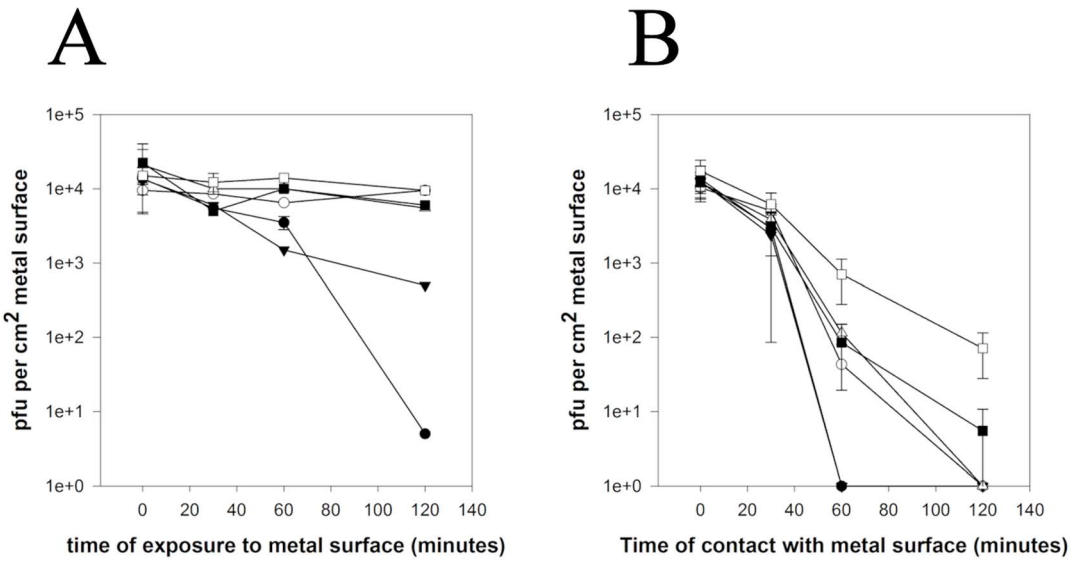

Figure 3. Efficacy of copper alloys to reduce infectivity of wet fomite contamination with MNV at 4°C (A) and 37°C (B). Approximately 5×10^4 pfu were applied to test surfaces that had been acclimatised to required temperature (copper (●), phosphor bronze (95% copper) (○), copper nickel (▼), cartridge brass (70% copper) (Δ), nickel silver (65% copper) (■), stainless steel (□)) in 20 µL ('wet' inoculum). Virus was removed and assessed for infectivity using plaque assay.
doi:10.1371/journal.pone.0075017.g003

in the copy number of virus removed from copper and brass (Figure 6A). Analysis of the PCR products by electrophoresis showed a reduction in intensity of amplified region that is proportional to percentage copper (Figure 6B). Virus that has been removed from stainless steel is similar to equivalent volume of virus not exposed to surfaces. Virus was also removed from all test surfaces immediately (time 0) and there was no significant difference in copy number from the stainless steel 2 hour sample shown (time 0 samples not shown).

Discussion

The human cost and also the economic burden of norovirus infection is a huge problem worldwide. In the UK, norovirus costs the National Health Service at least £100 million/year, in times of high incidence, and up to 3000 people admitted to hospital per year in England. The incidence in the community is thought to be about 16.5% of the 17 million cases of infectious intestinal disease, in England per year and there is evidence that this burden is increasing [38]. In the US the costs escalate to more than $2 billion per year on outbreaks with endemic costs of more than $500 million per year (reviewed in [1]). The low infectious dose, prolonged survival at a range of temperatures and humidity for up to 7 days on dry inanimate surfaces, resistance to commonly used disinfectants, long infectious period and prolonged shedding all increase the risk of disease spread. Approximately 30% of infections from norovirus are asymptomatic and the virus can be transmitted although at a lower frequency compared to symptomatic individuals who can shed up to 10^{10} copies of viral RNA per gram faeces [39]. Lopman, et al. [5] suggested the highest risk in transmission of infectious norovirus is initially, over the first 24 hours, from direct contact but subsequent contamination of the environment produces a risk that lasts much longer, for at least 2 weeks. In addition, direct hand contact or even cleaning cloths used to wipe contaminated surfaces can spread infectious virus to other environmental surfaces. Because norovirus is resistant to many commonly used cleaning agents it is necessary to use 0.1% hypochlorite (equivalent to 1000ppm chlorine) to disinfect surfaces. However, regular use of this biociode bleaches and degrades a range of commonly used touch surface materials and is hazardous in poorly ventilated areas [40].

In this study we have shown that murine norovirus is also rapidly inactivated on copper surfaces. A significant reduction occurred on copper, cartridge brass and nickel silver after 2 hours at room temperature. The rate of inactivation was approximately proportional to copper content but further studies are required to evaluate the effect of different metal surface finishes and the copper ion release rate from individual alloys. In dry touch contamination viral inactivation is extremely rapid, with the highest rate of inactivation occurring in the first 5 minutes. The process appears to be a result of a combination of copper action and drying process followed by a slower rate of inactivation; Li and Dennehy [41] observed that bacteriophages also lost infectivity on copper but as soon as the inoculum dried no further inactivation occurred and Abad et al. [42] observed that the drying process affected persistence of poliovirus and adenovirus on environmental fomites. Sharps et al. [43] observed that virus retained infectivity in transfer 'wet' fomite from stainless steel to fingertips and fruits but transfer was reduced if contaminating inoculum was allowed to dry. We have found that the rate of inactivation is also affected by temperature: inactivation occurs more slowly at 4°C and is faster over a 2 hour period at 37°C. It is unclear if the mechanism of copper inactivation of norovirus is different in wet or dry scenarios. The clear stages of inactivation may reflect populations of virions at various stages of replication (e.g. complete or incomplete capsids and packaging) with varying susceptibilities and rates of inactivation.

We have previously shown that although dry copper surfaces are efficacious against a range of bacteria the copper killing mechanism is different. In Gram-negative cells the outer membrane is the initial target and Fenton reactions between respiration generated oxygen radicals and copper ions results in generation of reactive oxygen species causing the cells to commit 'metabolic suicide' [25]. In this current study we have observed

