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Departamento de Ciências da Vida
Faculdade de Ciências e Tecnologia
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Molecular basis for antimicrobial properties

Christophe Ruis Espírito Santo

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Dissertação apresentada à Universidade de Coimbra para a obtenção do grau de Doutor em Bioquímica, especialidade em Microbiologia Molecular, realizada sob a orientação científica do Professor Doutor Gregor Grass, senior researcher at Bundeswehr Institute of Microbiology, Munique, Alemanha e da Professora Doutora Paula Maria de Melim Vasconcelos de Vitorino Morais, Professora Auxiliar do Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Resumo

Em todo o mundo, milhões de pessoas contactam com superfícies, sendo esta uma forma significativa de contaminação com agentes patogénicos microbianos em hospitais e outras instalações públicas. As infeções hospitalares constituem uma das principais causas de morte e morbilidade entre os pacientes. O *Centers for Disease Control and Prevention* (Centro de Controlo e Prevenção de Doenças - CDC) relatou um total de 100 000 mortes só nos Estados Unidos da América em 2009, com custos agravados superiores a quarenta e sete mil milhões de dólares. Na Inglaterra, em 2005, foi reportado que em cada ano 5 mil pessoas morrem de infeções hospitalares com custos associados acima de um mil milhão de libras esterlinas.

Os minérios de cobre foram utilizados ao longo dos séculos por muitas civilizações devido ao seu poder natural medicinal e de sanitização. No entanto, apenas recentemente, publicações científicas demonstram que a aplicação de ligas metálicas de cobre reduz fortemente a carga microbiana presente nas superfícies, tanto em condições laboratoriais como em ambientes hospitalares. Esta propriedade única antimicrobiana do cobre é cada vez mais reconhecida por microbiólogos e especialistas em higiene pública como uma nova ferramenta de combate a infecções hospitalares. O presente trabalho explora diferentes superficies de cobre que apresentam uma grande capacidade de inactivação de uma ampla variedade de micróbios em ambiente húmido (suspensões de células, imitando contaminação por gotículas) ou seco (contacto directo entre as células e as superfícies, imitando as superfícies de contacto). Em particular, foca-se na compreensão dos mecanismos moleculares que conduzem à morte microbiana em superfícies de ligas metálicas de cobre. Sob condições húmidas,

as células são apenas inactivadas quando os iões de cobre dissolvidos atingem uma concentração tóxica crítica que, por sua vez, interferem provavelmente no metabolismo geral e na respiração. Em contraste, o contacto directo das células em meio seco resulta num choque rápido por iões de cobre levando à formação de espécies tóxicas reactivas de oxigênio. Consequentemente, este processo conduz ao aparecimento de danos nas células, mais especificamente, nos lípidos membranares, que são as biomoléculas mais próximas. As membranas são, portanto, gravemente danificadas, culminando na instabilidade letal da estrutura celular. Assim, o potencial de membrana perde-se e o conteúdo citoplasmático é libertado. Após a morte da célula, outras biomoléculas são também degradadas por oxidação, levando à degradação do ADN. Este trabalho indica, ainda, que o processo denominado “contact-killing” (morte por contacto), não é causado por genotoxicidade.

Este trabalho vem contribuir para a compreensão das propriedades antimicrobianas das ligas metálicas do cobre. Igualmente, confirma-se aqui que a aplicação de ligas metálicas de cobre é bastante útil e segura em hospitais, como medida adicional para a prevenção de infeções hospitalares.

Summary

Around the world billions of people touch surfaces many times a day, an important way of transmission of contaminating microbial pathogens in public and healthcare facilities. Among hospital users, hospital acquired infections (HAI) are one of the leading causes of death and morbidity among hospital users. The Centers for Disease Control and Prevention (CDC) reported a total of one hundred thousand deaths only in the United States of America in the year of 2009 with aggravated costs over forty-seven billion dollars. In England, a report from 2005 estimates that each year there are approximately 5,000 people killed by hospital acquired infections and over one billion British pounds are spent in costs associated with HAI.

Copper and its minerals were used throughout the ages by many different civilizations for its natural medicinal and sanitizing powers. However, only recently, research has proven that applying metallic copper strongly reduces microbial surface-burden, both in laboratory settings and healthcare environments. This unique antimicrobial property of metallic copper is increasingly becoming recognized by microbiologists and hygiene specialists as a very promising novel tool for reducing hospital acquired infections. This present work explores different copper surfaces which have strong microbe-inactivating activities against a wide variety of microbes under moist (droplets of cell suspensions, mimicking splash-contamination) or dry (direct contact between cells and surfaces, mimicking touch surfaces) conditions. Here this work is focused on understanding the molecular mechanisms that lead to microbial death on solid copper surfaces. For instance, under wet conditions cells are killed within few hours. When cells are exposed, copper ions are released from the surface

into the droplet, inactivating the cells when dissolved copper ions reach a critical toxic level. By this method cells are killed most likely due to copper ion interference with general metabolism and respiration. In contrast, cells in dry contact suffer fast killing: cells are inactive after few minutes of exposure. Upon contact the small amount of buffer that separates the cells from the surface dries in seconds. This leads to surface oxidation releasing high amounts of copper and, in addition, generating toxic reactive oxygen species. Subsequently, this inflicts damage to the cell, more specifically to the membrane lipids which are the closest biomolecules. Eventually this in severe membrane damage results in lethal structural instability. The cell's membrane potential is lost and cytoplasmic content may be released. After cell death other biomolecules are also degraded by oxidation including degradation of cellular DNA. Furthermore, this work shows that this copper-mediated process of cell-inactivation termed "contact-killing" is not caused by genotoxicity.

Our understanding of the antimicrobial properties of metallic copper surfaces have made great strides in the last five years and this work at hand has contributed significantly to deciphering the molecular processes leading to cell death. In addition, this work strongly points to a safe, economical and sustainable application of metallic copper surfaces in healthcare to prevent hospital acquired infections.

Chapter 1

General Introduction

1.1. Hospital acquired infections: a threat to modern society

Hospital acquired infections (HAI) occur worldwide and affect both resource-poor and developed countries and the associated diseases are significant contributors to morbidity and mortality (Emmerson et al., 1996; Gastmeier et al., 1998; Mayon-White et al., 1988; Raymond & Aujard, 2000; Vaqué et al., 1999). These kinds of infections are defined as occurring more than 48 hours after admission to a healthcare facility and also include occupational infections contracted by healthcare staff (Benenson, 1995; Ducel et al., 1988; Horan et al., 1992). During hospitalization, patients are exposed to a variety of risk factors that play a role in development of HAI. These factors include the microbial agent itself (resistance to antimicrobials, inoculum size and intrinsic virulence), patient susceptibility (age, immune status, underlying disease, diagnostic and therapeutic interventions), and environmental factors (crowded hospital, frequent transfers and concentration of patients highly susceptible to infection in one area and touch-surfaces/equipments) (Ducel et al., 2002) (Figure 1).

Hospitals are environments where both infected and non-infected persons gather: people are the center of the phenomenon. Hospital users act as main reservoir and source of microorganisms, both as transmitter and as receptors for microorganisms, consequently becoming a new reservoir for microorganisms (Figure 1). Transmission of infectious material may occur by direct and indirect contact through aerosolized particles or fomites. Transmission can be exogenous cross–infection (acquired from another person), endogenous infection (patient own flora), endemic or epidemic exogenous / environmental infections (acquired from healthcare flora) (Ducel et al., 2002) (Figure 2).

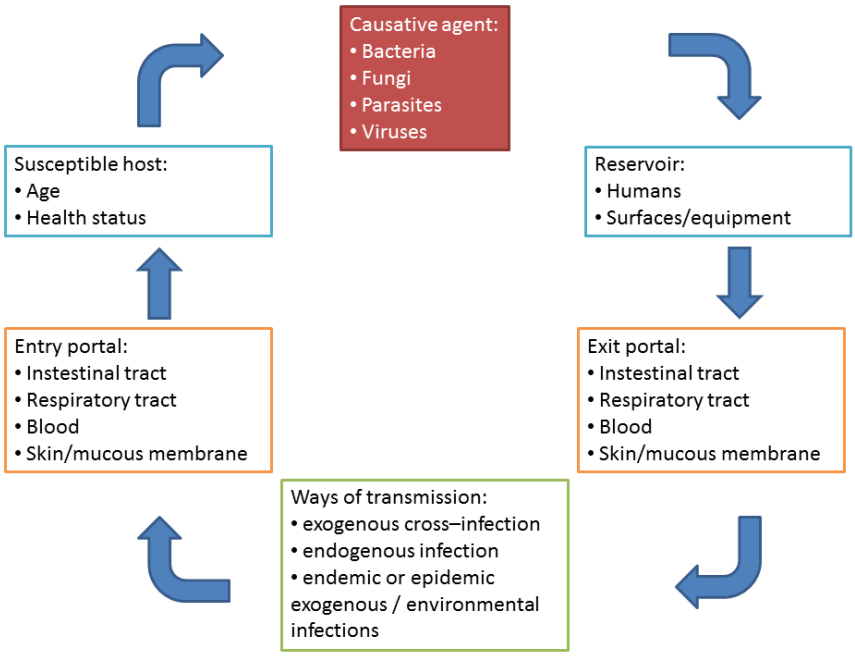

Figure 1 – Schematic of hospital acquired infections cycle. People are the center of the action: people act as the main reservoir which can lead of contamination of other people or contaminating equipment/surfaces making new reservoirs. The causative agent can be transmitted to another person by different kinds of transmission. Age and health status are key factors in susceptibility to acquire an HAI. (adapted from: http://medical-dictionary.thefreedictionary.com/Hospital+Acquired+Infections)

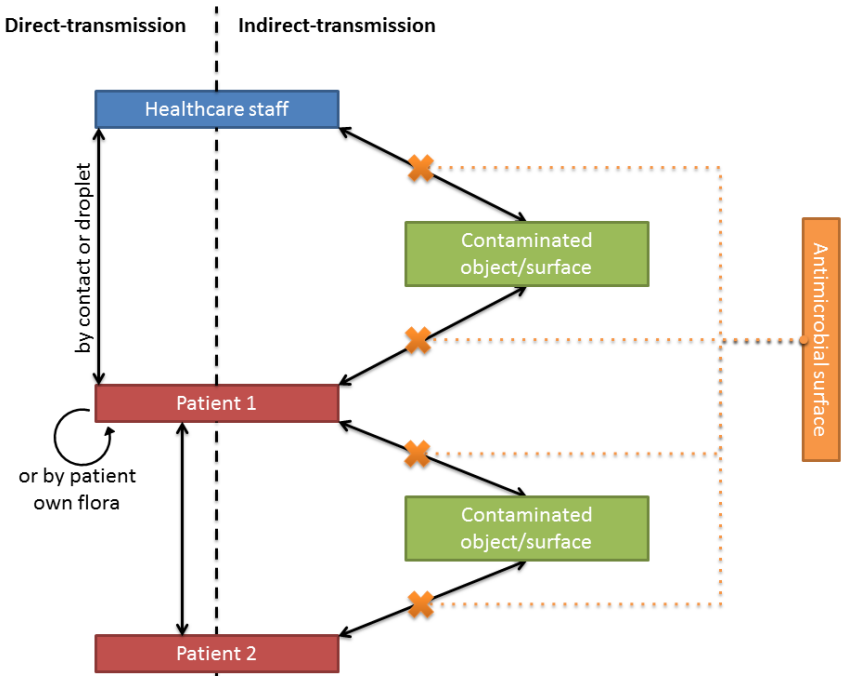

Figure 2 – Direct and indirect modes of transmission of microbes involved in hospital acquired infections. Microbes can be acquired from another person, from the own flora or from a contaminated surface/equipment. Antimicrobial surfaces are envisioned to help in diminishing transmission via objects/surfaces.

Prevalence surveys conducted throughout the world have shown the significant impact for patient and public health. Methods used to measure HAI rates differ from country to country (type of infections covered, definitions, health units surveyed, etc). The world health organization (WHO) conducted studies in 55 hospitals of 14 countries representing Europe, Eastern Mediterranean, South-East Asia and Western Pacific. According to their estimates, an average of 8.7% of hospitalized patients will contract a HAI. This corresponds to over 1.4 million people worldwide per year that will be infected while in healthcare (Tikhomirov, 1987). Eastern Mediterranean and South-Asian regions show the highest incidence, 11.8 % and 10.0%, respectively. In Europe and Western Pacific regions these numbers decrease to 7.7% and 9.0%, respectively (Mayon-White et al., 1988). Surgical wounds, urinary tract and lower respiratory tract infections are the most frequent HAI (Ducel et al., 2002). Prevalence has been identified to be higher in intensive care units and in acute surgical and orthopedic wards (Tikhomirov, 1987). Patients that are more susceptible of contracting an infection are old age, underlying disease or chemotherapy (Ducel et al., 2002).

The Centers for Disease Control and Prevention (CDC, USA) and the Health Protection Agency (HPA, England) are important references throughout the world for statistical data regarding HAI. In 2009 (Scott II, 2009) estimated that in the USA, each year, two million people contract a HAI. Among that, one out of twenty dies, corresponding to around 100,000 deaths in total. In intensive care units, this number rises to one in four. This makes HAI´s the fourth leading cause of death in the USA. In the case of England, a report from 2005 estimates that each year there are 300,000 cases of HAI, leading to approximately 5,000 deceased (House of Commons London, 2004).

In addition, HAI bring about significant economic impact to the healthcare budget. The most noteworthy contributor is the increase in the length of stay (Coello et al., 1993; Kirkland et al., 1999; Pittet et al., 1994). Other factors influence the HAI bill: use of therapeutic drugs, need of patient isolation and usage of additional diagnostic measures. According to the CDC, in the USA alone there are an additional $47 billion in added healthcare costs meaning that HAI add 208% to the hospital bill (Scott II, 2009). For England´s National Health Service, HAI cost each year as much as £1 billion (House of Commons London, 2004).

Solving this problem is a priority to the healthcare business. High frequency of HAI is evidence of a poor-quality health service, leading to additional costs that could have been prevented. Additionally, microorganisms causing HAI can be carried to the general population through staff, visitors and discharged patients. Moreover, if the culprit microbial agent is multi-resistant, the germ might be a major hazard to the community. Enforcing a strict hygiene policy, proper clothing and sterilization/disinfection of equipment, help preventing contamination between people, and between people and surfaces. However, all these efforts have limitations. Creating additional barriers between microbes and people is necessary. Touch-surfaces such as doorknobs, push-plates, equipment buttons, bed-rails, etc., are difficult to properly clean and disinfect. Therefore, in addition to strict hygiene condition, “self-cleaning” surfaces are part of an innovative field that presents a potential to help fighting HAI (Figure 1). This present work deals with metallic copper surfaces as an antimicrobial material. Why and how copper might be ideal for this purpose is outlined in the next section.