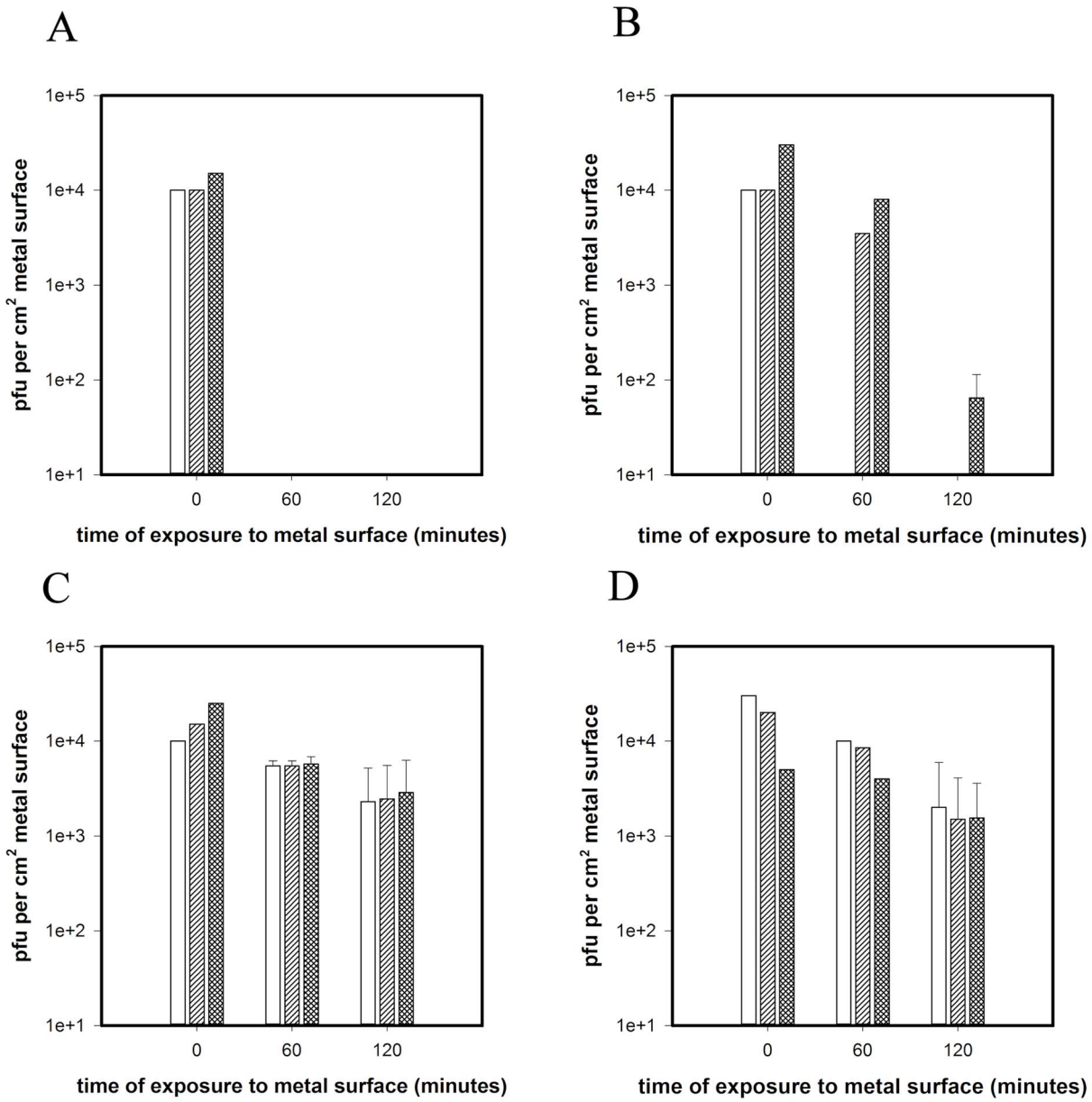

Figure 4. Inactivation of MNV on copper surfaces in the presence of quenchers D-mannitol or Tiron (A) or chelators EDTA or BCS (B) and to remove hydroxyl radical or superoxide, copper II or Cu I, respectively. Approximately 5×10^4 pfu MNV was inoculated onto metal surfaces in the presence of chelators or quenchers of reactive oxygen species and assessed for infectious virus using plaque assay as described in text. The results were compared to those obtained without chelators or quenchers to ascertain if there was a protective effect. No quenchers or chelators present is represented by white bars; D-mannitol (A) or EDTA (B) represented by diagonal striped bars; Tiron (A) or BCS (B) represented by cross hatched bars. No significant reduction of infectivity occurred in the presence of any quenchers or chelators on stainless steel surfaces (even though D-mannitol has been reported to interfere with HSV replication) (C and D respectively). Error bars represent ± SD and data are from multiple experiments.
doi:10.1371/journal.pone.0075017.g004

that Cu(II) was important in the short term but it is Cu(I) that is the primary effector of copper surface inactivation of norovirus. Shionoiri et al. [44] observed Cu(I) was important in inactivation of feline norovirus (FCV), but they investigated copper iodide nanoparticles in solution and Sagripanti et al. [45] discovered that superoxide was not involved in the destruction of the double stranded DNA of Herpes Simplex Virus on copper and only partial protection was seen for hydroxyl radicals. We also found that Cu(I) release on contact surfaces did not result in the generation of hydroxyl radicals or superoxide indicating Fenton chemistry is not important. This suggests that copper ions are having direct effect in virus inactivation. Copper ions have been observed to cause aggregation of virus particles [46].

We investigated if the viral RNA was affected by the copper because virus inactivation is possible without obvious effects on the genome [31]. The entire RNA genome was destroyed on copper suggesting the function of other genes could also be affected so the next step was to investigate if individual genes are affected by more sensitive and quantitative molecular methods. Norovirus replication is rapid and efficient because the positive strand genome does

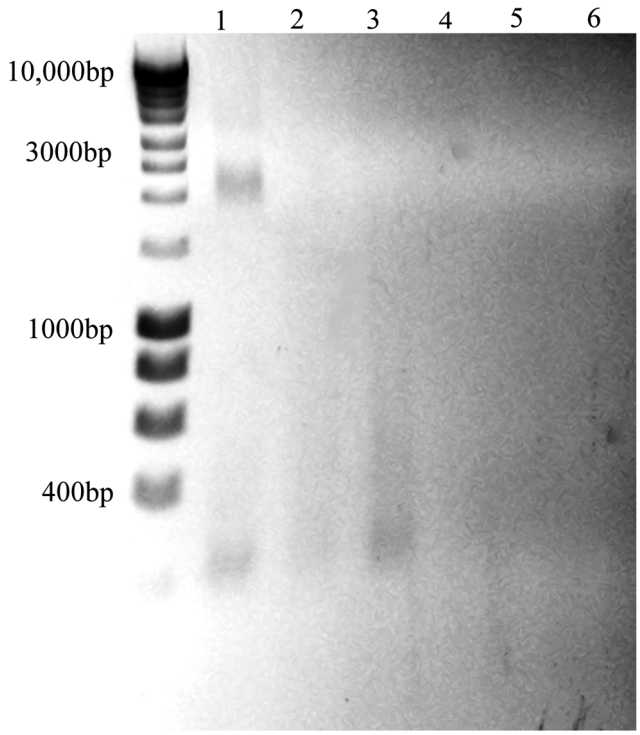

Figure 5. Destruction of entire MNV genome occurs on copper. MNV (PEG concentrate) was exposed to copper (lane 1), cartridge brass (lane 2) or stainless steel (lane 3) for 2 hours. Viral RNA was purified using Qiagen mini prep viral RNA kit and fragments separated on non-denaturing 1% agarose gel electrophoresis and visualised in UV light box. Viral RNA has degraded on copper, less on brass and not at all on stainless steel (see control RNA S2 Supplementary Information). Lanes 4, 5 and 6 are PEG precipitation of uninfected cells (mock) applied to stainless steel, brass and copper respectively. Virus added to all surfaces and removed immediately was similar to lane 1 although some reduction in intensity on copper was visible (not shown). DNA ladder is Bioline hyperladder I (HL1 1 Kb)
doi:10.1371/journal.pone.0075017.g005

not need a DNA stage, produces its own primer in VPg and a viral RNA polymerase and occurs within the cytoplasm without the need to traverse the cell's nuclear membrane. Exposure to copper and brass resulted in a reduction in the copy number of VPg gene that was unaffected on stainless steel. The extent of RNA destruction was proportional to the percentage of copper. Investigations into the effect on other genes and the viral capsid are now underway to determine the sequence of events as norovirus inactivates on copper surfaces and actual targets.

Recombination events resulting from small amino acid substitutions affecting antigenic domains (P2) and in the viral polymerase have led to the evolution of more virulent norovirus strains. GII.g/GII.12 was first isolated in Australia 2008 and results in an increased severity of disease that is not restricted to individuals with specific blood groups, unlike earlier strains, and is capable of zoonotic transmission [1,47]. The survival of infectious norovirus for long periods on surfaces and foods may contribute to interspecies transmission and the evolution of more virulent strains. The destruction of the viral genome we have observed on copper surfaces may mitigate against this as well as prevent the spread of infection.

There is now a considerable body of evidence from laboratory based studies that copper alloys are efficacious against a diverse range of pathogenic microorganisms. Earlier studies demonstrated a rapid kill of *Escherichia coli* O157 [17,19,26], *Listeria monocytogenes* [20] and methicillin-resistant *Staphylococcus aureus* (MRSA) [18] which evolved from commensals into a serious threat to world health. This was followed by observations that both vegetative cells and spores of virulent toxin producing *Clostridium difficile*, responsible for numerous hospital acquired infections (HAI), were also destroyed on copper [22]. The increased antimicrobial therapies required to combat MRSA has resulted in the evolution of potentially more serious multi-drug resistant bacterial pathogens including vancomycin-resistant enterococci and more recently the rise in serious, difficult to treat infections by Gram-negative *Enterobacteriaceae* producing extended spectrum β-lactamases and metallo-β-lactamases, including NDM-1(New Delhi metallo-β-lactamase) which is resistant to all β lactams. This is often located on plasmids containing many other resistance and virulence genes.

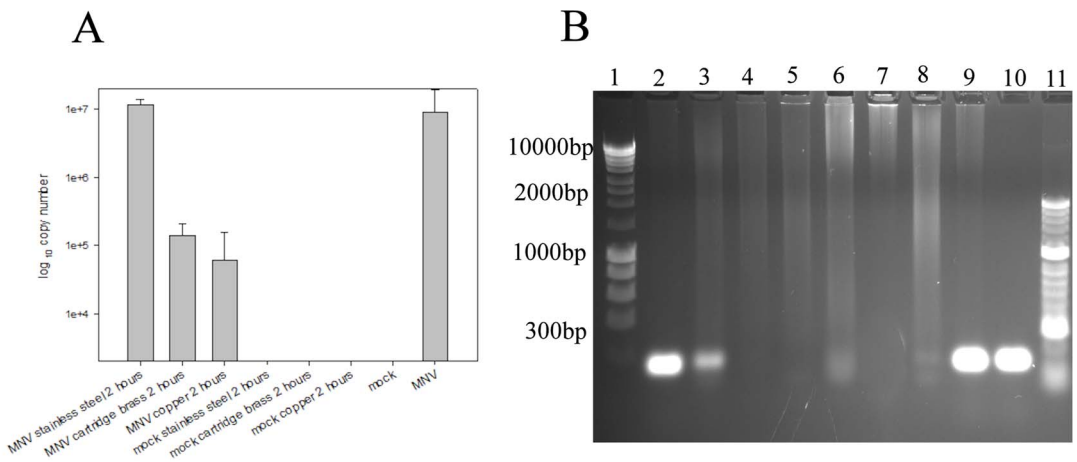

Figure 6. The degradation of viral RNA observed on copper and brass surfaces affects individual genes. cDNA was generated from the RNA of virus recovered from dry surfaces following 2 hours at room temperature. Detection of VPg which is essential for infectivity was performed by qPCR and copy numbers determined from standard curve (A). A large copy number is present in virus removed from stainless steel and untreated virus but greatly reduced from brass and copper. This is also evident from electrophoresis of PCR products (B). Lanes 2–4 is virus removed from stainless steel, brass and copper showing reduction in VPg intensity respectively. Lanes 5–7 are mock infected cells. Lane 8 and lane 9 are mock infected cells and infected cell lysate, respectively, that had not been applied to surfaces. Lane 10 is amplified gene from standard cDNA.
doi:10.1371/journal.pone.0075017.g006

However, all these emerging pathogens are destroyed on copper and copper alloy surfaces although we now know the killing mechanism is not universal [24–27]. We also observed in a previous study the rapid transfer of *bla* $_{NDM-1}$ to other bacterial contaminants on stainless steel surfaces which did not occur on copper [27]. Therefore copper surfaces could also help to prevent horizontal gene transfer (HGT) which is ultimately responsible for the spread in resistance to our existing antibiotics. We have now shown that MNV is also rendered non-infectious on dry copper alloy surfaces and we are currently investigating efficacy against a range of respiratory viral pathogens following an earlier study on influenza A [21].

There have been numerous clinical trials following encouraging results from laboratory studies [28,29]. The recent report by Salgado et al. [30] based on trials at 3 hospitals is extremely encouraging, that replacing only 6 items within a hospital ICU room could have such an impact on reducing the infection rate by more than 50%. This suggests that copper alloy surfaces may also be usefully employed in other high risk areas such as care homes, public transport and even in the home.

The use of copper alloy dry surfaces in health care and community environments could be invaluable in preventing the spread of bacterial, fungal and viral pathogens, including norovirus, that contaminate dry surfaces and perpetuate the infection cycle. The race to develop effective antimicrobials against pathogens that have evolved mechanisms to evade our existing ones is fierce and led to a fear that we are entering a pre-antibiotic era.

Copper alloys, although they provide a constant killing surface, should always be used in conjunction with regular and efficient cleaning and decontamination regimes using non-chelating reagents that could inhibit the copper ion activity.

Materials and Methods

Viral strains and cell lines

Murine norovirus 1, MNV-1, CW1, and the mouse monocyte macrophage line, RAW 264.7, were supplied by Professor Herbert Virgin IV, Washington University, US. The semi-adherent cell line was maintained at sub-confluence to prevent loss of characteristic phenotype and maintained in HEPES buffered Dulbecco's Modified Eagle Medium (DMEM) containing GlutaMAX, 25 mM D-glucose, 10% foetal bovine serum and without sodium pyruvate at 37°C in the presence of 5% CO_2. The cells adhere to tissue culture grade plastic through cation- dependant and independent receptors but can easily be removed by scraping.

To ensure at least 99% cells were infected virus stocks were prepared by infecting cells with multiplicity of infection of approximately 5. The inoculum was removed after 90 minutes incubation at 37°C in the presence of 5% CO_2, replaced with fresh medium and incubated for a further 48 hours or until characteristic cytopathic effect (cpe) was observed. Infected cells were exposed to 3 freeze/thaw cycles, cell debris was removed by low speed centrifugation and supernatant stored at −80°C. Conventional ultrapurification methods may affect structure and infectivity of murine norovirus so a further purification step was performed using polyethylene glycol(PEG) and NaCl precipitation (BioVision Inc, US) which concentrated the sample 100 times. Infected cell supernatants and PEG precipitated virus were used in infectivity assay, Mock infected cells were used as controls

Preparation of sample surfaces

Metal coupons (10×10×0.5 mm) were degreased in acetone, stored in absolute ethanol and flamed prior to use as described previously [24]. The constituents of each metal tested are detailed in Table 1 and all were supplied by the Copper Development Association.

Table 1. Composition of metals used in the study.

Metal type	UNS[a] no.	% composition					
		Cu	Zn	Sn	Ni	Fe	Cr
copper	C11000	100					
phosphor bronze (contains ~ 0.26% P)	C51000	95		5			
copper nickel	C70600	89			10	1	
cartridge brass	C26000	70	30				
nickel silver	C75200	65	17		18		
stainless steel	S30400				8	74	18

[a]Unified Numbering System.

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Inoculation of metal coupons with MNV-1 (to simulate wet fomite or dry touch contamination) and assessment of infectious virus by the detection of cytopathic effect in murine cell line (plaque assay)

The surfaces of coupons were inoculated with MNV−1 5×10^4 plaque forming units (pfu) in 20 µL or 5×10^4 pfu in 1 µL to represent wet fomite (dries in 30–40 minutes at 22°C) or dry touch contamination (dries in seconds), respectively. Drying time was included in the exposure time. For temperatures other than ambient coupons were allowed to acclimatise for 30 minutes prior to inoculation. Some modifications were made to the method previously described for removing bacteria from coupons [24]. Viruses were removed from the coupons at the required timepoint by vortexing for 15 s (half the time for bacteria to reduce frothing) in 5 ml complete DMEM with approximately 100×2 mm diameter glass beads (twice the number used for bacteria). A range of dilutions was prepared immediately in complete DMEM and 1 mL aliquots were plated onto monolayers of RAW 264.7 that had been seeded with 10^6 cells per well of 6 well plates (diameter 3.5 cm) 3 hours previously, and incubated at 37°C and 5% CO_2 for 90 minutes. The inoculum was aspirated and overlay of 3 mL per well of 3% low melting point (LMP) agarose in complete medium was added to prevent virus spreading to other cells. Plates were incubated for 15 minutes at 4°C until set and then at 37°C, 5% CO_2 for 72 hours. Monolayers were stained with 2 mL per well of a filtered 0.01% solution of the supravital stain, Neutral Red, which is pinocytosed by viable cells and accumulates in the cell lysosomes staining the cells red, in PBS for 2 hours at 37°C and 5% CO_2. Excess stain was removed and the plates re-incubated for a further hour. Concentrations of stain > 100 µg/mL can be cytotoxic and should not be used. Plates were stored overnight at 4°C to increase definition of plaques which were counted and used to calculate pfu recovered per coupon.

The rate of virus inactivation was calculated for the following time periods: 0–5, 5–10 and 10–30 minutes for 'dry' inoculum and 0–30, 30–60 and 60–120 minutes for the 'wet' inoculum according to the following formula:

$$K = (ln\ N(t)/N(0))/T$$

$$K = rate\ of\ inactivation$$

$$N(t) = pfu\ per\ coupon\ at\ end\ of\ selected\ time$$

$$N(0) = pfu\ per\ coupon\ at\ start\ of\ selected\ time\ period$$

$$T = length\ of\ selected\ time\ period\ i.e.\ rates\ expressed\ per\ minute$$

The effect of copper chelators and reactive oxygen species quenchers in infectivity of MNV exposed to copper and copper alloy surfaces

Incorporation of chelators ethylenediaminetetraacetic acid (EDTA) (20 mM) and bathocuproine disulfonic acid (BCS) (20 mM) to chelate Cu(II) or Cu(I), respectively, at the time of inoculation of virus to the metal surfaces was investigated using plaque assay. In addition 20 mM D-mannitol and 20 mM 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron) were used to quench hydroxyl radicals and superoxide, respectively. Stainless steel was used as a control surface and to determine if quenchers and chelators affect viral replication.

Survival of infectivity of MNV on metal surfaces at 37°C and 4°C

Metal surfaces were allowed to acclimatise to the test temperature for 30 minutes prior to inoculation with virus. Virus was removed from coupons and assessed for infectivity as described.

Purification of viral RNA and analysis of integrity by agarose gel electrophoresis

The total RNA of untreated virus or virus exposed to metal surfaces (5 coupons per test, virus removed from coupons by pipetting up and down in a small volume, 100 µL) was extracted using the Qiagen QIAamp viral RNA mini kit according to manufacturer's instructions and using carrier RNA provided to prevent degradation.

Purified RNA fragments were separated on a non-denaturing 1% agarose gel using GelRed Nucleic Acid Prestaining Kit (Biotium, UK) according to the manufacturer's instructions. The staining intensity is reduced because GelRed binds to ssRNA approximately half as much as double stranded nucleic acid. DNA ladders were supplied by Bioline. Gels were observed and photographed using GeneSnap software and a Syngene UV light box.

Detection and quantification of VPg in MNV exposed to copper and brass surfaces

cDNA was prepared from the purified viral RNA (RT-nanoscript) PrimerDesign, UK). Primers were designed to amplify a 70 bp region of VPg in ORF-1 from complete genome of MNV-1 CW1 (accession number DQ285629) (PrimerDesign Ltd., Southampton, UK)

sense primer GCGAGCGAGAAGAAGAACT (position 2761)

antisense primerTTCAACCCGAAGCCATCC (position 2380).

Amplification was performed on a BioRad iQ5 cycler and standard curves prepared from known copy number standards to determine copy number in test samples. A synthesised VPg cDNA was used to prepare standard curve and calculate copy number in equivalent volumes virus suspension applied to test surface samples. PCR products were analysed by gel electrophoresis as described.