1.2. Historical usage of copper as a biocide

Copper originates from the Latin word cuprum, meaning Cyprus, site of one of the largest ancient Mediterranean copper mines. However, copper was mined long before the word's origin. In history, copper was the first metal used by human civilizations, probably because it was easily extracted, available in great quantities and very malleable (Cowen). One of the uses for copper was to prevent and treat diseases and disinfect fluids and solids (Block, 2001; Dollwet & Sorenson, 1985). An Egyptian medical document, the Smith papyrus (circa 2400 B.C.) states that copper was used to sanitize drinking water and wounds. Copper oxide and malachite, a copper carbonate compound, was used in Mesoamerica by the Aztecs to treat skin conditions. In the ancient Greece, Hippocrates (400 BC), the "father" of medicine prescribed copper for pulmonary diseases and to disinfect drinking water (Dollwet & Sorenson, 1985). The Roman Empire used copper piping to improve public hygiene. Great traders like early Phoenicians needed clean ship hulls to travel faster so they fixed copper strips on the ship bodies to inhibit biofouling. Additionally, many cultures throughout the world dropped copper coins in water vessels to prevent diseases like dysentery (Dollwet & Sorenson, 1985). Until the 19th century, all these early civilizations were using copper without knowing the existence of microorganisms. Only when Antonie van Leeuwenhoek discovered microorganism and Louis Pasteur brought the notion that germs may lead to disease, in his *Germ theory of Disease*, copper usage gained a more specific meaning: copper as a biocide. During the same century, it was noted that, in Paris, copper workers were not affected during a raging cholera epidemic. The employment of the metal and its salts in the subsequent century became widespread in medicine: a variety of copper

compounds were used to treat diseases such as eczema, tubercular infections, and “The Great Pox”, syphilis. However, with the discovery of antibiotics and their commercialization, exploitation of copper as an antimicrobial became all but forgotten (Dick et al., 1973). Only today, when, antibiotic resistant bacteria become widespread a renewed need to use unorthodox means including copper surfaces to improve both health and hygiene in sensitive areas.

1.3. Copper as a bio-element

What makes copper an ideal metal to use as antimicrobial compound? In order to answer this question it is necessary to have a look at the intrinsic peculiarities of copper-related redox chemistry. In the primordial anaerobic Earth, early life used iron but not copper. Iron was bioavailable as the water-soluble ferrous Fe (II). Its natural abundance and its redox properties allowed the chemistry that was appropriate for life at the time. Conversely, copper was not bioavailable for it was largely in the water-insoluble cuprous Cu (I) state, in the form of highly insoluble sulfides. Copper was only available in acidic waters near hydrothermal vents. About 10^9 years ago, dioxygen started to accumulate in the atmosphere due to prokaryotic (cyanobacterial) metabolism. The arrival of dioxygen was dramatic for most living organisms because of its toxicity. The new atmosphere oxidized iron to the water-insoluble ferric iron (III) state. Thus, bioavailability of iron was lost. In its place, the oxidation of insoluble Cu (I) led to soluble cupric Cu (II). Anaerobic metabolism was designed to use proteins and enzymes that had a low redox potential. In the presence of an oxidizing atmosphere, a new redox active metal, copper, with a higher redox potential was generated. Copper was now available and quite ideal for life under oxygenated conditions, thus becoming used by living

organisms. A new era had started, the copper era (Crichton & Pierre, 2001; Fraústo Da Silva & Williams, 2001; Ochiai, 1986).

Copper is a transition metal that belongs to the group eleven of the periodic table. Along with silver and gold, copper is referred as a coinage metal due to the characteristic color, corrosion resistance and value. Copper has an atomic number of 29 and is present in Earth's crust in two stable isotopic forms: 63 (69,1%) and 65 (30,9%). The electronic distribution of its 29 electrons is $1s^2$ $2s^2$ $2p^6$ $3s^2$ $3p^6$ $4s^2$ $3d^9$, however, one electron from the 4s orbital is always allocated in the 3d orbital ($4s^1$ $3d^{10}$). The inner electronic layers (1s, 2s, 2p, 3s and 3p) are closer to the positively charged nucleus permitting the 4s electron to "escape" to the 3d orbital, characterizing a low energy state (Huheey et al., 1993). Copper can lose up to two electrons in one-electron step transfers resulting in cuprous (Cu (I)), and cupric (Cu (II)). All these physic-chemical characteristics are extremely important for organisms. In addition to the physic-chemical characteristics, the mechanism and rate of copper-catalysis depends on a complex multiplicity of factors: type of ion valence, complexes it forms (inner or outer sphere), chelator or complexing agent, redox potential of its complexes, solvents, phase localization and availability of oxygen or hydroperoxides. This multiplicity allows organisms to use copper in different types of reaction.

The major property of copper that organisms take advantage of, is to alternate oxidation states by one electron transfer, between Cu (I) and Cu (II), allowing them to handle a variety of oxidation-reduction processes (Karlin, 1993). This permits copper to function as a cofactor for a variety of enzymes involved in processes such as respiration (cytochrome c oxidase), photosynthesis (plastocyanin), reactive oxygen species (ROS) turnover (copper-zinc superoxide

dismutase), nitrite and nitrous oxide reductases and oxygen transport (hemocyanin) (Metzler, 2003). In oxidases, hydroxylases, and reductases copper functions as an electron donor/acceptor (Metzler, 2003). Or it functions as an electron carrier for instance in azurin and plastocyanin (Metzler, 2003).

Copper, in excess, is very toxic to cells. Copper is ranked fifth among the most toxic of seventeen metals to soil bacteria, behind silver, mercury, chromium, and cadmium (Drucker et al., 1979). In addition, it was found that copper is one of the most toxic metals to heterotrophic bacteria in aquatic environments (Albright & Wilson, 1974). Heavy metal sensitivity of water microflora was: Ag > Cu > Ni > Ba > Cr > Hg > Zn > Na> Cd. It is well known that in excess, copper is capable of forming stable complexes with a wide variety of ligands, thus, copper can bind to biomolecules such as proteins, lipids, and nucleic acids, regardless of its valence state (Fraústo Da Silva & Williams 2001).

Copper-induced toxicity can be very diverse depending on the environment surrounding this metal, provoking damage by multiple mechanisms. This means that measuring copper toxicity *in vivo* can be very challenging. Copper redox versatility, other than being beneficial for cells, is also the most important source of its toxicity. Under aerobic conditions, copper can alternate oxidation state and form ROS described by the Fenton like Haber-Weiss reactions (Figure 3) (Liochev & Fridovich, 2002). ROS, *per se*, are extremely reactive, when formed and ROS are quickly consumed causing damage to surrounding biomolecules: lipids, proteins and DNA (Yoshida et al. 1993).

$$H_2O_2 + O_2^{-} \longrightarrow O_2 + OH^{-} + OH^{\bullet} \quad (1)$$

$$Cu^{2+} + O_2^{-} \longrightarrow Cu^{+} + O_2 \quad (2)$$

$$Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + O_2 + OH^{-} + OH^{\bullet} \quad (3)$$

Figure 3 – **Reactive oxygen species formation by copper-mediated catalysis.**

Hydrogen peroxide reacts with superoxide as described in the equation 1 with low constant rate; this rate is increased when copper ions are added to the equations. Cupric ion is reduced to cuprous ion by superoxide anion (Figure 3, equation 2), oxidized back to cupric by hydrogen peroxide (Figure 3, equation 3), and the cycle repeats. At each cycle more hydroxyl radicals are formed, increasing cell damage. On the other hand, it has been suggested that copper ions can also lead to depletion of sulfhydryl groups (Figure 4). For instance, copper can react with glutathione (GSH) producing glutathione disulfide (GSSG) or with the amino-acid cysteine generating cystine groups (Figure 4, equation 4).

$$2\ Cu^{2+} + 2\ RSH \longrightarrow 2\ Cu^{+} + RSSR + 2\ H^{+} \quad (4)$$

$$2\ Cu^{+} + 2\ H^{+} + O_2 \longrightarrow 2\ Cu^{2+} + H_2O_2 \quad (5)$$

Figure 4 – **Reactions by which copper leads to sulfhydryl groups depletion.**

The resulting cuprous ions generated by sulfhydryl group oxidation are recycled back to cupric ion producing hydrogen peroxide. This hydrogen peroxide can be converted to more highly reactive ROS, hydroxyl radical and superoxide, by the equations 2 and 3 described above (Macomber & Imlay, 2009).

Lipid oxidation occurs ubiquitously in biological systems (Schaich, 2005), however this process is not well understood. The process by which lipids (L) suffer oxidation follows three steps: initiation, propagation, and termination (Figure 5) (Frankel, 1980; Schaich, 2005).

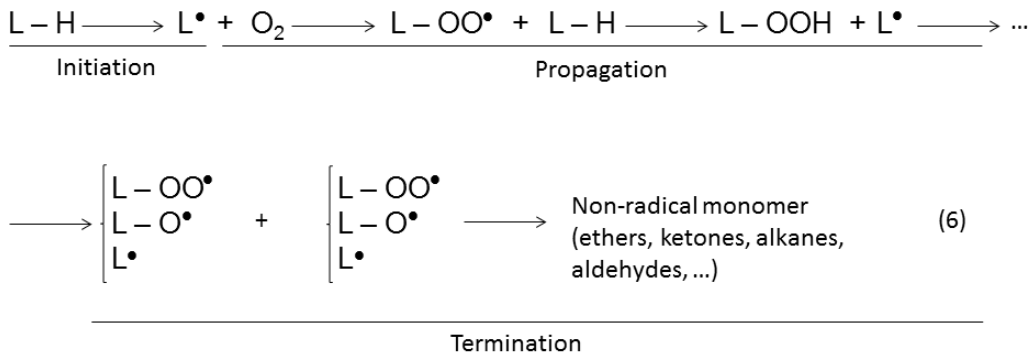

Figure 5 – **Schematic representing the three steps that characterize lipid oxidation.**

After initial generation of lipid radicals ($L^{\bullet}$), oxidized lipids rapidly oxidize other lipids leading to further lipid oxidation (propagation). Once started, lipid oxidation is self-propagating and self-accelerating, being designated as autocatalytic. A single initiating-event can lead to about 200 to 300 chain reactions, showing how effective one initiation-event is (Cosgrove et al., 1987; Hyde & Verdin, 1968). However, biological systems are able to use antioxidants that strongly repress this event, yet when lipid oxidation-events are overwhelming, organisms are totally or partially vulnerable.

Lipid oxidation can occur very easily, but it is not a spontaneous reaction. Initiation of lipid oxidation, e.g., directly by oxygen is not possible. In thermodynamic aspects oxygen cannot react directly with lipids due to different electron spin states. For oxidation to happen, catalysts are required to overcome the electron spin barrier. Copper with its one-electron transfer reactions is considered an active catalyst (Uri, 1961). Metals are able to initiate lipid oxidation forming lipid alkoxyl radicals ($LO^{\bullet}$) and lipid peroxyl radicals ($LOO^{\bullet}$). In case of redox-active metals, like copper, *in vitro* studies showed that trace quantities are sufficient for effective catalysis (Schaich, 2005; Uri, 1961). Trace amounts can oxidize lipids indirectly through ROS by the reactions 2 and 3 (Figure 3). Nevertheless, direct lipid initiation by copper can be achieved by various routes.

By the high valence ion, Cu (II), lipids can be oxidized by various routes (Figure 6) (Schaich, 2005). Reactions 7 and 8 (Figure 6) were proposed as primarily mode of catalysis for cobalt, manganese and chromium. However, other metals can induce catalysis with chelating agents that shift the redox potential; or with solvents, that alter acid/base properties and electron transfer efficiency. Generation of oxidized lipids by electron transfers is extremely rapid in non-polar media (Chalk & Smith 1957; Chalk & Smith 1957). Reaction 10 (Figure 6) is strongly catalyzed by Cu (II) as well as Co (III) and Mn (II). This reaction occurs primarily in non-polar solvents and is inhibited by water competition. In complexes with outer sphere coordination, electrons flow occurs between non-connected species, directly between the metal valence shell and the ligand; electron transfer is fast and selective. Inner sphere electron transfers require a covalent linkage between the ligand and the metal, and electron flow is through the ligands; electron flow is slow and less discriminating. Copper forms mostly inner sphere complexes with organic substrates, especially in non-polar solvents. On the other hand, most copper salts catalyze direct electron transfer through outer sphere complexes (Lippard & Berg, 1994).

In the case of lower valence state Cu (I), activation of oxygen is required, in order to start lipid oxidation. Cu (I) can form complexes with oxygen, thus forming an active complex capable of attacking lipids and form lipid radicals (Figure 7). These reactions are similar to reactions 1 to 3 (Figure 3) but with the difference that Cu (I) forms a complex with oxygen. These reactions are facilitated in hydrophobic environments (Copping & Uri, 1968). As it is shown by Figure 7, there is a multitude of ways to oxidize lipids leading to lipid radicals and ROS.

Resulting Cu (II) and ROS, like hydrogen peroxide can be cycled back to oxygen and Cu (I).

$$RCH = CHR + Cu^{2+} \longrightarrow R\overset{\bullet}{C}H - \overset{+}{C}HR + Cu^{+} \quad (7)$$

$$RH + Cu^{2+} \longrightarrow R^{\bullet} + H^{+} + Cu^{+} \quad (8)$$

$$RCOOH + Cu^{2+} \longrightarrow RCOO^{\bullet} + Cu^{+} \longrightarrow R^{\bullet} + CO_2 \quad (9)$$

$$RCHO + Cu^{2+} \longrightarrow RCO^{\bullet} + H^{+} + Cu^{+} \quad (10)$$

Figure 6 – **Multiple reaction by which lipid initiation occurs in the presence of the high valence ion, Cu (II).** The aliphatic chain is represented by R.