Statistical analysis

Data are expressed as mean ± standard errors of the mean (SEM) and are from multiple independent experiments. 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 and graphical representations were performed using Sigma Plot version 12.

Supporting Information

Figure S1 Linear regression analysis of virus inactivation rate (0–30 minutes, 'wet' inoculum) and percentage copper in alloys tested resulted in a coefficient of determination (R^2) of 0.926 suggesting a good correlation. The result for phosphor bronze was removed from this analysis. (including phosphor bronze reduced the R^2 to 0.541). Further investigations into the efficacy of phosphor bronze to inactivate norovirus are planned including determining the influence of different metal surface finishes and other metal constituents.
(TIF)

Figure S2 The entire RNA genome of untreated MNV (PEG concentrate) was purified as described in the text and fragments separated by electrophoresis on a non-denaturing 1% agarose gel (lane 2). Lane 3 shows 18 s and 28 s cellular RNA from uninfected RAW 264.7 cells and lanes 1 and 4 are Bioline Hyperladders I and II, respectively.
(TIF)

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Author Contributions

Conceived and designed the experiments: SLW CWK. Performed the experiments: SLW. Analyzed the data: SLW CWK. Wrote the paper: SLW.

References

1. Donaldson EF, Lindesmith LC, Lobue AD, Baric RS (2010) Viral shape-shifting: norovirus evasion of the human immune system. Nat Rev Microbiol 8: 231–241.
2. Griffiths PD (2012) Norovirus: tribulation and possible trial. Rev Med Virol 22: 67–68.

3. Weber DJ, Rutala WA, Miller MB, Huslage K, Sickbert-Bennett E (2010) Role of hospital surfaces in the transmission of emerging health care-associated pathogens: norovirus, *Clostridium difficile*, and *Acinetobacter* species. Am J Infect Control 38: S25–S33.
4. Kawada J, Arai N, Nishimura N, Suzuki M, Ohta R, et al. (2012) Clinical characteristics of norovirus gastroenteritis among hospitalized children in Japan. Microbiol Immunol 56: 756–759.
5. Lopman B, Gastanaduy P, Park GW, Hall AJ, Parashar UD, et al. (2012) Environmental transmission of norovirus gastroenteritis. Curr Opin Virol 2: 96–102.
6. Rodriguez-Lazaro D, Cook N, Ruggeri FM, Sellwood J, Nasser A, et al. (2012) Virus hazards from food, water and other contaminated environments. FEMS Microbiol Rev 36: 786–814.
7. Beersma MF, Sukhrie FH, Bogerman J, Verhoef L, Mde MM, et al. (2012) Unrecognized norovirus infections in health care institutions and their clinical impact. J Clin Microbiol 50: 3040–3045.
8. Turcios-Ruiz RM, Axelrod P, St John K, Bullitt E, Donahue J, et al. (2008) Outbreak of necrotizing enterocolitis caused by norovirus in a neonatal intensive care unit. J Pediatr 153: 339–344.
9. Takahashi H, Ohuchi A, Miya S, Izawa Y, Kimura B (2011) Effect of food residues on norovirus survival on stainless steel surfaces. PLoS One 6: e21951. 10.1371/journal.pone.0021951 [doi]
10. Clay S, Maherchandani S, Malik YS, Goyal SM (2006) Survival on uncommon fomites of feline calicivirus, a surrogate of noroviruses. Am J Infect Control 34: 41–43.
11. D'Souza DH, Sair A, Williams K, Papafragkou E, Jean J, et al. (2006) Persistence of caliciviruses on environmental surfaces and their transfer to food. Int J Food Microbiol 108: 84–91.
12. Seitz SR, Leon JS, Schwab KJ, Lyon GM, Dowd M, et al. (2011) Norovirus infectivity in humans and persistence in water. Appl Environ Microbiol 77: 6884–6888.
13. Gerhardts A, Hammer TR, Balluff C, Mucha H, Hoefer D (2012) A model of the transmission of micro-organisms in a public setting and its correlation to pathogen infection risks. J Appl Microbiol 112: 614–621.
14. Thornley CN, Emslie NA, Sprott TW, Greening GE, Rapana JP (2011) Recurring norovirus transmission on an airplane. Clin Infect Dis 53: 515–520.
15. Barker J, Vipond IB, Bloomfield SF (2004) Effects of cleaning and disinfection in reducing the spread of Norovirus contamination via environmental surfaces. J Hosp Infect 58: 42–49.
16. Gibson KE, Crandall PG, Ricke SC (2012) Removal and transfer of viruses on food contact surfaces by cleaning cloths. Appl Environ Microbiol 78: 3037–3044.
17. Wilks SA, Michels H, Keevil CW (2005) The survival of *Escherichia coli* O157 on a range of metal surfaces. Int J Food Microbiol 105: 445–454.
18. Noyce JO, Michels H, Keevil CW (2006) Potential use of copper surfaces to reduce survival of epidemic meticillin-resistant *Staphylococcus aureus* in the healthcare environment. J Hosp Infect 63: 289–297.
19. Noyce JO, Michels H, Keevil CW (2006) Use of copper cast alloys to control *Escherichia coli* O157 cross-contamination during food processing. Appl Environ Microbiol 72: 4239–4244.
20. Wilks SA, Michels HT, Keevil CW (2006) Survival of *Listeria monocytogenes* Scott A on metal surfaces: implications for cross-contamination. Int J Food Microbiol 111: 93–98.
21. Noyce JO, Michels H, Keevil CW (2007) Inactivation of influenza A virus on copper versus stainless steel surfaces. Appl Environ Microbiol 73: 2748–2750.
22. Weaver L, Michels HT, Keevil CW (2008) Survival of *Clostridium difficile* on copper and steel: futuristic options for hospital hygiene. J Hosp Infect 68: 145–151.
23. Weaver L, Michels HT, Keevil CW (2010) Potential for preventing spread of fungi in air-conditioning systems constructed using copper instead of aluminium. Lett Appl Microbiol 50: 18–23.
24. Warnes SL, Green SM, Michels HT, Keevil CW (2010) Biocidal efficacy of copper alloys against pathogenic enterococci involves degradation of genomic and plasmid DNAs. Appl Environ Microbiol 76: 5390–5401
25. Warnes SL, Keevil CW (2011) Mechanism of copper surface toxicity in vancomycin-resistant enterococci following wet or dry surface contact. Appl Environ Microbiol 77: 6049–6059.
26. 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.
27. Warnes SL, Highmore CJ, Keevil CW (2012) Horizontal transfer of antibiotic resistance genes on abiotic touch surfaces: implications for public health. MBio 3. mBio.00489-12 [pii];10.1128/mBio.00489-12 [doi].
28. Casey AL, Adams D, Karpanen TJ, Lambert PA, Cookson BD, et al. (2010) Role of copper in reducing hospital environment contamination. J Hosp Infect 74: 72–77.
29. Schmidt MG, Attaway HH, Sharpe PA, John J, Jr., Sepkowitz KA, et al. (2012) Sustained reduction of microbial burden on common hospital surfaces through introduction of copper. J Clin Microbiol 50: 2217–2223.
30. Salgado CD, Sepkowitz KA, John JF, Cantey JR, Attaway HH, et al. (2013) Copper surfaces reduce the rate of healthcare-acquired infections in the intensive care unit. Infect Control Hosp Epidemiol 34: 479–486.
31. Hamza IA, Jurzik L, Uberla K, Wilhelm M (2011) Methods to detect infectious human enteric viruses in environmental water samples. Int J Hyg Environ Health 214: 424–436.
32. Baert L, Wobus CE, Van CE, Thackray LB, Debevere J, et al. (2008) Detection of murine norovirus 1 by using plaque assay, transfection assay, and real-time reverse transcription-PCR before and after heat exposure. Appl Environ Microbiol 74: 543–546.
33. Duizer E, Schwab KJ, Neill FH, Atmar RL, Koopmans MP, et al. (2004) Laboratory efforts to cultivate noroviruses. J Gen Virol 85: 79–87.
34. Wobus CE, Karst SM, Thackray LB, Chang KO, Sosnovtsev SV, et al. (2004) Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages. PLoS Biol 2: e432. 10.1371/journal.pbio.0020432 [doi].
35. Wobus CE, Thackray LB, Virgin HW (2006) Murine norovirus: a model system to study norovirus biology and pathogenesis. J Virol 80: 5104–5112.
36. McFadden N, Bailey D, Carrara G, Benson A, Chaudhry Y, et al. (2011) Norovirus regulation of the innate immune response and apoptosis occurs via the product of the alternative open reading frame 4. PLoS Pathog 7: e1002413. 10.1371/journal.ppat.1002413 [doi]
37. Goodfellow I (2011) The genome-linked protein VPg of vertebrate viruses - a multifaceted protein. Curr Opin Virol 1: 355–362.
38. Tam CC, Rodrigues LC, Viviani L, Dodds JP, Evans MR, et al. (2012) Longitudinal study of infectious intestinal disease in the UK (IID2 study): incidence in the community and presenting to general practice. Gut 61: 69–77.
39. Lee N, Chan MC, Wong B, Choi KW, Sin W, et al. (2007) Fecal viral concentration and diarrhea in norovirus gastroenteritis. Emerg Infect Dis 13: 1399–1401.
40. Norovirus Working Party (Health Protection Agency (now Public Health England); British Infection Association; Healthcare Infection Society; Infection Prevention Society; National Concern for Healthcare Infections; NHS Confederation) March 2012 (to be reviewed December 2016) Guidelines for the management of norovirus outbreaks in acute and community health and social care settings http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1317131639453
41. Li J, Dennehy JJ (2011) Differential bacteriophage mortality on exposure to copper. Appl Environ Microbiol 77: 6878–6883.
42. Abad FX, Pinto RM, Bosch A (1994) Survival of enteric viruses on environmental fomites. Appl Environ Microbiol 60: 3704–3710.
43. Sharps CP, Kotwal G, Cannon JL (2012) Human norovirus transfer to stainless steel and small fruits during handling. J Food Prot 75: 1437–1446.
44. Shionoiri N, Sato T, Fujimori Y, Nakayama T, Nemoto M, et al. (2012) Investigation of the antiviral properties of copper iodide nanoparticles against feline calicivirus. J Biosci Bioeng 113: 580–586.
45. Sagripanti JL, Routson LB, Bonifacino AC, Lytle CD (1997) Mechanism of copper-mediated inactivation of herpes simplex virus. Antimicrob Agents Chemother 41: 812–817.
46. Abad FX, Pinto RM, Diez JM, Bosch A (1994) Disinfection of human enteric viruses in water by copper and silver in combination with low levels of chlorine. Appl Environ Microbiol 60: 2377–2383.
47. Giammanco GM, Rotolo V, Medici MC, Tummolo F, Bonura F, et al. (2012) Recombinant norovirus GII.g/GII.12 gastroenteritis in children. Infect Genet Evol 12: 169–174.