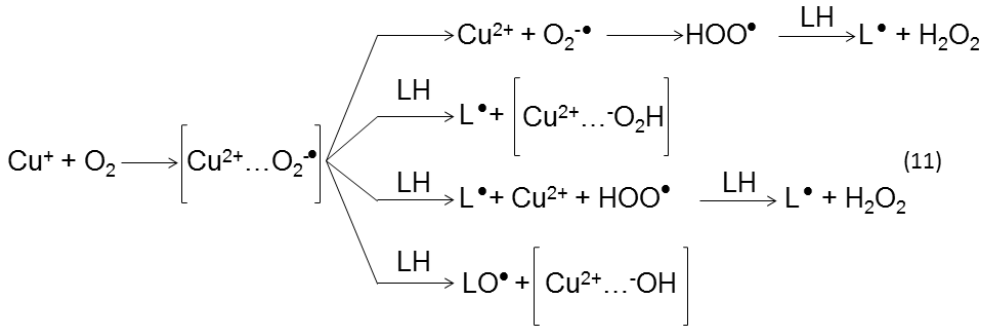

Figure 7 – **First steps of lipid oxidation by the low valence ion, Cu (I).**

Cu (I) and (II) is also able to form complexes with newly formed lipid peroxides (Kochi, 1962; Schaich, 2005), leading to further lipid radicals, thus enhancing propagation. These reactions happen particularly at low concentrations of hydroperoxide and in non-polar solvents.

$$Cu^{+} + ROOH \longrightarrow Cu^{2+} + RO^{\bullet} + OH^{-} \quad (12)$$

$$Cu^{2+} + ROOH \longrightarrow Cu^{+} + ROO^{\bullet} + H^{+} \quad (13)$$

Figure 8 – **Propagation of lipid oxidation by copper ions.**

Transposing *in vitro* knowledge to *in vivo* environments is a difficult task, given the multitude of possible reactions and products that can be formed. For that reason, *in vivo* studies are still lacking in this field.

Damage to nucleic acids, lipids, and proteins by previously described mechanisms have been demonstrated *in vitro* in many studies (e.g. Bittner et al., 2002; Cervantes-Cervantes et al., 2005; Cooke et al., 2003; Dizdaroglu et al., 2002; Imlay & Linn, 1988; Kim et al., 2000; Yoshida et al., 1993). There are some recent findings suggesting an alternative mechanism responsible for the primary toxic effects of copper *in vivo*. At first, the majority of copper inside the cell is bound to biomolecules, while free copper is at extremely low levels or even nonexistent, thus making the Fenton chemistry and sulfhydryl depletion very unlikely mechanisms (Changela et al., 2003). Another study by (Macomber et al., 2007) showed that *Escherichia coli* cells grown without copper are more sensitive to killing by hydrogen peroxide than *E. coli* pretreated with copper. In addition, copper decreased the rate of DNA damage induced by hydrogen peroxide. The authors suggested that copper exerts its toxicity by mechanisms other than oxidative stress. Furthermore, (Macomber & Imlay, 2009) showed *in vivo* as well as *in vitro* that when intracellular copper rises, copper can lead to the displacement of iron from iron-sulfur clusters. Copper specifically damaged the iron–sulfur clusters of various dehydratases involved in branched amino acid biosynthesis from *E. coli* cells. Further investigations in this area are needed, in order to have a clear conception on the mechanism of copper-induced toxicity in cells.

1.4. **Copper homeostasis**

Cells that are challenged with rising copper concentrations need to control intracellular copper concentration, in order to ensure their survival. For that, cells developed systems that can expel copper out of the cell, sequester excess

copper or additionally may oxidize Cu (I) to the less toxic Cu (II). Among Gram-negative bacteria, *Escherichia coli* is one of the most studied on copper homeostasis. *E. coli* possess multiple systems that confer resistance against rising concentrations of copper ions. Surprisingly, it is not yet clear how copper enters most Gram-negative cells. There are multiple possible ways: either by diffusion across the membranes, either through porines, or by an unknown specific or unspecific transport of copper ions across the cytoplasmic membrane. When copper is present in the cytoplasm, copper is mainly in its reduced state, as Cu (I). Cytoplasmic Cu (I) can be transported into the periplasm by CopA, a P-type ATPase, energized by ATP hydrolysis (Rensing et al. 2000). The Cus efflux system is a complex of three proteins (CusCBA), which expels periplasmic Cu (I) out of the cell, depending on proton motive force (PMF). CusA is a member of the resistance-nodulation-division (RND) protein superfamily of proton-driven cation symporters and antiporters. CusC is an outer membrane factor (OMF), and CusB belongs to the family of membrane fusion proteins (MFP). A copper chaperon CusF binds Cu (I) and delivers it to the CusCBA complex (Franke et al., 2003). Alternatively, Cu (I) can be oxidized to Cu (II) by a multicopper oxidase, CueO (Grass & Rensing 2001), hence protecting the periplasm space from Cu (I) toxicity (Singh et al., 2004). Genes that encode these copper homeostasis system are regulated by two independent routes. The *copA* and *cueO* genes are under control of CueR, a cytoplasmic MerR-family repressor, that when bound to Cu (I) activates gene expression of *cueO and copA* (Stoyanov et al. 2001). The *cusCFBA* operon is induced by a periplasmic two-component system, CusRS (Munson et al. 2000; Outten et al. 2001). Recently, an outer membrane protein, ComC (copper-induced outer membrane component), was found to be involved

in copper permeability. When this protein is not present, copper concentration was higher inside the periplasm and cytoplasm. Additionally, a novel TetR-like copper-responsive repressor, ComR, controls the expression of ComC (Mermod et al., 2012).

In addition to these chromosome-encoded genes, there are plasmid-encoded copper resistances. The best studied is the plasmid-encoded copper resistance determinant, Pco system of plasmid pRJ1004, isolated from *E. coli* present in the gut flora of pigs, which were fed with a diet supplemented with copper sulphate as a growth promotant (Brown et al. 1995). This plasmid encodes seven genes, *pcoABCDRSE*. PcoA is a multicopper oxidase related to CueO. PcoC and PcoE are two periplasmic copper chaperones. PcoB and PcoD have unknown functions. The expression is dependent on copper and is accomplished by PcoRS, a two-component system that is a paralog of CusRS (Brown et al. 1995).

Among Gram-positive bacteria, *L. lactis, Bacillus subtilis*, and *Enterococcus hirae* are the most studied species on copper homeostasis. The model organism for metal handling is *E. hirae* (Solioz & Stoyanov 2003). An operon of four genes, *copYZAB*, is responsive to copper stress. Excess of Cu (I) leads to binding to CopY, a copper-responsive repressor, resulting on derepression of the *cop* operon. When transcription starts, more CopY is produced, as well as CopZ, a copper chaperone, and the ATPases copper transporters, CopA and CopB (Odermatt et al., 1992). CopB extrudes excess of copper and silver (Solioz & Odermatt 1995). Conversly, Δ*copA* mutants showed poor growth in media where copper is limited by complexation with copper chelators (Odermatt et al., 1994). *E. hirae* CopA-mediated copper import system still awaits rigorous experimental confirmation.

L. lactis is widely used in the food industry. In particular, *L. lactis* in copper vats serves for traditional Swiss cheese making because these bacteria are able to withstand copper release from the vats. In order to cope with copper concentrations, *L. lactis* developed copper homeostasis systems. Similarly to *E. hirae*, *L. lactis* has a copper-inducible operon, *copRZA*. CopR is a CopY-type repressor, CopZ is a copper chaperone, and CopA is a copper ATPase. The CopB is encoded separately and repressed by CopR, but a copper export function has not been determined (Solioz et al. 2010).

Without copper-detoxifying mechanisms, cells suffer copper-induced toxicity that might compromise survival. Nevertheless there is another, related challenge: stress caused by contact to metallic copper. Knowledge related to this so-called "contact-killing" (Grass et al., 2011) by metallic copper surfaces, is reported in the following section.

1.5. Testing the antimicrobial properties of metallic copper surfaces.

Copper surfaces were put to the test both in laboratory studies and hospital trials. The first method put in practice, explores the antimicrobial activity of metallic copper surfaces against cells suspended in a buffer solution (wet method). In the presence of buffer solution, cells are not directly in contact with the surfaces but instead suspended away from the metallic copper (e.g. Faúndez et al. 2004; Wilks et al. 2005; Noyce et al. 2006b; Wilks et al. 2006; Noyce et al. 2006a). Wet method mimics moist environments, such as food processing, public baths, water conservation, pipelines, and bathrooms. Here droplets containing germs fall on top of surfaces and can be picked up by a person carrying the suspended germs. The first studies on antimicrobial copper surfaces accessed the killing capacity to

various microorganisms. In 2004 (Faúndez et al., 2004), demonstrated under laboratory conditions that copper surfaces are able to reduce bacterial counts of *Salmonella enterica* and *Capylobacter jejuni*, two notorious human pathogens, mainly transmitted by food ingestion. This study was the first peer-reviewed publication confirming the antimicrobial efficacy of copper surfaces versus steel control surfaces. But earlier that same year, conference papers by Harold Michels and colleagues (Michels et al. 2004; Michels et al. 2003) reported the killing kinetics of various copper alloys against *E. coli* O157:H7, an enterohemorrhagic strain found in ground beef. In addition, this was the first study pointing out that temperature played a role for the killing process. At lower temperatures cells took longer to be killed. At 20˚C cells were inactivated on 99% pure copper under one hour and a half, and at 4˚C this process took three hours. Surfaces without copper failed to inactivate *E. coli* O157:H7. Diminishing the copper content in the alloys was accompanied by a reduction of the killing rate. These results were published in a peer-review journal, in 2005, by Wilks and co-workers (Wilks et al. 2005). These studies confirmed the ancient-knowledge that early civilizations had applied but not understood. Later on, many publications showed killing efficiencies of copper surfaces versus control surfaces against a variety of microorganisms. Much of the work was done in Bill Keevil's group. Methicillin-resistant *Staphylococcus aureus* (Noyce et al. 2006; Michels et al. 2009; Mehtar et al. 2008; Gould et al. 2009), *Listeria monocytogenes* Scott A (Wilks et al. 2006), Influenza A Virus (Noyce et al. 2007), *Clostridium difficile* (Weaver et al., 2008; Wheeldon et al., 2008), *Candida albicans* (Mehtar et al., 2008; Weaver et al., 2010), *Pseudomonas aeruginosa* (Elguindi et al. 2009; Mehtar et al. 2008; Gould et al. 2009), *Mycobacterium tuberculosis*, *Klebsiella*

pneumoniae and *Acinetobacter baumannii* (Mehtar et al., 2008), Vancomycin resistant *Enterococci* (VRE) (Warnes & Keevil 2011; Gould et al. 2009) and Methicillin sensitive *Staphylococcus aureus* (MSSA) (Gould et al., 2009), *Aspergillus niger, A. flavus*, *A. fumigatus*, *Fusarium culmonium*, *F. oxysporium, F. solani*, *Penicillium chrysogenum* (Weaver et al., 2010), *Enterococcus hirae* (Molteni et al., 2010), *E. faecium* (Elguindi et al. 2011), were killed within hours on copper surfaces under wet conditions. Backup by all this results, the Environmental Protection Agency (EPA) registered almost 300 different copper alloys as antimicrobial in 2008 (http://www.epa.gov/pesticides/factsheets/copper-alloy-products.htm).

Prior to 2008, all the work was to test the antimicrobial efficiency against multiple microbes but there was no progress in elucidating the mechanism on how bacteria die on copper surfaces. Only in 2008 (Espírito Santo et al., 2008), made an initial step towards understanding the mechanisms for metallic copper surface-mediated killing of bacteria. For the first time, an alternative method was developed to mimic touch to dry surfaces. In dry copper exposure cells are applied directly on the surface within very little buffer, which evaporates very rapidly, within seconds, mediating immediate contact between cells and surface. Using this method cells were killed within minutes (Figure 3A) (Espírito Santo et al., 2008). This method may be applied as a laboratory model to simulate surfaces being contaminated by touch or air particles in hospitals or other public places, coins, and air conducts. The Grass group did most of the work using this dry copper exposure model. *E. coli W3110* (Espírito Santo et al., 2011, Espírito Santo et al., 2008), *Acinetobacter johnsonii*, *Pantoea stewartii*, *Pseudomonas oleovorans*, *Staphylococcus warnerii*, *Brachybacterium conglomeratum* (Espírito

Santo et al., 2010), *Candida albicans*, *Saccharomyces cerevisiae* (Quaranta et al., 2011), *Staphylococcus haemolyticus* (Espírito Santo et al., 2012), *Francisella tularensis*, *Bacillus cereus*, *B. anthracis*, *Brucella melitensis*, *Burkholderia mallei*, *B. pseudomallei*, *Yersinia pestis* (Bleichert, Espírito Santo & Grass, unpublished results), were all inactivated within minutes under dry copper surfaces.

There are several factors that were identified to influence killing rates: temperature (Figure 8B), alloy copper content (Figure 8), copper ion toxicity, copper chelators (Figure 9A), osmotic stress (Figure 9B), and reactive oxygen species (Figure 9B), but surprisingly not anaerobiosis. Strains genetically deleted in their copper detoxification systems were only slightly more sensitive than wild-type strain (Figure 8 and 5). Finally, pre-adaptation to copper enhanced survival rates upon copper surface exposure (Figure 5) to some extend but did not prevent killing.

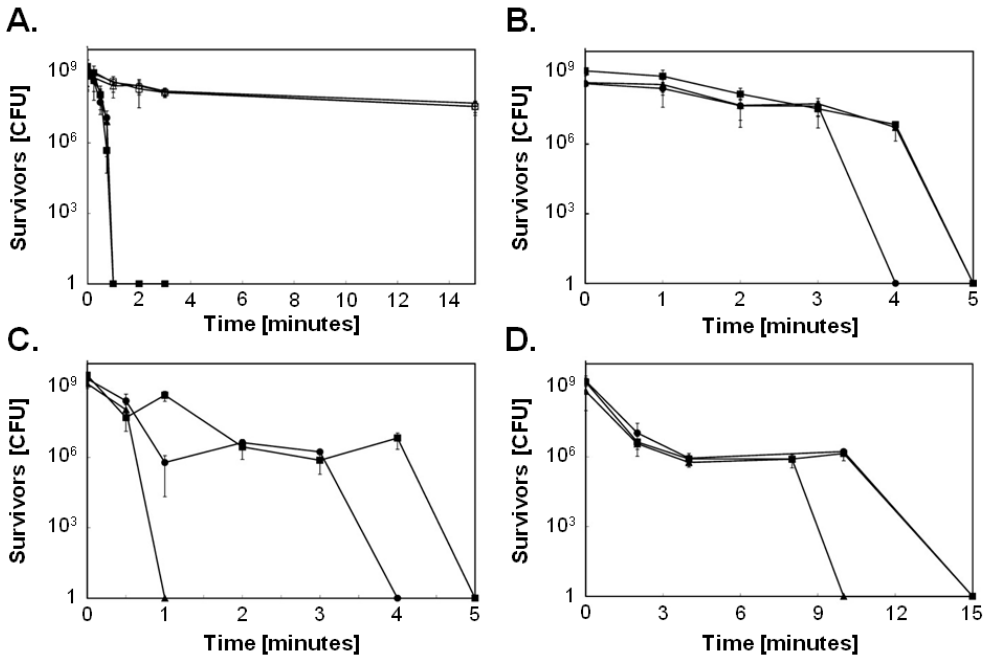

Figure 8 – Survival of copper-resistant or -sensitive *E. coli* strains on copper surfaces and those of its alloys and stainless steel. Cells of *E. coli* wild-type strain W3110 (■), its copper-sensitive derivative Δ*copA* Δ*cus* Δ*cueO* (▲) or W3110 harboring the high-level copper resistance system Pco (●) were streaked on copper alloy surfaces (filled symbols) or stainless steel (open sympols). After the indicated time periods at ambient conditions (23°C [A, C, and D] or 5.5°C [B])

cells were removed from metal surfaces, diluted, and plated on LB agar. Surviving cells were counted as CFU after 16 h at 37°C. The alloys were pure copper (C11000, 99.9%) (A and B), "nickel-silver" (C75200, maximum of 62% Cu) (C), Muntz metal (C28000, maximum of 62% Cu) (D), and stainless steel (AISI 304) (A). Shown are averages with standard deviations (error bars) from three independent experiments (Espírito Santo et al., 2008).

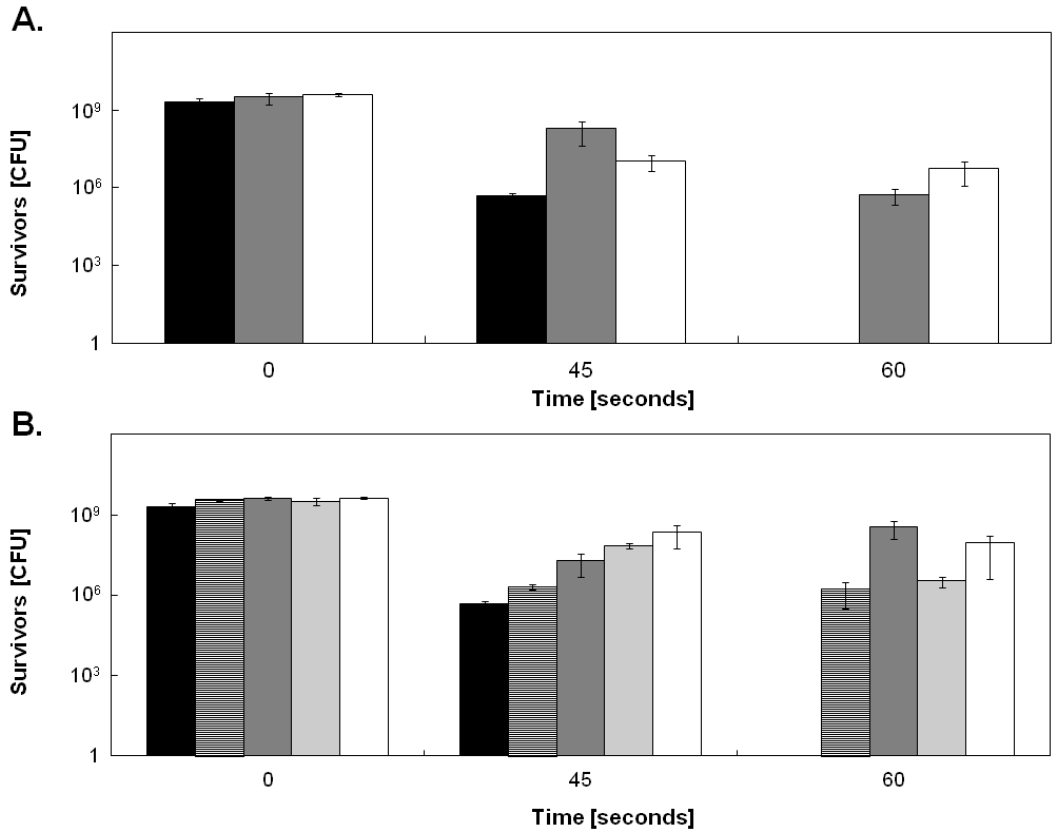

Figure 9 – Protective effects of metal chelators (A) and reactive oxygen species quenchers or sucrose (B) on the survival of *E. coli* on copper surfaces. Cells were treated as described in the legend for Figure 3 except samples were withdrawn only after 0, 45, and 60 s. Cells were mixed with Cu(II) chelator EDTA ([A], gray bars), Cu(I) chelator BCS ([A], white bars), ROS quenchers mannitol ([B], horizontally striped bars), catalase ([B], dark gray bars), superoxide dismutase ([B], light gray bars) sucrose ([B], white bars), or no additive ([A, B], black bars) prior to application onto copper surfaces. Shown are averages with standard deviations (error bars) from three independent experiments (Espírito Santo et al., 2008).

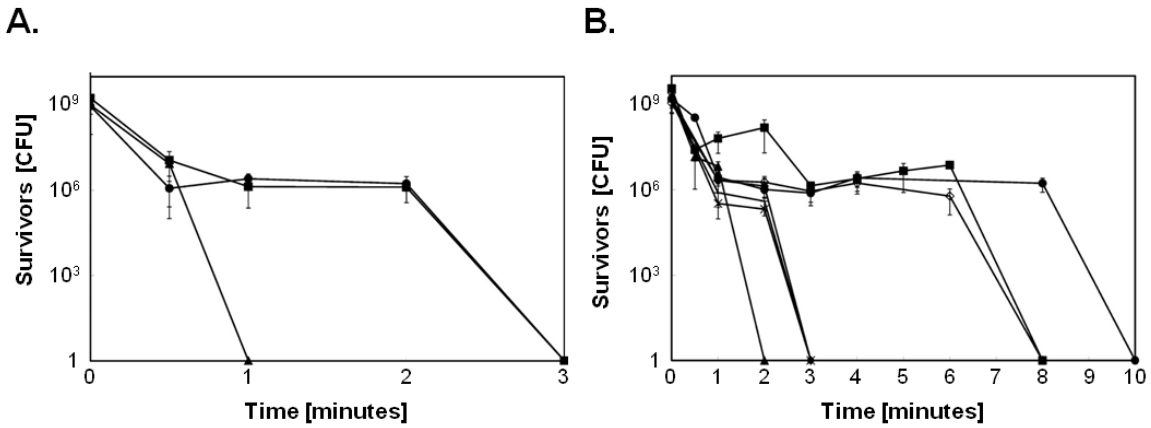

Figure 10 – Survival of preadapted *E. coli* strains on copper alloy surfaces. *E. coli* cultures were grown overnight in the presence of nontoxic concentrations of $CuCl_2$ to induce copper detoxification systems. Washed cells of *E. coli* wild-type strain W3110 (■) or its copper-sensitive Δ*copA* Δ*cus* Δ*cueO* (▲), Δ*cus* Δ*cueO* (♦), Δ*copA* (+), Δ*cueO* (X), Δ*cus* (◊), or W3110 harboring the high-level copper resistance system Pco (●) were streaked on 99.9% copper (A) or "nickel silver" alloy (C75200, maximum of 62% Cu) (B) surfaces and treated as described in the legend for Figure 3. Shown are averages with standard deviations (error bars) from three independent experiments (Espírito Santo et al., 2008).

In 2009, it was suggested that DNA was the target for copper surface toxicity but no experiments were made to prove this hypothesis (Michels et al. 2009). Molteni and co-workers (2010) showed that also in Gram-positive *Enteroccoccus hirae*, mutants lacking the cellular copper-export system were more sensitive to killing by "wet platting" on copper than on stainless steel (control surface). Furthermore, it was established that media composition influences the killing rate. When *E. hirae* was suspended in a Tris-buffer, cells became more sensitive to copper surface toxicity than if cells were in water or phosphate-based buffer. Likewise, copper ion release was higher when Tris-buffer was used but water was the media that provoked lower copper ion release. In the same year, Bill Keevil group (Warnes et al., 2010; Warnes & Keevil, 2011) that previously suggested that DNA might be the target in copper surface toxicity, published their findings explaining this hypothesis. Experiments were performed using the wet method, involving

DNA stains, gel electrophoresis and genomic DNA fragmentation assays that were intended to support the claim that copper surfaces induce DNA damage, thus effectively killing cells. Additionally, Warnes and co-workers (2011) claim that membranes do not suffer damage, however respiration is impaired.

In 2010, Gram-positive bacteria was reported to survive longer than Gram-negative bacteria on dry copper surfaces (Espírito Santo et al., 2010). Bacteria isolated from copper alloy coins include strains that are able to survive and handle the toxic properties exerted by dry metallic copper surfaces. Several isolates survived on copper surfaces for forty-eight hours or more. But when the same isolates were exposed to moist surfaces or dissolved copper ions, isolates exhibited resistance levels close to those of the copper surface-sensitive type strains (Espírito Santo et al., 2010). These results suggest that some bacteria have possibly resistance mechanisms against dry copper surfaces different to the well-characterized copper ion homeostasis systems. Elguindi and colleagues (2011), reached similar conclusions to previous published data: killing kinetics of copper surfaces were influenced by the amount of moisture present, copper content of alloys, type of medium used, and type of bacteria. Furthermore, presence of corrosion inhibitors such as benzotriazole (BTA) lowers the copper ion release from copper surfaces thus diminishing the killing capacity (Elguindi et al., 2011).

Copper surface killing mechanisms were recently studied in detail (Espírito Santo et al., 2011): *E. coli* cells were exposed by the wet and dry methods. Cells accumulated high copper concentration in both methods, but by dry exposure the accumulation was virtually immediate. In addition, molecular targets for dry copper surface toxicity were investigated. Exposure to dry copper surfaces

caused cells to suffer extensive membrane damage within minutes and, after prolonged exposure, cells underwent structural disintegration. Furthermore, a short sharp shock from contact with copper surfaces did not provoke mutations or DNA fragmentation (Espírito Santo et al., 2011). Similar results were obtained using yeast cells (Quaranta et al., 2011). Yeast cells rapidly accumulated copper, but mutations were not observed. Membranes became depolarized and damaged. While exposed to copper surfaces, yeast vacuoles first became enlarged and then disappeared altogether indicating massive cell damage. Additionally, oxidative stress in the cytoplasm and mitochondria was elevated during copper surface exposure. Also, copper homeostasis systems influenced killing kinetics. Faster inactivation was obtained by a hyperactive mutant copper uptake transporter from *Saccharomyces cerevisae* Ctr1p (ScCtr1p) that is genetically rendered unregulated for copper-uptake. Similarly, lack of *Candida albicans* Crp1p (CaCrp1p) copper-efflux P-type ATPase or of the metallothionein CaCup1p caused mutant cells to be killed more rapidly than wild-type cells (Quaranta et al., 2011). The controversy about DNA as a target of copper surface toxicity was further debated by (Warnes & Keevil 2011). Authors proposed that the first stages of Gram-positive enterococci cell death via copper surfaces involves release of ionic copper and generation of superoxide, resulting in impaired respiration and DNA breakdown. This study reported that hydroxyl radicals, produced by the Fenton reaction, were not the major toxic factor for DNA damage. Additionally, membranes would remain intact by wet and dry exposure to copper surfaces. However, other conclusions were obtained with Gram-positive bacteria such as *Staphylococcus haemolyticus* (Espírito Santo et al., 2012) on dry copper surfaces: it was harder to effectively kill Gram-positive

bacteria when comparing with Gram-negative; cells accumulated high copper levels very quickly, however, no DNA mutations were observed; finally, cells underwent membrane damage when exposed to copper surfaces. In 2012, another group has clearly demonstrated that membranes went through lipid peroxidation when cells were exposed to a hybrid of moist/dry copper surfaces, and DNA degradation was not the primary cause of copper-mediated surface killing (Hong et al., 2012).

1.6. Antimicrobial copper in healthcare settings.

Dr. Phyllis J Kuhn (Kuhn, 1983) was one of the first to notice the bacteriostatic effect of copper. Dr. Kuhn trained housekeeping and maintenance personnel at the Hamot Medical Center in Pennsylvania. To raise students' awareness on ways of infection transmission, students were given blood agar plates and they sampled diverse sources: toilet bowl water (remarkably clean), salad from the employees' cafeteria (heavily colonized), and doorknobs. Brass (67% copper and 33% zinc) doorknob cultures showed scarce staphylococcal and streptococcal growth while stainless steel (about 88% iron and 12% chromium) doorknob cultures showed heavy growth of Gram-positive organisms and an array of Gram-negative organisms. Under laboratory conditions, antimicrobial properties of copper surfaces have been well established as outlined in the previous section. On the other hand, antimicrobial copper surfaces must also show efficacy as an additional barrier against microbes in healthcare settings. As an important caveat it should be mentioned that metallic copper surfaces cannot replace strict hygienic conditions but instead act as an additional approach that may help reducing microbial surface burden and consequently can be envisioned to

diminish infection rates in patients. It is known that regular cleaning and proper hygiene conditions help to lower transmission-rates of infectious diseases, but complete elimination of germs appears to be unrealistic (Dancer, 2008). Hospital surfaces are highly contaminated with microorganisms, as was shown for *C. difficile*, *Acinetobacter spp., Enterococcus spp* and *S. aureus* that are able to persist on regular surfaces for months (Kramer et al., 2006). Therefore, the usage of an actively antimicrobial surface might strongly diminish transmission of microbes to humans by reducing fomite contamination (Figure 2). Worldwide hospital trials confirmed metallic copper as an antimicrobial surface (Casey et al., 2010; Karpanen et al., 2012; Marais et al., 2010; Mikolay et al., 2010). Trials were able to validate that applying metallic copper surfaces effectively reduced surface burden compared with control surfaces (such as stainless steel, aluminum and plastic). During the 2010 trial in the Selly Oak Hospital in Birmigham, United Kingdom (Casey et al., 2010), recovery of microbes was between 90 to 100% lower from copper surfaces compared to control surfaces. Copper surfaces remained active even when these surfaces were oxidized (“aged”) over time. Similar positive results were obtained by Karpanen et al. (2012), copper alloys (greater than or equal to 58% copper) reduced microbial quantity on the surface compared with control surfaces. A reduction of 71% on copper surfaces compared to control surfaces was obtained in the South African trial (Marais et al., 2010). The German trial also reported a surface burden reduction in the magnitude of 63% (Mikolay et al., 2010). Furthermore, the repopulation rate of copper surfaces was less than half compared to the control surfaces.

There are still ongoing trials worldwide. Promising results were obtained on the trial that involves three hospitals: the Memorial Sloan-Kettering Cancer Center in

New York City, and the Medical University of South Carolina, and the Ralph H. Johnson VA Medical Center, both in Charleston (Michael Schmidt, personal communication). Application of metallic copper, lowered infection-rates by 40% when patients were in rooms with copper objects compared with patients in rooms with no copper objects (Michael Schmidt, personal communication).

These recent trials are promising signs that copper surfaces appear to be active and sustainable under the conditions of healthcare environments. Copper surfaces are in fact able to reduce the microbial burden; subsequently, there is a lower risk of exposure that seems to lead to lower infection-rates. Further trials and additional studies need to address the challenge of durable spores of endospore formers, eukaryotic microbes and the longer-term sustainability of copper-surfaced appliances.