Inactivation of Norovirus on Dry Copper Alloy Surfaces

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Supplementary data

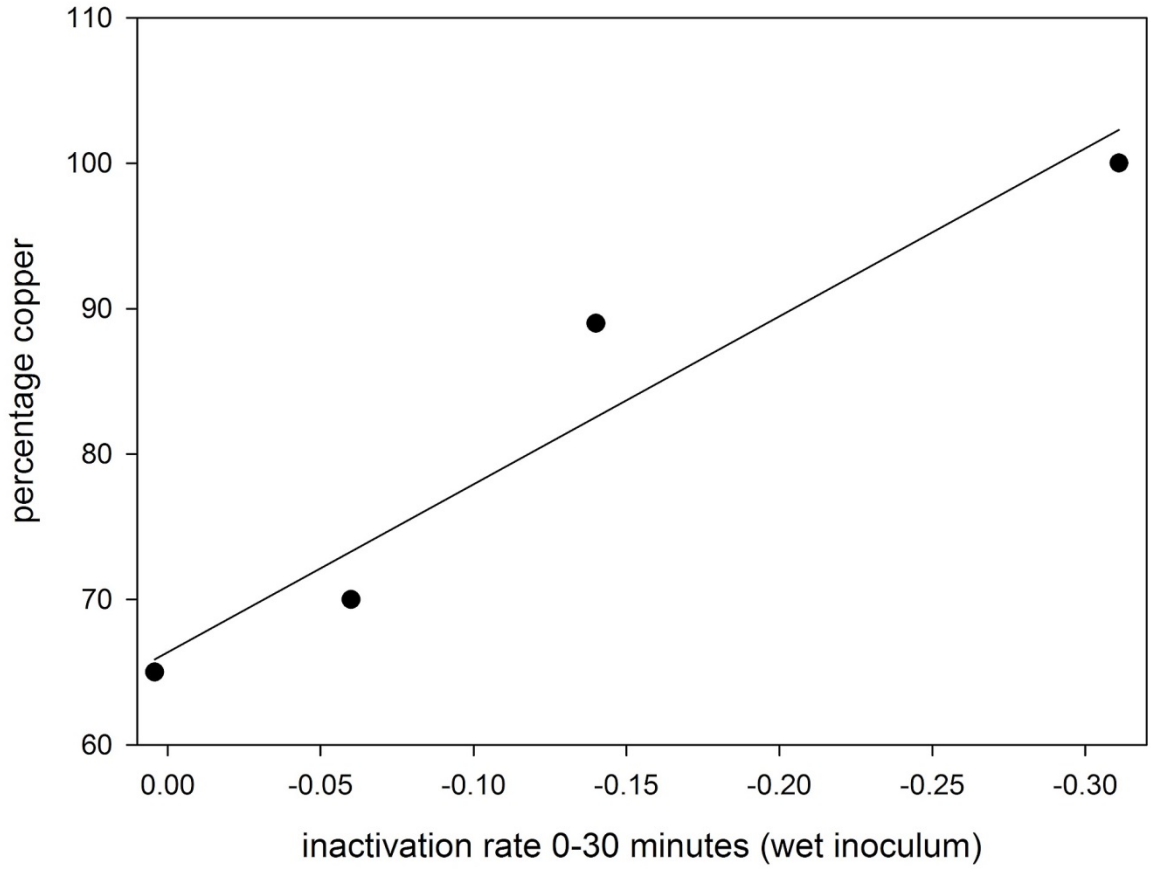

Figure S1
Linear regression analysis of virus inactivation rate (0–30 minutes, 'wet' inoculum) and percentage copper in alloys tested resulted in a coefficient of determination (R^2) of 0.926 suggesting a good correlation. The result for phosphor bronze was removed from this analysis. (including phosphor bronze reduced the R^2 to 0.541). Further investigations into the efficacy of phosphor bronze to inactivate norovirus are planned including determining the influence of different metal surface finishes and other metal constituents.

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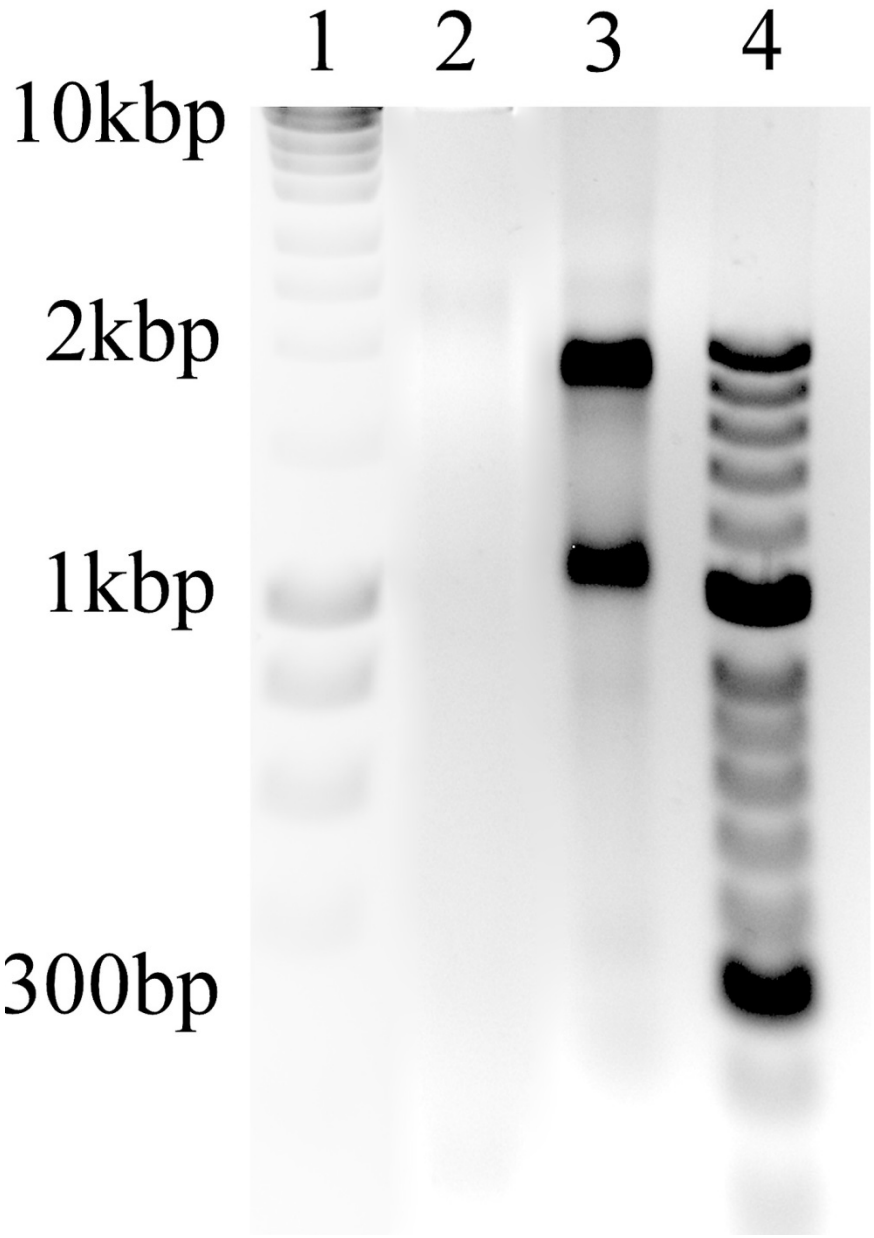

Figure S2

The entire RNA genome of untreated MNV (PEG concentrate) was purified as described in the text and fragments separated by electrophoresis on a non-denaturing 1% agarose gel (lane 2). Lane 3 shows 18 s and 28 s cellular RNA from uninfected RAW 264.7 cells and lanes 1 and 4 are Bioline Hyperladders I and II, respectively.