2. Objective

This work aims to understand the toxic factors involved and the molecular targets of metallic copper surfaces toxicity in bacteria. The outcomes can be expected to help build a clear picture on the killing mechanism of microbes by metallic copper surfaces. This process is paramount to be determined, in order to make predictions on the safe use of appliance mode of metallic copper surfaces in healthcare. These surfaces should be active against microbes and remain active on the long-term with no risk of microbial resistance. Finally, this knowledge might aid in development of further improved copper alloy surfaces with long-life span and high efficacy.

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Chapter 2

Isolation and Characterization of Bacteria Resistant to Metallic Copper Surfaces

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Abstract

Metallic copper alloys have recently attracted attention as a new antimicrobial weapon for areas where surface hygiene is paramount. Currently it is not understood on a molecular level how metallic copper kills microbes, but previous studies have demonstrated that a wide variety of bacteria, including *Escherichia coli*, *Staphylococcus aureus*, and *Clostridium difficile*, are inactivated within minutes or a few hours of exposure. In this study, we show that bacteria isolated from copper alloy coins comprise strains that are especially resistant against the toxic properties exerted by dry metallic copper surfaces. The most resistant of 294 isolates were Gram-positive staphylococci and micrococci, *Kocuria palustris*, and *Brachybacterium conglomeratum* but also included the proteobacterial species *Sphingomonas panni* and *Pseudomonas oleovorans*. Cells of some of these bacterial strains survived on copper surfaces for 48 hours or more. Remarkably, when these dry-surface-resistant strains were exposed to moist copper surfaces, resistance levels were close to those of control strains and minimum inhibitory concentration (MIC) for copper ions were at or below control strain levels. This suggests that mechanisms conferring resistance against dry metallic copper surfaces in these newly isolated bacterial strains are different from well-characterized copper ion detoxification systems. Furthermore, staphylococci on coins did not exhibit increased levels of resistance to antibiotics, arguing against coselection with copper surface resistance traits.

Keywords: copper surface, *Staphylococcus*, *Bacillus*, skin microflora, metal toxicity, 16S rDNA analysis.

Introduction

Copper in its ionic form is a required trace element for most pro- and eukaryotic organisms, including humans. While needed in small amounts, copper can easily become toxic when in surplus. This toxicity is caused mainly by the intrinsic properties of copper, as free copper ions undergo redox cycling reactions alternating between Cu(I) and Cu(II). This also results in the transfer of electrons to hydrogen peroxide and the concomitant generation of hydroxyl radicals that readily attack and damage cellular biomolecules. Recently, it was found that the majority of copper stress in *Escherichia coli*, as indicated by hydroxyl radical formation, occurs within the periplasm, away from the cytoplasmic DNA, and is thus copper-mediated oxidative stress (Macomber et al., 2007). The cytoplasm might thus be better protected from copper-mediated oxidative stress, and indeed cells usually prevent accumulation of significant intracellular concentrations of free copper ions either by producing copper-binding chaperones (Magnani et al., 2008; Singleton & Le Brun, 2007) or unspecific chelators such as glutathione (Helbig et al., 2008; Miras et al., 2008) or by efflux (Franke et al., 2003; Rensing et al., 2000). Nevertheless, copper ions within the cytoplasm also cause damage. Surprisingly, this damage is not related to oxidative stress but is exerted directly by the metal ions. It seems that copper ions attack and displace iron atoms from enzymes with solvent-exposed iron sulfur clusters such as those of hydratases (Macomber & Imlay, 2009). Thus, the presence of oxygen is not needed for this reaction, and there is no copper-mediated oxidative stress involved in this damage (Macomber & Imlay, 2009).

While we are now gaining a more detailed picture of why copper ions are toxic to cells, we do not understand why metallic copper surfaces kill single-celled

organisms such as bacteria and yeasts. Earlier studies have demonstrated that metallic copper surfaces efficiently inactivate microbes upon contact (Elguindi et al., 2009; Faúndez et al., 2004; Noyce et al., 2006), especially when exposed to dry surfaces (Espírito Santo et al., 2008). These beneficial properties led to the official registration of copper alloys as antimicrobials through the U.S. Environmental Protection Agency in 2008. There is now great hope that metallic copper surfaces will be able to help control hospital-acquired (nosocomial) infections (HAI). Indeed, there are ongoing trials in which dry touch surfaces in hospitals around the world are replaced by copper alloys. Results from a German hospital trial indicate that copper surfaces such as door knobs, light switches, and push plates diminished the bacterial load by up to 30% compared to stainless steel control surfaces (Mikolay et al., 2010). Similar studies in Great Britain and South Africa found that the numbers of bacteria on the surfaces of copper-containing items such as trolleys, desks, toilet seats, tap handles, or push plates were 71% (Marais et al., 2010) or 90% to 100% (Casey et al., 2010) lower than those on their stainless steel, wood, or tile control equivalents.

A potential challenge when applying metallic copper might be the probable emergence and spread of resistant bacteria, similar to what was observed after the introduction of antibiotics. The goal of this study was to investigate if bacteria that can withstand dry metallic copper surfaces can be isolated and if there is a link to multiple drug resistance. Where can potentially pathogenic bacteria that are in contact with both humans and metallic copper surfaces be found? Actually, people handle copper surfaces every day. Most coins around the world are made from copper or copper alloys. This includes the U.S. penny, which is

composed of copper plated over a zinc core, and the nickel, dime, and quarter, which are cupronickel alloys (www.usmint.gov/). Coins of the European Union, such as the 50-cent coin, are made from an 89% copper alloy, as are the bicolored one- and two-Euro coins, which consist of different copper alloys (http://www.copperinfo.co.uk/coins/).

In the present study we isolated and initiated characterization of aerobic heterotrophic bacteria from copper alloy coins as an example of heavily used copper surfaces and person-to-person vectors. We believe that knowledge of the physiology and resistance mechanisms of these microbes will help us to adapt our strategies for using metallic copper surfaces in hygiene-sensitive areas. This might not only diminish total bacterial numbers but also prevent the emergence and spread of multiple-drug-resistant strains in hospitals equipped with copper surfaces.

Materials and methods

Bacterial strains and growth media. The following type and collection strains were used in this study: *Staphylococcus haemolyticus* DSM 20263, *Staphylococcus hominis* DSM 20328, *Staphylococcus warnerii* DSM 20316, *Staphylococcus epidermidis* DSM 20044, *Acinetobacter johnsonii* DSM 6963, *Micrococcus luteus* DSM 20030, *Bacillus anthracis* Sterne 34F2 ($pXO1^+$ $pXO2^-$) (Sterne, 1937), *Bacillus cereus* DSM 31, *Pantoea stewarti* DSM 30176, *Brachybacterium conglomeratum* DSM 1-241, *Massilia timonae* DSM 16850, *Kocuria marina* JCM 13363, *Psychrobacter faecalis* DSM 14664, *Pseudomonas oleovorans* DSM 1045, and *Sphingomonas panni* (DSM 15761).

Strains were grown in Luria-Bertani broth (Difco BD, Sparks, MD), nutrient broth (Difco BD, Sparks, MD), or R2A medium (Difco BD, Sparks, MD) as required at 30°C with shaking to stationary growth phase (16 to 32 hours (h) of incubation). Bacto Agar (Difco BD, Sparks, MD) was added at 15 g/liter for solid media.

Isolation procedures. Aerobic heterotrophic bacteria were isolated from regular European 50-cent coins collected in Germany (27 coins) and Portugal (25 coins) in October 2007. Before use, coins were incubated in sterile plastic bags or petri dishes for at least 24 h at room temperature to reduce contamination with adventitious environmental germs. Two independent isolation procedures were performed. For method one, both sides of the coins were stamped on solid agar medium plates using sterile forceps. The plates were incubated at 30°C until colonies formed (1 to 3 days). Colonies were purified by streaking repeatedly on the same media. Alternatively, coins were incubated in liquid media and shaken for 2 days at 30°C. Mixed cultures were then diluted in the same growth media

and plated. Colonies were purified by repeatedly streaking on the same media. Overall, each of the three growth media was used for about one-third of copper surface bacteria for selection and isolation.

DNA extraction, 16S rDNA amplification and sequencing. Total DNA of the isolates was extracted from stationary cultures either by the freeze thaw method (Nielsen et al., 1995) or with the Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Amplification of the 16S rRNA gene was accomplished by PCR with the High-Fidelity DNA-Polymerase Mix (Roche, Indianapolis, IN) or Taq polymerase (Sigma, St. Louis, MO.), 1.5 mM $MgCl_2$, 100 µM dNTPs and 10 pmol of the primers 27F (5'-AGAGTTTGATCMTGGCTCAG; corresponding to *E. coli* 16S rDNA bases 8 to 27) (Johnson, 1994) and 1525R 5'-AAGGAGGTGWTCCARCC-3' (corresponding to *E. coli* 16S rDNA bases 1525 to 1541) (Johnson, 1994) with about 100 ng template DNA. PCR reactions were performed for 32 cycles, each consisting of a 30 s denaturation step at 96°C, a 30 s annealing step at 52°C, and a 1 minute extension step at 72°C.

PCR products were purified and partially sequenced on an ABI 3730xl automated sequencer by GATC Biotech AG (Konstanz, Germany) in a 96-well set-up using primer 16S rDNA 27F 5'-AGAGTTTGATCMTGGCTCAG-3' (Johnson, 1994). Alternatively, PCR products were sequenced at Laboratory Microbiology (Faculdade de Ciências e Tecnologia, Universidade de Coimbra, Portugal) on an ABIPrism310 automated sequencer with the same primer.

Taxonomic and phylogenetic analysis. The quality of 16S rRNA gene sequences were checked manually using Bioedit editor (Hall, 1999) and aligned

against representative reference sequences of the most closely related members, obtained from the Ribosomal Database Project (Cole et al., 2007) and European Molecular Biology Laboratory (EMBL), using the multiple-alignment CLUSTAL X software package (Aiyar, 2000). The method of Jukes and Cantor (Jukes & Cantor, 1969) was used to calculate evolutionary distances. Sequences were also checked for chimeric properties by using CHECK_CHIMERA routine of the Ribosomal database Project II (RDP-II) (Maidak et al., 2001). Phylogenetic analysis was conducted using the neighbor-joining method as implemented in the computer program Molecular Evolutionary Genetics Analysis (MEGA4) (Tamura et al., 2007) to determine the phylogenetic placement of each isolate relative to the type strains. Trees topologies were evaluated by performing bootstrap analysis (Felsenstein, 1985) of 1,000 data sets by using the MEGA4 package (Kumar et al., 2004). An isolate was considered to be a member of a particular species if the isolate and the particular type strain clustered with a bootstrap value of 90% or greater and displayed a similarity of 97% or more with respect to their 16S rRNA gene sequence divergence.

Dry metallic copper surface testing. For identification of metallic copper surface resistant bacteria approximately 10^9 cells of the isolates were applied to 1 x 1 inch copper coupons (C1100) as described previously (Espírito Santo et al., 2008). Cells were left on the coupons for 1 day, 2 days, 7 days and 31 days under ambient conditions in sterile plastic Petri dishes. In contrast to the original protocol (Espírito Santo et al., 2008) bacteria were not removed with phosphate buffered saline (PBS) but stamped directly on solid agar media and the coupon removed. Resistance to copper surfaces was counted negative if less or equal to

10 colonies were observed after incubation for 2 days. However, in most incidences there was a clear cut between resistant and sensitive isolates because resistant strains yielded a high density of colonies. For comparison, the bacteria were also tested on stainless steel (S304) surfaces to address sensitivity against desiccation. Results were compared with type strains and strain-collection bacteria of the same species. Experiments were repeated three times.

Moist metallic copper surface testing. Frequently, bacteria are also tested for resistance on metallic copper surfaces that have kept moist for the duration of the experiment (Elguindi et al., 2009; Faúndez et al., 2004; Noyce et al., 2006a; Wheeldon et al., 2008). We also tested the isolates for the occurrence of copper surface resistances under these conditions. For this, 40 µL aliquots of approximately 10^9 cells in PBS of each isolate were applied as a standing droplet on copper coupons in triplicates and 10 µL were removed after 1, 3, 24, 48 hours and 7 days and plated on solid agar media. Survivors were counted as colony forming units. Experiments were repeated three times.

Determination of $CuCl_2$ Minimal Inhibitory Concentrations (MIC). Cultures were grown until stationary growth phase (24-48 hours) at 30°C with shaking. Cultures were diluted 1:100 in fresh media and streaked onto solid media containing increasing $CuCl_2$ concentrations and incubated at 30°C. Growth was examined after 2 days. Experiments were repeated three times.

Evaluation of antibiotic resistance levels amongst Staphylococci. Antibiotics susceptibility of all Staphylococcal isolates was determined by the agar diffusion

method, using the BioMérieux antibiotic disks (Bio-discs, BioMérieux, Marcy l'Etoile, France; for details see Table A1 in the Annex A). In short *Staphylococci* were streaked on fresh solid agar media and grown at 30ºC for 24 hours. With the help of a sterile loop, cells were removed and resuspended in sterile deionized water. 100 µL of the cell suspension were transferred and spread on Mueller Hinton agar plates (Difco BD, Sparks, MD). After the plates were dried, antibiotic disks were applied on the surface of the agar. Plates were incubated at 30ºC for 24 hours. The growth inhibition diameters were measured and bacteria classified as sensitive, intermediate or resistant according to established French national guidelines (Cavallo et al., 2005).

Results

Bacteria can be isolated from copper coins. We reasoned that copper coins are ideal starting materials for the natural selection of metallic copper surface-resistant bacteria. Also, copper coins from general circulation are a suitable contact interface with the human skin microbiome because coins are handled by a wide variety of people with diverse sets of personal skin microbes. Thus, we hypothesized that there is significant overlap between human-associated bacteria and bacteria exposed to copper coins. These germs in turn could be potentially transferred to antimicrobial copper surfaces in hospitals.

For isolation of copper surface-resistant bacteria, we employed two different methods. Both the liquid and the solid medium selection procedures yielded an assortment of (facultative) aerobic heterotrophic bacteria. The advantage of the solid media was that the number of bacteria from each face of the coins could be directly counted. This amounted to an average of about six colony forming units (CFU) per coin (independent of the origin of the coins). Including the liquid procedure, a total of 294 isolates were isolated from 52 coins (Table 1). As the goal was to isolate copper surface-resistant bacteria, complete coverage of the bacterial coin population was not paramount. Therefore, colonies obtained from the liquid medium procedure were selected by colony morphology, whereas all isolates from the solid medium procedure were used for further analysis. This might have resulted in a small bias toward a higher incidence of species from different groups of bacteria and toward fewer species from the same genus.

Table 1 - Strains isolated from copper coins.

Species	Family	No. of isolates	No. of isolates surviving at day:		
			1	2	7
Brachybacterium conglomeratum	*Dermabacteraceae*	2	1	1	1
Dermacoccus nishinomiyaensis		2	0	0	0
Agrococcus jenensis	*Microbacteriaceae*	1	0	0	0
Curtobacterium flaccumfaciens		2	0	0	0
Frigoribacterium faeni		1	0	0	0
Microbacterium insulae, Microbacterium lacticum, Microbacterium oxydans, Microbacterium ulmi		4	1	0	0
Micrococcus luteus, Micrococcus lylae	*Micrococcaceae*	59	23	8	5
Pseudoclavibacter helvolus		1	1	0	0
Arthrobacter chlorophenolicus, Arthrobacter oxydans		2	0	0	0
Kocuria marina, Kocuria palustris, Kocuria rhizophila, Kocuria sp.		10	4	1	0
Aeromicrobium sp.	*Nocardioidaceae*	1	0	0	0
Propioniferax innocua	*Propionibacteriaceae*	1	0	0	0
Bacillus anthracis, Bacillus benzoevorans, Bacillus cereus, Bacillus circulans, Bacillus insolitus, Bacillus licheniformis, Bacillus macroides, Bacillus megaterium, Bacillus mycoides, Bacillus nealsonii, Bacillus phychrodurans, Bacillus pumilus, Bacillus silvestris, Bacillus simplex, Bacillus sp., *Bacillus thuringiensis, Bacillus weihenstephanensis*	*Bacillaceae*	50	0	0	0
Paenibacillus cineris, Paenibacillus favisporus, Paenibacillus rhizosphaerae	*Paenibacillaceae*	1	0	0	0
Lysinibacillus fusiformis, Lysinibacillus sphaericus	*Planococcaceae*	1	1	0	0
Staphylococcus capitis, Staphylococcus equorum, Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus hominis, Staphylococcus lugdunensis, Staphylococcus pasteuri, Staphylococcus sp.,*Staphylococcus vitulinus, Staphylococcus warneri*	*Staphylococcaceae*	115	27	11	0
Leuconostoc citreum	*Leuconostocaceae*	2	1	0	0
Brevundimonas bullata	*Caulobacteraceae*	4	0	0	0
Roseomonas pecunia	*Acetobacteraceae*	1	0	0	0
Sphingomonas panni, Sphingomonas sp.	*Sphingomonadaceae*	2	1	1	0
Cupriavidus metallidurans	*Burkholderiaceae*	1	0	0	0
Massilia aurea, Massilia timonae	*Oxalobacteraceae*	2	0	0	0
Enterobacter cowanii	*Enterobacteriaceae*	1	1	0	0
Erwinia persicina		1	1	0	0
Pantoea agglomerans, Pantoea ananatis, Pantoea stewartii, Pantoea vagans		13	5	0	0
Acinetobacter iwoffii, Acinetobacter johnsonii, Acinetobacter ursingii	*Moraxellaceae*	4	1	0	0
Moraxella osloensis		2	1	0	0
Psychrobacter faecalis		3	1	0	0
Pseudomonas asplenii, Pseudomonas fragi, Pseudomonas oleovorans, Pseudomonas putida	*Pseudomonadaceae*	5	1	1	0
Stenotrophomonas maltophilia	*Xanthomonadaceae*	1	0	0	0

Gram-positive bacteria are the predominant group of bacteria from copper coins and are the most resistant to copper surfaces. The majority of the isolates were Gram-positive bacteria (Figure 1 and Table 1). The largest group comprised the staphylococci (115 isolates), followed by micrococci (59 isolates) and bacilli (50 isolates). The remainder belonged to the *Actinobacteria* (except micrococci), including other *Micrococcineae* (24 isolates) and *Propionibacterineae* (2 isolates). One species, *Roseomonas pecunia*, has not been characterized before; a complete species description has been published (Lopes et al., 2011).

Finally, four non-Bacillus Firmicutes isolates were identified. Surprisingly, no corynebacteria or flavobacteria were among the isolates from coins.

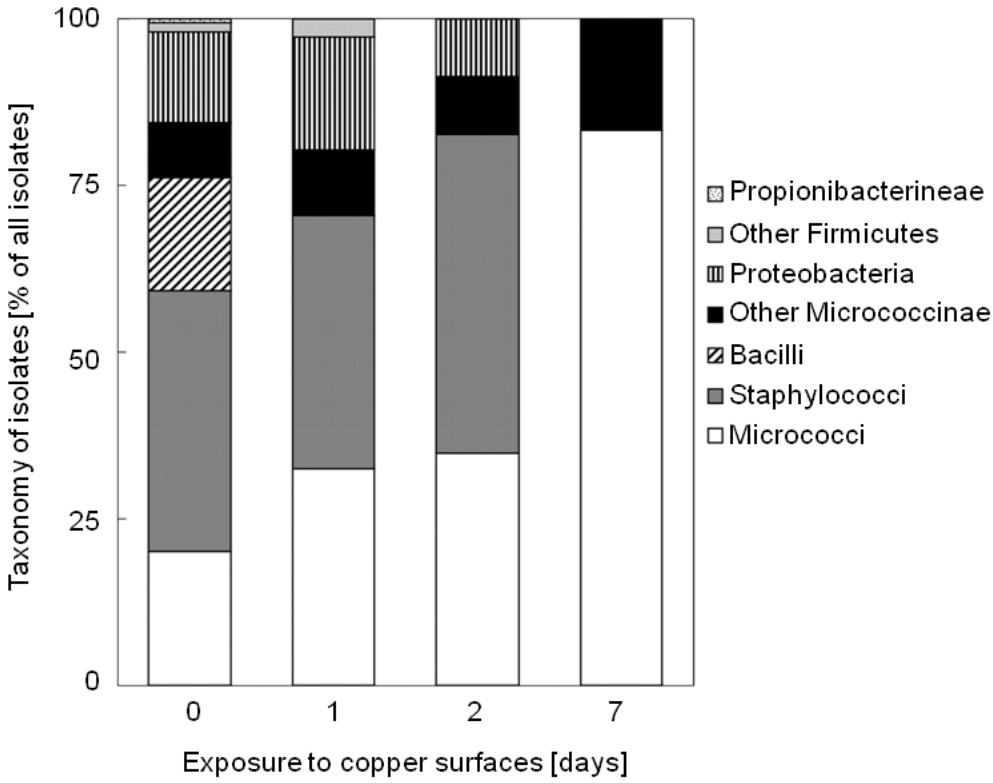

Figure 1 – Relative abundance of bacteria isolated from copper coins and their resistance to metallic copper surfaces. The relative abundance of 294 bacterial strains isolated from European copper coins is shown in time point 0. Relative abundance of copper surfaces resistant isolates after exposure to experimental pure metallic copper surfaces for different periods of time is indicated (1, 2, 7).

Only 40 isolates were Gram negative (Figure 1). The biggest group among these were the gammaproteobacteria (30 isolates), including *Pseudomonas oleovorans* (this strain was renamed *Pseudomonas psychrotolerans* L19 after sequencing of the complete 16S rRNA gene which has a 99.5% similarity to *P. oleovorans*), *Pantoea stewartii*, and *Acinetobacter johnsonii*. Other proteobacteria were from the alphaproteobacteria subgroup (seven isolates), including two species of the genus *Sphingomonas* (two isolates), and a few betaproteobacteria (three isolates), with two Massilia species.

Upon testing for survival on pure copper surfaces (99% Copper, C110), a total of 71 isolates were found to survive 1 day of exposure, 23 survived 2 days of exposure, and 6 survived 1 week of exposure, but none survived 1 month of exposure (Figure 1). The predominant genus of the 1-day survivors was *Staphylococcus* (27 strains), followed by *Micrococcus* (23 strains), the Gram-negative bacteria (proteobacteria) (12 strains), and *Micrococcineae* other than micrococci (7 strains). A similar trend was observed after 2 days of exposure, but the staphylococci (11 strains) and the micrococci (8 strains) had similar numbers of surviving strains. Only two actinomycetales other than micrococci, (*Kocuria palustris* and *Brachybacterium conglomeratum*) and two proteobacteria (*Sphingomonas panni* and *Pseudomonas oleovorans*) remained among the survivors. Finally, after 7 days only four micrococcal strains (all *M. luteus*) and one *Brachybacterium conglomeratum* strain survived. In general, the isolates survived from 16 times (*M. luteus*) up to 5,760 times (*P. oleovorans*) longer on copper surfaces than their type strains or controls (Table 2). Genome of *P. oleovorans* L19 was sequenced and published as a draft genome (Annex B).

Remarkably, most but not all control and type strains of the respective copper

coin-resistant isolates were killed much faster than the isolates. There were two exceptions to this trend. One was *Kocuria marina*, which survived 48 h, whereas the coin strain of *K. marina* survived for only 24 h. The other exception was *Sphingomonas panni*. Each *S. panni* strain survived for 48 h on metallic copper (Table 2). This suggests that resistance against metallic copper surfaces is rare but not absent from bacteria, especially among other strains of species that were found on copper coins.

Table 2 – Resistance of representative copper coin isolates to dry or moist copper surfaces and ionic copper.

Type or control strain or coin isolate[a]	Survival time on:		MIC (mM $CuCl_2$)
	Dry copper surfaces	Moist copper surfaces	
Escherichia coli W3110[b]	>30 s	>1 h	3.5
Pantoea stewartii DSM 30176	>30 s	>48 h	1.5
P. stewartii L10	>24 h	>1 h	4.5
Acinetobacter johnsonii DSM 6963	>1 min	>1 h	2.5
A. johnsonii L18	>24 h	>1 h	3.0
Pseudomonas oleovorans DSM 1045	>30 s	>1 h	2.5
P. oleovorans L19	>48 h	>24 h	3.5
Sphingomonas panni DSM 15761	>48 h	>48 h	2.0
S. panni R65P	>48 h	>1 h	0.75
Staphylococcus haemolyticus DSM 20263	>1 h	>3 h	3.5
S. haemolyticus L70	>48 h	>3 h	2.0
Staphylococcus epidermidis DSM 20044	>1 h	>1 h	1.0
S. epidermidis L77	>24 h	>24 h	1.5
Staphylococcus warnerii DSM 20316	>1 min	>1 h	2.5
S. warnerii L47	>48 h	>24 h	2.0
Brachybacterium conglomeratum DSM 10241	>10 min	>1 h	0.5
B. conglomeratum N96	>7 days	>1 h	0.5
Micrococcus luteus DSM 20030	>3 h	>24 h	2.0
M. luteus L51	>48 h	>3 h	1.5
Kocuria marina JCM 13363	>48 h	>1 h	2.0
K. marina L73	>24 h	>24 h	2.0
K. palustris R40	>48 h	>1 h	0.75

- [a] In each group, the first strain is a type or control strain and the second and/or third strain is a coin isolate.
- [b] Lab strain of *E. coli*, included as an example of a copper-sensitive bacterium.

All strains that were resistant to metallic copper surfaces survived for at least 1 month on stainless steel surfaces (data not shown). Most of the representative strains shown in Table 2 also survived for 1 month on plastic or aluminum, surfaces that cannot serve as a vital iron source. Unexpectedly, some Gram-negative strains were sensitive to aluminum or plastic surfaces. The type strain of *Pseudomonas oleovorans* was inactivated within 1 day upon exposure to plastic and aluminum but not to stainless steel (data not shown). The *Pantoea stewartii* type strain was inactivated on aluminum after 1 day but not on plastic or stainless steel surfaces. In general, all strains shown in Table 2 survived longer on plastic, stainless steel, or aluminum than on copper surfaces. Thus, the differences in survival time on dry metallic copper observed among the strains cannot be attributed to resistance against desiccation but are specific to dry metallic copper.

Coin bacteria are sensitive to wet copper surfaces and to copper ions. Recent studies demonstrated that the toxicity exerted by dry metallic copper surfaces is different from that of wet copper surfaces and that the mechanism of killing differs from that of copper ions (Elguindi et al., 2009; Espírito Santo et al., 2008). We therefore reasoned that the isolates found to be resistant to dry metallic copper (Figure 1 and Table 1) might carry new resistance mechanisms unrelated to defense systems against moist copper surfaces or ionic copper. When strains that survived dry metallic copper for 2 days were tested on moist copper coupons, some of them were more sensitive than their control strains. The coin isolate of *P. stewartii*, strain L10, was dead after1h of exposure, but the control survived for 1 week of exposure (Table

2). Similarly, *S. panni* R65P from coins was killed by moist exposure after 1 h, but the type strain survived for 48 h. The type strain of *M. luteus* survived eight times longer on moist copper than the *M. luteus* L51 isolate. Other coin isolates were as resistant to moist exposure as their controls (*A. johnstonii*, *S. haemolyticus*, and *B. conglomeratum*). This lower resistance against moist metallic copper suggests that the resistance mechanisms that have evolved to withstand dry copper surfaces are different from those needed for survival on moist copper.

Likewise, there was no correlation between the dry copper surface resistance of the 2-day survivors and their $CuCl_2$ MICs on solidified agar media (Table 2). Virtually all dry copper surface-resistant isolates were as sensitive to copper ions as their control strains and exhibited resistance levels comparable to those of *E. coli* (Table 2). Exceptions were *S. panni* R65P and *K. palustris* R40, which were about three times less resistant against $CuCl_2$, and *P. stewartii* L10, which was three times more resistant than its type strain (Table 2).

Staphylococci isolated from coins are no more antibiotic resistant than their type strains. It might be argued that prolonged use of metallic copper surfaces not only would select for resistance traits against this challenge but also would favor coselection with innate antibiotic resistance genes. We investigated if our staphylococcal strains from copper coins already had undergone such an evolutionary process by testing these bacteria in the bioMérieux antibiotic disk assay. Overall, the isolates did not exhibit an increased resistance to the antibiotics tested (Table 1). Most of the strains were scored as sensitive. This suggests that at least in the case of copper

coins, there has been no coselection of metallic copper resistance and resistance against antibiotics. However, this possibility might become an issue once copper surfaces are widely used in health-related areas, making constant evaluation of antibiotic resistance in exposed microbes obligatory.

Discussion

Bacteria from coins and copper surface resistance. Surprisingly little attention has been paid to the microbial load of copper coinage. The antimicrobial properties of coins have been demonstrated, and coins have been found to have a lower bacterial load than paper currency (Havas, 2000; Pachter et al., 1997). Nevertheless, coins have been shown to carry opportunistic pathogens, such as a variety of species of the genera *Staphylococcus*, *Bacillus*, and *Corynebacterium* (Pachter et al., 1997; Xu et al., 2005). An earlier study identified *S. aureus*, *E. coli*, and *P. aeruginosa* on coins (Abrams & Waterman, 1972). The most comprehensive study on the bacterial flora from coins was published in 2005, comprising a total of 25 isolates from coinage collected from 17 countries (Xu et al., 2005).