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(TIF)

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PLOS ONE

Correction

Correction: Inactivation of Norovirus on Dry Copper Alloy Surfaces

The *PLOS ONE* Staff

There is an error in the primer position under the sub-heading "Detection and quantification of VPg in MNV exposed to copper and brass surfaces" in the "Materials and Methods" section. The correct antisense primer position is TTCAACCCGAAGC-CATCC (position 2830).

In addition, lanes 1 and 3 are labeled incorrectly in the legend for Figure 5. Please view the correct legend and figure here:

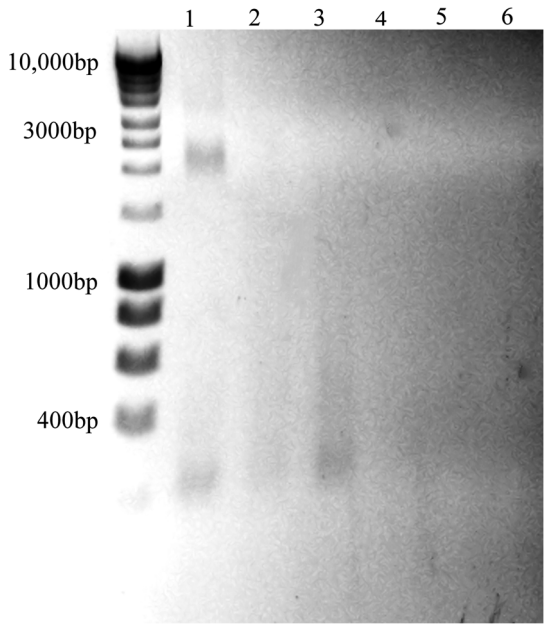

Figure 5. Destruction of entire MNV genome occurs on copper. MNV (PEG concentrate) was exposed to copper (lane 3), cartridge brass (lane 2) or stainless steel (lane 1) for 2 hours. Viral RNA was purified using Qiagen mini prep viral RNA kit and fragments separated on non-denaturing 1% agarose gel electrophoresis and visualised in UV light box. Viral RNA has degraded on copper, less on brass and not at all on stainless steel (see control RNA S2 Supplementary Information). Lanes 4, 5 and 6 are PEG precipitation of uninfected cells (mock) applied to stainless steel, brass and copper respectively. Virus added to all surfaces and removed immediately was similar to lane 1 although some reduction in intensity on copper was visible (not shown). DNA ladder is Bioline hyperladder I (HL1 1 Kb)
doi:10.1371/journal.pone.0075017.g005

Reference

1. Warnes SL, Keevil CW (2013) Inactivation of Norovirus on Dry Copper Alloy Surfaces. PLoS ONE 8(9): e75017. doi:10.1371/journal.pone.0075017

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References

1. Pecanac M, Janjic Z, Komarcevic A, Pajic M, Dobanovacki D, Miskovic SS (2013) Burns treatment in ancient times. Med Pregl **66**: 263-267

2. Hippocrates, Volume IX: Coan prenotions.Anatomical and Minor Clinical writings. Loeb Classical library 509 (Paul Potter, translator) ISBN 06749996402

3. Antibiotic Resistance Threats in the United States, 2013. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, CDC stacks http://stacks.cdc.gov/view/cdc/20705. Available at http://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf

4. Healthcare-associated Infection and Antimicrobial resistance: 2010/11. Health Protection Agency (now Public Health England) http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1317136146912

5. Rodriguez-Rojas A, Rodriguez-Beltran J, Couce A, Blazquez J (2013) Antibiotics and antibiotic resistance: a bitter fight against evolution. Int J Med Microbiol **303**: 293-297. S1438-4221(13)00019-2 [pii];10.1016/j.ijmm.2013.02.004 [doi].

6. Gootz TD (2010) The global problem of antibiotic resistance. Crit Rev Immunol **30**: 79-93. 42750210589e65a5,574f265f0176fca5 [pii].

7. Livermore DM (2012) Fourteen years in resistance. Int J Antimicrob Agents **39**: 283-294. S0924-8579(12)00044-1 [pii];10.1016/j.ijantimicag.2011.12.012 [doi].

8. Otto M (2012) MRSA virulence and spread. Cell Microbiol **14**: 1513-1521. 10.1111/j.1462-5822.2012.01832.x [doi].

9. Seiffert SN, Hilty M, Perreten V, Endimiani A (2013) Extended-spectrum cephalosporin-resistant Gram-negative organisms in livestock: an emerging problem for human health? Drug Resist Updat **16**: 22-45. S1368-7646(13)00002-2 [pii];10.1016/j.drup.2012.12.001 [doi].

10. Hall-Stoodley L, Stoodley P (2009) Evolving concepts in biofilm infections. Cell Microbiol **11**: 1034-1043. CMI1323 [pii];10.1111/j.1462-5822.2009.01323.x [doi].

11. Donlan RM (2002) Biofilms: microbial life on surfaces. Emerg Infect Dis **8**: 881-890. 10.3201/eid0809.020063 [doi].

12. Potera C (1999) Forging a link between biofilms and disease. Science **283**: 1837, 1839.

13. Keevil CW (2004) The physico-chemistry of biofilm-mediated pitting corrosion of copper pipe supplying potable water. Water Sci Technol **49**: 91-98.

14. Lu J, Buse HY, Gomez-Alvarez V, Struewing I, Santo DJ, Ashbolt NJ (2014) Impact of drinking water conditions and copper materials on downstream biofilm microbial communities and *Legionella pneumophila* colonization. J Appl Microbiol . 10.1111/jam.12578 [doi].

15. Otter JA, Yezli S, Salkeld JA, French GL (2013) Evidence that contaminated surfaces contribute to the transmission of hospital pathogens and an overview of strategies to address contaminated surfaces in hospital settings. Am J Infect Control **41**: S6-11. S0196-6553(13)00004-7 [pii];10.1016/j.ajic.2012.12.004 [doi].

16. Wellington EM, Boxall AB, Cross P, Feil EJ, Gaze WH, Hawkey PM, Johnson-Rollings AS, Jones DL, Lee NM, Otten W, Thomas CM, Williams AP (2013) The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. Lancet Infect Dis **13**: 155-165. S1473-3099(12)70317-1 [pii];10.1016/S1473-3099(12)70317-1 [doi].

17. Perry JA, Wright GD (2013) The antibiotic resistance "mobilome": searching for the link between environment and clinic. Front Microbiol **4**: 138. 10.3389/fmicb.2013.00138 [doi].

18. Weber DJ, Anderson D, Rutala WA (2013) The role of the surface environment in healthcare-associated infections. Curr Opin Infect Dis **26**: 338-344. 10.1097/QCO.0b013e3283630f04 [doi].

19. Weber DJ, Anderson DJ, Sexton DJ, Rutala WA (2013) Role of the environment in the transmission of *Clostridium difficile* in health care facilities. Am J Infect Control **41**: S105-S110. S0196-6553(13)00020-5 [pii];10.1016/j.ajic.2012.12.009 [doi].

20. Weber DJ, Rutala WA (2013) Self-disinfecting surfaces: review of current methodologies and future prospects. Am J Infect Control **41**: S31-S35. S0196-6553(13)00011-4 [pii];10.1016/j.ajic.2012.12.005 [doi].

21. Juhas M (2013) Horizontal gene transfer in human pathogens. Crit Rev Microbiol . 10.3109/1040841X.2013.804031 [doi].

22. Lindsay JA (2014) *Staphylococcus aureus* genomics and the impact of horizontal gene transfer. Int J Med Microbiol **304**: 103-109. S1438-4221(13)00183-5 [pii];10.1016/j.ijmm.2013.11.010 [doi].

23. Brinton CC, Jr., Gemski P, Jr., Carnahan J (1964) A new type of bacterial pilus genetically controlled by the fertility factor of *E. coli* K 12 and its role in chromosome transfer. Proc Natl Acad Sci U S A **52**: 776-783.

24. Microbiology, Fifth Edition (2002), Editors L. M. Prescott, J. P. Harley and D. A. Klein, publishers McGraw-Hill Higher Education (ISBN 0-07-112259-1)

25. Stevenson J, Barwinska-Sendra A, Tarrant E, Waldron KJ (2013). Microbial pathogens and strategies for combating them: science, technology and education. Book series ISBN-13 Vol.1: 978-84-939843-9-7

26. Lemire JA, Harrison JJ, Turner RJ (2013) Antimicrobial activity of metals: mechanisms, molecular targets and applications. Nat Rev Microbiol **11**: 371-384. nrmicro3028 [pii];10.1038/nrmicro3028 [doi].