In the present study we isolated 294 strains from two countries, Germany and Portugal. Though the goal of the study was not an inventory of the bacterial diversity of copper coins, our results generally follow those of previous studies. Xu et al. reported 100% Gram-positive isolates from coins, with the majority belonging to the genera Bacillus (40%) and Staphylococcus (28%) (Xu et al., 2005). Other studies also found predominantly staphylococci and bacilli but also corynebacteria (Pachter et al., 1997). The predominant groups in the present study were the staphylococci (115 isolates) and bacilli (50 isolates), but a significant number of other bacteria, including Gram-negative strains (40 isolates) were also identified. However, corynebacteria, which are typical skin symbionts, were not observed. Compared to studies assessing the bacterial diversity of human skin, significant overlap with our isolates can be observed. A recent inventory of the human skin microbiome also identified a mixed

population of bacteria from the dry sites of the palm of the hand proximal to the little finger, with a higher prevalence of betaproteobacteria and *Flavobacteriales* (Grice et al., 2009). There were no flavobacterial isolates derived from our copper coins. Bacteria of this phylum may have not been able to grow on the media used. Alternatively, flavobacteria might be especially sensitive to exposure to copper surfaces and consequently could not be selected.

Another study found propionibacteria to predominate human palms, followed by *Streptococcaceae* and *Staphylococcaceae* (Fierer et al., 2008). Streptococci were also absent from our isolates, suggesting a high sensitivity against metallic copper. Thus, overall the bacteria derived from copper coins constitute a subgroup of the typical skin surface bacteria, with a bias toward staphylococci, bacilli, and betaproteobacteria.

Previous work by us (Espírito Santo et al., 2008) and others (Elguindi et al., 2009; Faúndez et al., 2004; Noyce et al., 2006b) has clearly demonstrated that metallic copper surfaces have strong antimicrobial properties against both Gram-positive and -negative bacteria. While coins probably provide a strong selective force for bacteria accidently exposed to these copper surfaces, 76% of our isolates were copper surface sensitive upon retesting on copper coupons; i.e., cells were killed in less than 1 day. The coins used for this study were regular coinage from general circulation and therefore probably were soiled with organic matter. Soiling has previously been demonstrated to enable bacteria to withstand copper surfaces for an extended time, rendering the biocidal surfaces inactive (Airey & Verran, 2007; Tolba et al., 2007; Wheeldon et al., 2008). The isolated coin bacteria that failed to exhibit copper surface resistance were thus probably protected by soiled patches or particles from the surface. Nevertheless,

retesting of the strains surviving the initial selection provided evidence that dry copper surface resistance is not rare among bacteria related to the human skin.

One unexpected complication involved the bacilli. While scoring resistant to copper surfaces in our initial screens (data not shown), a closer examination of these Firmicutes elucidated that survival was because of the production of endospores. Endospores of *Clostridium difficile* have been demonstrated to withstand contact with copper surfaces (Wheeldon et al., 2008). Another study, however, found a 5-log-unit reduction of dormant endospores of *C. difficile* following 24 to 48 h of exposure to copper surfaces (Weaver et al., 2008). In our research, some of the *Bacillus* spores from the copper coin isolates were able to germinate and form colonies even after 1 month of exposure on pure copper surfaces (data not shown), but vegetative cells of all coin-derived bacilli proved to be sensitive (Table 1).

Dry copper surfaces provide different antimicrobial properties than ionic copper, with potential implications for the challenge posed by multiple-drug-resistant germs. The aim of this study was to identify bacteria able to withstand dry copper surfaces. On first glance these bacteria might pose a future complication for a more general application of metallic copper surfaces. However, knowledge of the taxonomic identity of these bacteria is the first step in adapting existing hygiene procedures to deal with this challenge. Dry copper surface resistance and resistance against copper ions do not go hand in hand. Most of the isolates that were resistant to surfaces were as sensitive to copper ions as their respective type strains. However, resistance systems that confer an increased level of resistance

against copper ions compared to strains of the same species lacking these determinants have been studied frequently. The most prominent representatives are Pco from an *E. coli* strain isolated from a piggery (Williams, et al., 1993), Cop from *Pseudomonas syrigae* (Cha & Cooksey, 1991), and Tcr from enterococci (Hasman, 2005). These very efficient copper ion resistance systems fail, however, in protecting cells from exposure to dry metallic copper, as exemplified by Pco. An *E. coli* strain harboring the *pco* genes was almost as sensitive to copper surfaces as a strain lacking *pco* or a strain lacking all major copper ion resistance systems, i.e., CopA, CueO, and Cus (Espírito Santo et al., 2008).

Frequently, the antimicrobial properties of metallic copper are tested with aqueous bacterial cultures exposed to the surfaces (Elguindi et al., 2009; Faúndez et al., 2004; Noyce et al., 2006a; Wheeldon et al., 2008). We believe that this experimental setup does not reflect the situation in a clinical environment where most touch surfaces are dry. Therefore, we have recently adapted a method for studying bacteria exposed to dry copper surfaces (Espírito Santo et al., 2008). Bacteria in a protocol mimicking dry touch surfaces were killed within minutes, in contrast to about 1 to 8 h under wet conditions (Elguindi et al., 2009; Noyce et al., 2006a). Recent results from hospital trials conducted in three countries (South Africa, Great Britain, and Germany) confirm the efficient antimicrobial properties of copper surfaces in a real-life setting (Casey et al., 2010; Marais et al., 2010; Mikolay et al., 2010). Therefore, studying copper surface-resistant bacteria and their mechanisms of survival will probably strengthen our comprehension for use of copper surfaces and their further development. The results reported in this present work suggest that resistance mechanisms

against dry metallic copper differ from those responsible for defense of bacteria against wet surfaces or dissolved copper ions. Thus, it is unsurprising that our isolates where as sensitive as their respective type or control strains when survival on copper ion-containing solid agar media was examined (Table 2). There is further indication that dry copper surfaces pose a significantly different kind of stress to bacteria than copper surfaces that are left wet. Under moist conditions, the dry copper surface-resistant coin isolates exhibited inactivation rates more similar to those of the sensitive controls (Table 2).

Dry metallic copper surfaces are not a habitat on which bacteria actually can grow and propagate. This is in sharp contrast to environments such as piggeries or orchards, where both antibiotics and copper compounds are applied to an actively growing and interacting microbial community, including microbial biofilms. Animal guts, biofilms, and clinical environments are recognized as settings where coselection of antibiotic and metal resistances may or may not occur (reviewed in reference Baker-Austin et al. (2006)). Therefore, a major concern when applying metal ions such as copper salts as antimicrobials is the potential of very efficient copper ion resistance systems related to Pco, Cop, or Tcr driving the coselection with resistances against antimicrobial agents and antibiotics. In fact, Tcr-like systems were found quite frequently when copper ions were used as a growth supplement in husbandry. These copper ion-detoxifying systems were also found to be genetically linked to genetic determinants conferring multiple drug resistance (to macrolides and glycopeptides) in livestock (Baker-Austin et al., 2006; Hasman & Aarestrup, 2002). However, the appearance of copper and antibiotic resistance in bacteria isolated from pigs could not be correlated, and the available data did not

support coselection of these traits (Hasman & Aarestrup, 2005).

Given that dry copper surfaces do not support growth of microbes, coselection of different resistance mechanisms is expected to be of minor concern. Nevertheless, antibiotic and copper surface resistance systems could co-occur, facilitating intra- and interspecies spread. Yet, at least for the staphylococci that we have isolated, copper surface resistance and resistance against common antibiotics do not seem to be intrinsically coupled (Table 1). Because dry copper surfaces also inactivate bacteria expressing efficient copper ion resistance determinants such as *pco*, previous reports of the co-occurrence of metal (ion) and antibiotic resistances in staphylococci likely have little consequence for the safe use of metallic copper (Ug & Ceylan, 2003). Further work is currently in progress to unravel the mechanisms that enable some bacteria to withstand the toxic properties of metallic copper surfaces. We expect to find mechanisms that go beyond resistance against copper ions. This knowledge might then be applied to develop new strategies involving the use of copper surfaces in the battle against bacteria responsible for nosocomial infections. Ideally, these efforts will lead to minimization, if not prevention, of the spread of such pathogens in hospitals and other places where human health is at risk.

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Chapter 3

Bacterial Killing by Dry Metallic Copper Surfaces

Results published in:

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Abstract

Metallic copper surfaces rapidly and efficiently kill bacteria. Cells exposed to copper surfaces accumulated large amounts of copper ions, and this copper uptake was faster from dry copper than from moist copper. Cells suffered extensive membrane damage within minutes of exposure to dry copper. Further, cells removed from copper showed loss of cell integrity. Acute contact with metallic copper surfaces did not result in increased mutation rates or DNA lesions. These findings are important first steps for revealing the molecular sensitive targets in cells lethally challenged by exposure to copper surfaces and provide a scientific explanation for the use of copper surfaces as antimicrobial agents for supporting public hygiene.

Keywords: copper surface, copper uptake, *Escherichia coli, Deinococcus radiodurans*, *Bacillus cereus*, metal toxicity, membrane damage.

Introduction

For many organisms, the trace element copper is an essential nutrient. It serves as a cofactor in respiration, and thus copper is required for aerobic metabolism. However, when copper is in excess, it is also highly toxic (Rensing & Grass, 2003). This is because accumulation of copper ions or intracellular release of free copper ions from proteins causes cell damage. Copper readily catalyzes reactions that result in the production of hydroxyl radicals through the Fenton and Haber-Weiss reactions (Halliwell & Gutteridge, 1984; Sies, Abelson, & Simon, 1990). The highly reactive oxygen intermediates cause lipid peroxidation and oxidation of proteins (Imlay & Linn, 1988; Sies et al., 1990; Stadtman, 2006). Free copper ions are able to oxidize sulfhydryl groups, such as cysteine, in proteins or the cellular redox buffer glutathione (Helbig et al., 2008; Stohs & Bagchi, 1995). Specifically, copper ions inactivate proteins by damaging iron-sulfur clusters in cytoplasmic hydratases. In *Escherichia coli*, these are dihydroxy-acid dehydratase (IlvD) in the branched-chain amino acid synthesis pathway, isopropylmalate dehydratase (LeuC) in the leucine-specific branch, fumarase A (FumA) in the tricarboxylic acid cycle, and 6-phosphogluconate dehydratase in the pentose phosphate pathway (Edd). All were recently found to be damaged by copper ions (Macomber & Imlay, 2009). Thus, these proteins constitute specific targets for copperinduced toxicity. In Bacillus subtilis, copper ion toxicity was shown to interfere with the biosynthesis of iron-sulfur clusters and increased production of cluster scaffold and target proteins (Chillappagari et al., 2010). *In vitro* exposure of DNA to copper ions causes mutations (Tkeshelashvili et al., 1992). It has also been thought that *in vivo* copper ion toxicity in bacteria is mediated by oxidative DNA damage, but this view was

challenged because the growth rate of *E. coli* was found to be more strongly suppressed by copper ions under anaerobic conditions than when oxygen was present (Outten, Huffman, Hale, & O'Halloran, 2001). Copper ions even decreased oxidative DNA damage when E. coli cells were exposed to hydrogen peroxide (Macomber, Rensing, & Imlay, 2007).

In recent years, it has become evident that copper surfaces with which pathogenic agents may come in contact, i.e., metallic copper touch surfaces, may help diminish surface-related hygiene problems. Dry copper surfaces in laboratory settings and in hospital trials proved to have great killing efficiency against a wide range of microbes (Casey et al., 2010; Espírito Santo et al., 2010; Noyce et al., 2006). In most laboratory studies, cells suspended in buffer were applied to copper surfaces and incubated under ambient conditions. Usually, these cells were killed within hours (Elguindi et al., 2009; Molteni et al., 2010). We recently established a method that mimics contact of microbes with dry copper touch surfaces. Under these conditions, most microbes are killed within minutes (Espírito Santo et al., 2010; Espírito Santo et al., 2008). Copper ions are released from metallic copper upon contact with bacteria (Espírito Santo et al., 2008) or with buffer alone (Molteni et al., 2010). However, direct copper ion-mediated toxicity, targeting metabolic enzymes such as hydratases involved in amino acid biosynthesis (Macomber & Imlay, 2009), is unlikely to be the reason for contact killing because of the fast killing kinetics. Furthermore, extracellular supplementation with substances known to protect against oxidative stress, such as catalase, superoxide dismutase, or the hydroxyl radical quencher mannitol, delayed the killing of *E. coli* cells on dry copper surfaces (Espírito Santo et al., 2008). Thus, while we have some insight into the molecular mode of action

exerted by copper ions on bacteria, the specific modes of stress exerted by metallic copper surfaces and the identity of sensitive cellular targets have not yet been elucidated. Such knowledge is needed to better understand why surfaces made from copper alloys exhibited efficient antimicrobial properties in recent successfully completed hospital trials (Casey et al., 2010; Marais et al., 2010; Mikolay et al., 2010).

In this study, we investigated the mode of action of dry metallic copper surfaces against *E. coli* and other bacterial model organisms. Our results demonstrate that exposed cells accumulated copper ions and exhibited membrane and cell envelope damage. It is likely that membrane proteins or the membrane lipids constitute the major targets of copper surface toxicity, but contact killing did not involve lethal damage to the cellular DNA through mutations and lesions.

Materials and methods

Bacterial strains and growth media. The strains used in this study were *Escherichia coli* W3110, *Bacillus cereus* L8, and *Deinococcus radiodurans* DSM 20539. *E. coli* was grown in Luria-Bertani (LB) broth (Difco BD) at 37°C for 16 h, Bacillus cereus (Espírito Santo et al., 2010) in LB broth at 30°C, and *D. radiodurans* (Brooks et al., 1980) in LB broth with 0.5% glucose at 30°C with rotary shaking (250 RPM) until stationary growth phase (approximately 24 h of incubation). Exponential-growth-phase cultures of *D. radiodurans* and *E. coli* were grown as described above, but cells were harvested after 16 h for *D. radiodurans* or after 3 h for *E. coli*. Bacto agar (Difco BD) was added at 15 $g.liter^{-1}$ for solid media.

Assay for contact killing on metal surfaces. Metal surfaces used in this study were 2.5- by 2.5-cm copper coupons (C11000, 99.9% copper) and stainless steel control coupons (AISI 304, approximately 67 to 72% Iron, 17 to 19.5% Chromium, and 8 to 10.5% Nickel) (C11000 and AISI 304 coupons were supplied by the International Copper Association). All copper alloy coupons were treated prior to each experiment to standardize the surface properties. Coupons were incubated for 30 s in 3% (wt/vol) NaOH solution at 70°C and rinsed in distilled water. After transfer into 10% (vol/vol) sulfuric acid solution for5sat room temperature (RT), coupons were immediately washed with distilled water. All coupons were disinfected and cleaned by immersion in ethanol and kept in a sterile container. To prevent surface reoxidation, cleaned coupons were not flamed after immersion in 95% ethanol.