27. Grass G, Rensing C (2001) Genes involved in copper homeostasis in *Escherichia coli*. J Bacteriol **183**: 2145-2147. 10.1128/JB.183.6.2145-2147.2001 [doi].

28. Solioz M, Abicht HK, Mermod M, Mancini S (2010) Response of gram-positive bacteria to copper stress. J Biol Inorg Chem **15**: 3-14. 10.1007/s00775-009-0588-3 [doi].

29. Rensing C, Grass G (2003) Escherichia coli mechanisms of copper homeostasis in a changing environment. FEMS Microbiol Rev **27**: 197-213. S0168644503000494 [pii].

30. Fu Y, Tsui HC, Bruce KE, Sham LT, Higgins KA, Lisher JP, Kazmierczak KM, Maroney MJ, Dann CE, III, Winkler ME, Giedroc DP (2013) A new structural paradigm in copper resistance in *Streptococcus pneumoniae*. Nat Chem Biol **9**: 177-183. nchembio.1168 [pii];10.1038/nchembio.1168 [doi]

31. Kuhn, PJ (1983) Doorknobs: a source of nosocomial infection? Diagnostic Medicine November/December: 62-63

32. Wilks, S. A., H. Michels, and C. W. Keevil. 2005. The survival of *Escherichia coli* O157 on a range of metal surfaces. Int. J. Food Microbiol. **105**: 445-454.

33. Wilks, S. A., H. T. Michels, and C. W. Keevil. 2006. Survival of *Listeria monocytogenes* Scott A on metal surfaces: Implications for cross-contamination. Int. J. Food Microbiol.**111**: 93-98.

34. Noyce, J. O., H. Michels, and C. W. Keevil. 2006. Potential use of copper surfaces to reduce survival of epidemic methicillin-resistant *Staphylococcus aureus* in the healthcare environment. J. Hosp. Infect. **63**: 289-297.

35. Weaver, L., Michels, H. T., Keevil, C. W. 2008. Survival of *Clostridium difficile* on copper and steel. J. Hosp. Infect. **68**: 145-151.

36. Wheeldon, L. J., Worthington, T., Lambert, P. A., Hilton, A. C., Lowden, C. J. and T. S. Elliot 2008. Antimicrobial efficacy of copper surfaces against spores and vegetative cells of *Clostridium difficile*: the germination theory. J. Antimicrob. Chemother. **62**: 522-5.

37. Mehtar, S., Wiid, I. and S. D. Todorov. 2008. The antimicrobial activity of copper and copper alloys against nosocomial pathogens and *Mycobacterium tuberculosis* isolated from healthcare studies in the Western Cape: an *in vitro* study. J. Hosp. Infect. **68**:45-51.

38. Weaver, L., Michels, H. T. and C. W. Keevil. 2009. Potential for preventing spread of fungi in air conditioning systems constructed using copper instead of aluminium. Lett. Appl. Microbiol. **50**: 18-23.

39. Noyce, J. O., H. Michels, and C. W. Keevil. 2007. Inactivation of influenza A virus on copper versus stainless steel surfaces. Appl. Environ. Microbiol. **73**: 2748-2750.

40. Espirito SC, 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. AEM.01938-07 [pii];10.1128/AEM.01938-07 [doi].

41. Arias CA, Murray BE (2012) The rise of the Enterococcus: beyond vancomycin resistance. Nat Rev Microbiol **10**: 266-278. nrmicro2761 [pii];10.1038/nrmicro2761 [doi].

42. Murray BE (2000) Vancomycin-resistant enterococcal infections N Engl J Med **342**: 710-721 DOI: 10.1056/NEJM200003093421007

43. Rice, L. B. 2007. Emerging issues in the management of infections caused by multidrug-resistant Gram-negative bacteria. Cleveland Clinic Journal of Medicine **74**: S12-20

44. Woodford N, Wareham DW, Guerra B, Teale C (2013) Carbapenemase-producing *Enterobacteriaceae* and non-*Enterobacteriaceae* from animals and the environment: an emerging public health risk of our own making? J Antimicrob Chemother . dkt392 [pii];10.1093/jac/dkt392 [doi].

45. Woodford, N., J. F. Turton, and D. M. Livermore. 2011. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol.Rev. **35**:736-755.

46. Bushnell G, Mitrani-Gold F, Mundy LM (2013) Emergence of New Delhi metallo-beta-lactamase type 1-producing *Enterobacteriaceae* and non-*Enterobacteriaceae*: global case detection and bacterial surveillance. Int J Infect Dis **17**: e325-e333. S1201-9712(12)01318-5 [pii];10.1016/j.ijid.2012.11.025 [doi].

47. Johnson AP, Woodford N (2013) Global spread of antibiotic resistance: the example of New Delhi metallo-beta-lactamase (NDM)-mediated carbapenem resistance. J Med Microbiol **62**: 499-513. jmm.0.052555-0 [pii];10.1099/jmm.0.052555-0 [doi].

48. Goren, M. G., Y. Carmeli, M. J. Schwaber, I. Chmelnitsky, V. Schechner, and S. Navon-Venezia. 2010. Transfer of carbapenem-resistant plasmid from *Klebsiella pneumoniae* ST258 to Escherichia coli in patient. Emerg.Infect.Dis. **16**:1014-1017

49. Kruse, H. and H. Sørum.1994. Transfer of drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Appl. Environ. Microbiol. **60**: 4015-4021

50. Griffiths PD (2012) Norovirus: tribulation and possible trial. Rev Med Virol **22**: 67-68. 10.1002/rmv.1705 [doi].

51. Wobus CE, Thackray LB, Virgin HW (2006) Murine norovirus: a model system to study norovirus biology and pathogenesis. J Virol **80**: 5104-5112. 80/11/5104 [pii];10.1128/JVI.02346-05 [doi].

52. Stohs, S.J. and D. Bagghi. 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radic. Biol. Med. **18**: 321-336

53. Swoboda JG, Campbell J, Meredith TC, Walker S (2010) Wall teichoic acid function, biosynthesis, and inhibition. Chembiochem **11**: 35-45. 10.1002/cbic.200900557 [doi].

54. Breeuwer, P, Abee, T (2004) Assessment of membrane potential, intracellular pH and respiration of bacteria employing fluorescent techniques. Mol Microb Ecol Man **8**:1563-1580

55. Berney M, Hammes F, Bosshard F, Weilenmann HU, Egli T (2007) Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry. Appl Environ Microbiol **73**: 3283-3290. AEM.02750-06 [pii];10.1128/AEM.02750-06 [doi].

56. Peng, L., Lifang, R., Hongyu, X., Xi, L. and Z. Chaocan. 2007. Study on the toxic effect of lead (II) ion on *Escherichia coli*. Biol. Trace Elem. Res. **115**: 195-202

57. Fernandez, J. L., Cartelle, M., Muriel, L., Santiso, R., Tamayo, M., Goyanes, V., Gosálvez, J. and G. Bou. 2008. DNA fragmentation in Microorganisms assessed *in situ*. Appl. Environ. Microbiol. **74**: 5925-5933.

58. 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 introduction of copper. J Clin Microbiol **50**: 2217-2223. JCM.01032-12 [pii];10.1128/JCM.01032-12 [doi].

59. Espirito SC, 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. AEM.01599-10 [pii];10.1128/AEM.01599-10 [doi].

60. Noyce, J. O., H. Michels, and C. W. Keevil. 2006. Use of copper cast alloys to control *Escherichia coli* O157 cross-contamination during food processing. Appl. Environ. Microbiol. **72**: 4239-4244.

61. Molteni, C., Abicht, H. K. and M. Solioz. 2010. Killing of bacteria by copper surfaces involves dissolved copper. Appl. Environ. Microbiol. **76**: 4099-101

62. Kirakosyan G, Trchounian K, Vardanyan Z, Trchounian A (2008) Copper (II) ions affect *Escherichia coli* membrane vesicles' SH-groups and a disulfide-dithiol interchange between membrane proteins. Cell Biochem Biophys **51**: 45-50. 10.1007/s12013-008-9014-7 [doi].

63. 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. AEM.07068-11 [pii];10.1128/AEM.07068-11 [doi].

64. Mathews S, Hans M, Mucklich F, Solioz M (2013) Contact killing of bacteria on copper is suppressed if bacterial-metal contact is prevented and is induced on iron by copper ions. Appl Environ Microbiol **79**: 2605-2611. AEM.03608-12 [pii];10.1128/AEM.03608-12 [doi].

65. Strickertsson JA, Desler C, Martin-Bertelsen T, Machado AM, Wadstrom T, Winther O, Rasmussen LJ, Friis-Hansen L (2013) *Enterococcus faecalis* infection causes inflammation, intracellular oxphos-independent ROS production, and DNA damage in human gastric cancer cells. PLoS One **8**: e63147. 10.1371/journal.pone.0063147 [doi];PONE-D-12-26162 [pii].

66. Sagripanti JL, Routson LB, Bonifacino AC, Lytle CD (1997) Mechanism of copper-mediated inactivation of herpes simplex virus. Antimicrob Agents Chemother **41**: 812-817.

67. Ramos GBA, Rosato YB (1996) Copper accumulation in *Xanthomonas campestris* pv. vesicatoria Brazilian J of Genetics **19**(4): 551-554

68. Halliwell, B Gutteridge, JMC (1989) Free Radicals in Biology and Medicine, 2nd edn. Oxford, UK; Clarendon Press

69. Lloyd, D. R. and D. H. Phillips. 1999. Oxidative DNA damage mediated by copper (II), iron (II) and nickel (II) Fenton reactions: evidence for site-specific mechanisms in the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intrastrand cross-links. Mutation Res. **424**: 23-36.

70. Santo CE, Morais PV, Grass G (2010) Isolation and characterization of bacteria resistant to metallic copper surfaces. Appl Environ Microbiol **76**: 1341-1348. AEM.01952-09 [pii];10.1128/AEM.01952-09 [doi].

71. Gutierrez H, Portman T, Pershin V, Ringuette M (2013) Evaluation of biocidal efficacy of copper alloy coatings in comparison with solid metal surfaces: generation of organic copper phosphate nanoflowers. J Appl Microbiol **114**: 680-687. 10.1111/jam.12094 [doi].

72. Hans M, Erbe A, Mathews S, Chen Y, Solioz M, Mucklich F (2013) Role of copper oxides in contact killing of bacteria. Langmuir **29**: 16160-16166. 10.1021/la404091z [doi].

73. Kumar P, Imam B (2013) Footprints of air pollution and changing environment on the sustainability of built infrastructure. Sci Total Environ **444**: 85-101. S0048-9697(12)01482-9 [pii];10.1016/j.scitotenv.2012.11.056 [doi].

74. Chawla SK Payer JH 1990 The early stage of atmospheric corrosion of copper by sulphur dioxide. J. Electrochem. Soc., 137:60-64

75. Brimblecombe P, Grossi CM (2012) Carbonyl compounds indoors in a changing climate. Chem Cent J **6**: 21. 1752-153X-6-21 [pii];10.1186/1752-153X-6-21 [doi].

76. Valkirs AO, Seligman PF, Haslbeck E, Caso JS (2003) Measurement of copper release rates from antifouling paint under laboratory and in situ conditions: implications for loading estimation to marine water bodies. Mar Pollut Bull 46: 763-779. S0025-326X(03)00044-4 [pii];10.1016/S0025-326X(03)00044-4 [doi].

77. Hedberg YS, Hedberg JF, Herting G, Goidanich S, Odnevall W, I (2014) Critical review: Copper runoff from outdoor copper surfaces at atmospheric conditions. Environ Sci Technol 48: 1372-1381. 10.1021/es404410s [doi].

78. Beveridge, T. J., and W. S. Fyfe. 1985. Metal fixation by bacterial cell walls. Can. J. Earth Sci. **22**:1892-1898.

79. Letelier ME, Lepe AM, Faundez M, Salazar J, Marin R, Aracena P, Speisky H (2005) Possible mechanisms underlying copper-induced damage in biological membranes leading to cellular toxicity. Chem Biol Interact **151**: 71-82. S0009-2797(04)00216-9 [pii];10.1016/j.cbi.2004.12.004 [doi].

80. Letelier ME, Sanchez-Jofre S, Peredo-Silva L, Cortes-Troncoso J, Aracena-Parks P (2010) Mechanisms underlying iron and copper ions toxicity in biological systems: Pro-oxidant activity and protein-binding effects. Chem Biol Interact **188**: 220-227. S0009-2797(10)00395-9 [pii];10.1016/j.cbi.2010.06.013 [doi].

81. Letelier ME, Faundez M, Jara-Sandoval J, Molina-Berrios A, Cortes-Troncoso J, Aracena-Parks P, Marin-Catalan R (2009) Mechanisms underlying the inhibition of the cytochrome P450 system by copper ions. J Appl Toxicol **29**: 695-702. 10.1002/jat.1460 [doi].

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

83. Macomber, L. and J. A. Imlay. 2009. The iron-sulfur clusters of dehydratases are primary targets of copper toxicity. P. N. A. S. **106**: 8344-8349

84. Macomber, L., Rensing, C., and J. A. Imlay. 2006. Intracellular copper does not catalyse the formation of oxidative DNA damage in *E. coli*. J. Bacteriol. **189**: 1616-1626.

85. Santo CE, Quaranta D, Grass G (2012) Antimicrobial metallic copper surfaces kill *Staphylococcus haemolyticus* via membrane damage. Microbiologyopen **1**: 46-52. 10.1002/mbo3.2 [doi].

86. Nandakumar, R., Espirito Santo, C., Madayiputhiya, N. and G. Grass. 2011. Quantitative profiling of the *Escherichia coli* response to metal stress. Biometals **24**: 429-44

87. Amachawadi RG, Scott HM, Alvarado CA, Mainini TR, Vinasco J, Drouillard JS, Nagaraja TG (2013) Occurrence of the transferable copper resistance gene *tcrB* among fecal enterococci of U.S. feedlot cattle fed copper-supplemented diets. Appl Environ Microbiol **79**: 4369-4375. AEM.00503-13 [pii];10.1128/AEM.00503-13 [doi].

88. 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. JAM4148 [pii];10.1111/j.1365-2672.2009.04148.x [doi].

89. Orell A, Navarro CA, Arancibia R, Mobarec JC, Jerez CA (2010) Life in blue: copper resistance mechanisms of bacteria and archaea used in industrial biomining of minerals. Biotechnol Adv **28**: 839-848. S0734-9750(10)00096-0 [pii];10.1016/j.biotechadv.2010.07.003 [doi].

90. 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. S0195-6701(09)00408-3 [pii];10.1016/j.jhin.2009.08.018 [doi].

91. Casey AL, Karpanen TJ, Adams D, Lambert PA, Nightingale P, Miruszenko L, Elliott TS (2011) A comparative study to evaluate surface microbial contamination associated with copper-containing and stainless steel pens used by nurses in the critical care unit.

Am J Infect Control **39**: e52-e54. S0196-6553(11)00113-1 [pii];10.1016/j.ajic.2010.12.012 [doi].

92. Karpanen TJ, Casey AL, Lambert PA, Cookson BD, Nightingale P, Miruszenko L, Elliott TS (2012) The antimicrobial efficacy of copper alloy furnishing in the clinical environment: a crossover study. Infect Control Hosp Epidemiol **33**: 3-9. 10.1086/663644 [doi].

93. Schmidt MG, Attaway Iii HH, Fairey SE, Steed LL, Michels HT, Salgado CD (2013) Copper continuously limits the concentration of bacteria resident on bed rails within the intensive care unit. Infect Control Hosp Epidemiol **34**: 530-533. 10.1086/670224 [doi].

94. Salgado CD, Sepkowitz KA, John JF, Cantey JR, Attaway HH, Freeman KD, Sharpe PA, Michels HT, Schmidt MG (2013) Copper surfaces reduce the rate of healthcare-acquired infections in the intensive care unit. Infect Control Hosp Epidemiol **34**: 479-486. 10.1086/670207 [doi].

95. Michels HT, Noyce JO, Keevil CW (2009) Effects of temperature and humidity on the efficacy of methicillin-resistant *Staphylococcus aureus* challenged antimicrobial materials containing silver and copper. Lett Appl Microbiol **49**: 191-195. LAM2637 [pii];10.1111/j.1472-765X.2009.02637.x [doi].

96. Jeffrey DJ (1995) Chemicals used as disinfectants: active ingredients and enhancing additives. Rev Sci Tech **14**: 57-74.

97. Barclay L, Park GW, Vega E, Hall A, Parashar U, Vinje J, Lopman B (2014) Infection control for norovirus. Clin Microbiol Infect . 10.1111/1469-0691.12674 [doi].

98. Cromeans T, Park GW, Costantini V, Lee D, Wang Q, Farkas T, Lee A, Vinje J (2014) Comprehensive Comparison of Cultivable Norovirus Surrogates in Response to Different Inactivation and Disinfection Treatments. Appl Environ Microbiol . AEM.01532-14 [pii];10.1128/AEM.01532-14 [doi].

99. Harrison JJ, Turner RJ, Joo DA, Stan MA, Chan CS, Allan ND, Vrionis HA, Olson ME, Ceri H (2008) Copper and quaternary ammonium cations exert synergistic bactericidal and antibiofilm activity against *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 52: 2870-2881. AAC.00203-08 [pii];10.1128/AAC.00203-08 [doi].

100. Copper Development Association, 2013, Publication 213: Antimicrobial Copper Alloy Touch Surfaces: Guidance on Cleaning and Disinfection http://www.copperalliance.org.uk/docs/librariesprovider5/resources/213-cleaning-guidelines.pdf?sfvrsn=0

101. Gold KM, Hitchins VM (2013) Cleaning assessment of disinfectant cleaning wipes on an external surface of a medical device contaminated with artificial blood or *Streptococcus pneumoniae*. Am J Infect Control **41**: 901-907. S0196-6553(13)00192-2 [pii];10.1016/j.ajic.2013.01.029 [doi].