To quantify cell survival on dry metal surfaces, cultures were concentrated 10-

fold and tested as described previously (Espírito Santo et al., 2008), with minor changes. Aliquots of 10^6 cells were streaked out on coupons using sterile cotton swabs. All samples dried completely within 5 s after contact with the surfaces. Unless indicated otherwise, this time point is considered time zero (t_0) throughout this study. To avoid contamination from the laboratory environment, coupons were incubated in sterile petri dishes at 23°C for different times. Coupons were transferred into 10 mL ice-cold phosphate-buffered saline (PBS) with approximately 20 glass beads (2 mm; Sigma-Aldrich) (PBSG buffer), and samples were vortexed for 1 min. Samples were diluted in PBS buffer and plated on LB agar. Surviving bacteria were counted as colony forming units (CFU) using an automatic counter (Acolyte; Synbiosis) and the associated software (version 2.0.8).

For “moist” copper exposure, 10^9 cells in 40 µL aliquots in PBS buffer were applied as a standing droplet on coupons, and samples from droplets were removed after specified time intervals for plating on solid agar media. Survivors were counted as CFU.

Mutagenicity assay. D-Cycloserine is an inhibitor of bacterial cell wall biosynthesis, but mutations in the *cycA* gene render cells resistant to this antimicrobial agent. CycA is a D-alanine, D-serine, and glycine permease that also transports D-cycloserine. D-Cycloserine uptake leads to cell wall toxicity and finally bacteriostasis but not to cell death. Mutagens increase the overall mutation rate in *E. coli*, thus leading to the increased appearance of D-cycloserine-resistant clones by inactivation of the *cycA* gene (Fehér et al., 2006). The advantage of this system is that any inactivation mutation (point, frameshift,

deletion, or insertion mutation) will generate resistant cells that can be scored.

A previously described method (Fehér et al., 2006) that tests mutagenesis of growing cells was adapted for use with nongrowing, surface-exposed cells. Cells were applied for 5 s (an exposure period shorter than that required for massive onset of lethal damage) to the surface of the metal coupons, removed as described above, concentrated (6-fold), and spread on solidified minimal medium (Mergeay et al., 1985) with glycerol as the sole carbon source for determination of total CFU and on minimal medium containing glycerol and 20 $\mu g.mL^{-1}$ D-cycloserine (Sigma-Aldrich) to select for cycA mutants. Colonies were counted after 24 h of incubation. They were assumed to have originated from mutations in the cycA gene. The percentage of cycA mutants was calculated by dividing the number of CFU of cycA mutants by the total number of CFU. As controls, cells were exposed for the same period of time to stainless steel or stainless steel with 0.25% (wt/vol) formaldehyde. This concentration of formaldehyde was used because it did not negatively affect overall survival rate of the challenged cells. To assess statistical significance, a t test was performed with the data from copper-exposed cells, stainless steel-exposed cells, and formaldehyde-exposed cells on stainless steel (positive control). The two-tailed probability (P) values were ≤ 0.05.

Comet assay. A comet, or single-cell gel electrophoresis (SCGE), assay was performed with *E. coli* as described previously (Singh, McCoy, Tice, & Schneider, 1988), with modifications. In short, cells were surface challenged and removed as described above, with minor changes. To prevent any further copper-mediated damage after removal from the surfaces, cells were removed

with 10 mL PBSG buffer containing 20 µM EDTA to sequester copper ions. For fixation, cells were treated with 20 mg/mL of lysozyme at 37°C for 15 min, mixed with low-melting 0.8% agarose, and applied to a glass slide precoated with 1.5% agarose. Further steps were performed at 4°C, and ice-cold reagents were used to minimize DNA damage after surface exposure. Complete gelling and solidification of agarose cell suspensions were allowed before addition of lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris-HCl, pH 10, 10% Triton X-100, and 1% dimethyl sulfoxide [DMSO] to prevent oxidation during lysis). Slides were immersed in lysis buffer and carefully agitated (25 RPM). Slides were washed with deionized water, and DNA unwinding was promoted by incubation with denaturation buffer (300 mM NaOH and 1 mM EDTA, pH > 13). The samples were neutralized by a short incubation with excess Tris-borate-EDTA (TBE) buffer (Sambrook, Fritsch, & Maniatis, 1989). Slides were subjected to electrophoresis at 25 mV at 10 mA for 3 min at RT, removed, washed with ice-cold deionized water, immersed into absolute ethanol, and air dried overnight. Slides were stained with 1 × SYBR® Gold (Invitrogen) in TBE and incubated for 1 min in the dark. Fluorescence was then observed (excitation wavelength [λ_{Ex}] of ≈ 495 nm, emission wavelength [λ_{Em}] of ≈ 537 nm) with an inverted confocal fluorescence microscope (Olympus IX 81) under oil immersion and with an argon laser at 488 nm (Olympus). The image capture software used was Fluoview 500 (Olympus).

Inductively coupled plasma mass spectrometry (ICP-MS) analysis. Cells were applied as droplets (moist method) or spread directly (dry method) on surfaces of copper coupons as described above. At different time points, cells

were removed from the surfaces and excess copper was removed by washing cells with ice-cold PBSG buffer containing 20 µM EDTA. Initial cell numbers were determined by plating as described above, and samples were mineralized with concentrated 70% (vol/vol) nitric acid (trace metal grade; Mallinckrodt) for 2h at 70°C. Samples were diluted to adjust to a final concentration of 5% (vol/vol) nitric acid. Gallium as $Ga(NO_3)_3$ was added at a final concentration of 50 ppb as an internal standard. Inductively coupled plasma mass spectroscopy (ICP-MS) analysis was performed using an Agilent ICP-MS model 7500cx operating with a collision cell with a flow of 3.5 $mL.min^{-1}$ of H_2 and 1.5 $mL.min^{-1}$ of He. Data for each sample were accumulated in triplicate for 100 ms. An external calibration curve was recorded with gallium in 5% nitric acid. Samples were loaded onto 96-well plates prior to analysis, and an autosampler (Elemental Scientific) was used to inject samples.

General staining methods. *B. cereus* and *E. coli* were applied to surfaces of metal coupons as described above, and cells were resuspended on the coupon with 100 µL PBS buffer, transferred onto a glass slide, and air dried. Staining of B. cereus endospores was performed with malachite green and counterstaining with safranin (Schaeffer & Fulton, 1933). *E. coli* was stained only with safranin. Glass slides were examined under oil immersion using light microscopy (Olympus AX70 fluorescence microscope). Live/Dead® staining to evaluate membrane damage. A Live/Dead® staining technique was employed to differentiate cells on copper and control surfaces with undamaged and damaged permeable membranes (Live/Dead® BacLight™ bacterial viability kit; Invitrogen). This kit employs two nucleic acid stains: a green-fluorescent SYTO® 9 stain and

a red-fluorescent propidium iodide stain. These stains differ in their abilities to penetrate healthy bacterial cells. When used alone, the SYTO® 9 stain labels DNA of both live and dead bacteria. In contrast, propidium iodide penetrates only bacteria with damaged membranes, reducing SYTO® 9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes fluoresce green, while bacteria with damaged membranes fluoresce red. Cells were applied to and removed from surfaces as described above. For staining, cells were suspended in 100 µL of 0.9% NaCl, and 1 µL of the staining mixture (1 part SYTO® 9 and 1 part propidium iodide in 60 µL DMSO) was added. Cell suspensions were incubated in the dark for 15 min and then transferred onto glass slides and immediately examined by fluorescence microscopy (λ_{Ex} of 488/ 543 nm, λ_{Em} of 522/590 nm) under oil immersion using an inverted confocal microscope (Olympus IX 81). For SYTO® 9, the laser used was argon at 488 nm, and for propidium iodide, the laser used was HeNe_G at 543 nm. The image capture software used was Fluoview 500 (Olympus).

Visualization of labile intracellular Cu(I) pools. Coppersensor-1 {CS1; 8-[N,N-bis (3',6'-dithiaoctyl)-aminomethyl]-2,6-diethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene} (Miller et al., 2006; Zeng et al., 2006) was synthesized and employed to investigate changing intracellular Cu(I) concentrations. CS1 is a membrane-permeable fluorescent dye, which after binding to Cu(I) increases its red fluorescence 10-fold. The dye binds Cu(I) stably and selectively over other metal cations in aqueous solution. The apparent Kd (dissociation constant) for Cu(I) binding to CS1 is 3.6×10^{-12} M (Zeng et al., 2006). CS1 is not a ratiometric dye, but higher copper ion

concentrations result in increasing red fluorescence signals as long as the concentration of CS1 is higher than that of Cu(I). As such, CS1 can be used to monitor changes in cellular labile copper levels (Miller et al., 2006). To visualize intracellular labile Cu(I), cells exposed to a metal surface were removed as described above and stained with CS1 in the dark at RT for 20 min according to a previously described method (Zeng et al., 2006). Copper accumulation within cells was examined (λ_{Ex} of 543 nm, λ_{Em} of 555/600 nm) under oil immersion with an upright fluorescence microscope (Olympus AX70). The laser used was HeNe_G at 543 nm. The image capture software used was Fluoview 500 (Olympus).

Results

***E. coli* cells release and accumulate copper ions from moist metallic copper surfaces.** Copper ions released from copper surfaces contribute to contact killing (Espírito Santo et al., 2008; Molteni et al., 2010). However, it is currently not known whether cells exposed to metallic copper actually accumulate copper ions intracellularly. In the present experiments, we found that moist plating of E. coli cells on copper coupons resulted in markedly increased copper ion concentrations over time in the buffer in which the cells were suspended compared to concentrations in buffer alone. At t_0 , only about (1.2 ± 1.0) × 10^{16} atoms.mL^{-1} (4.6 × 10^{-4} M) copper was detected in buffer-alone samples, and this value increased to (6.4 ± 2.9) × 10^{17} atoms.mL^{-1} (0.02 M) after 3 h, with an initial release rate of 9 × 10^{15} atoms.mL^{-1}.min^{-1} (3.74 ± 10 M.min^{-1}). In contrast, we found that the copper content of buffer-alone samples on stainless steel remained constant at (3.8 ± 0.8) × 10^{14} atoms.mL^{-1} (1.6 × 10^{-5} M).

Buffer with suspended cells accumulated (1.2 ± 1) × 10^{16} atoms.mL^{-1} (4.6 × 10^{-4} M) copper at t_0 and 3.6 × 10^{18} (± 7.2 × 10^{16}) atoms.mL^{-1} (0.15 M) copper after 3 h, yielding an initial release rate of 3 × 10^{16} atoms.mL^{-1}.min^{-1} (1.25 × 10^{-3} M.min^{-1}). Conversely, in samples after contact with stainless steel coupons, the concentration of copper remained constant at (1 ± 0.3) × 10^{15} atoms.mL^{-1} (4.3 × 10^{-5} M) during the course of the 3 h experiment.

Next, we quantified copper accumulation in cells exposed to moist copper surfaces. At t0, cells contained (1.8 ± 0.5) × 10^4 copper atoms/cell. The amount of intracellular copper increased linearly for the next 60 min at a rate of 1.0 × 10^6 atoms/cell/minute and reached a maximum of (1.5 ± 0.3) × 10^8 copper

atoms/cell at the end of the experiment at 3 h (Figure 1A). In contrast, copper contents of cells on stainless steel remained virtually constant during these 3 h, at (7.9 ± 0.5) × 10^4 atoms/cell.

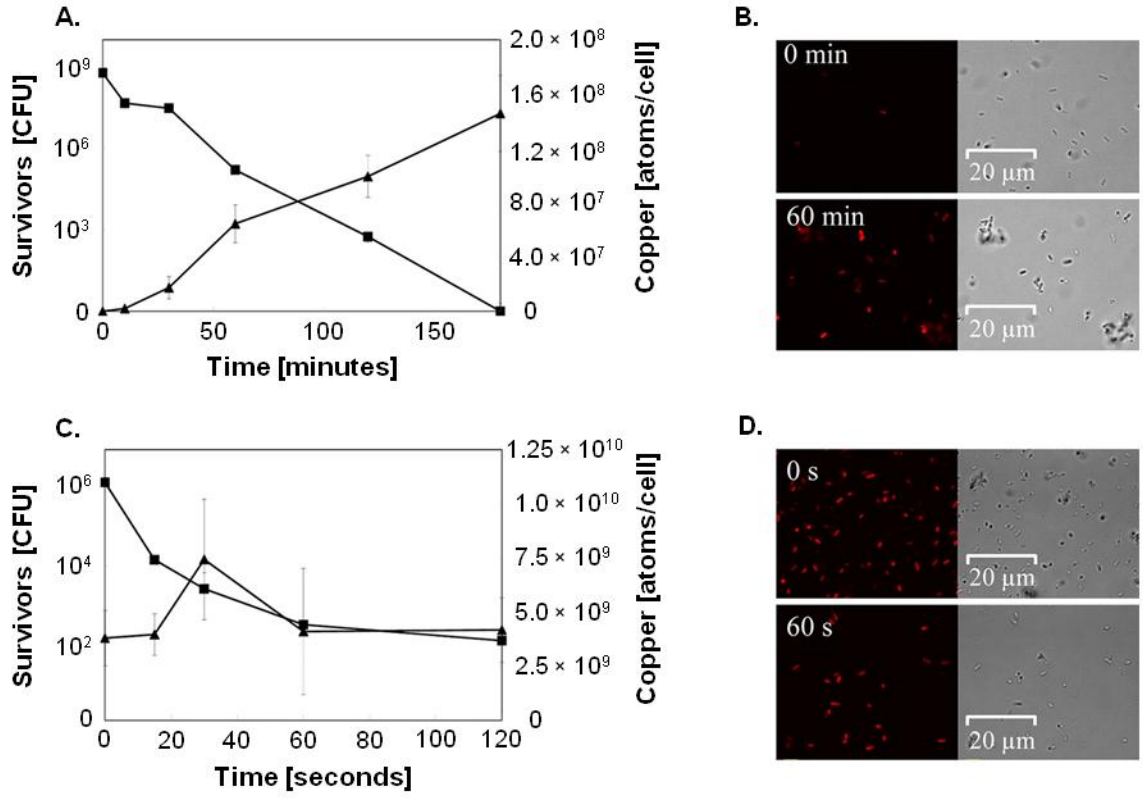

Figure 1 - Copper uptake into cells exposed to moist or dry copper surfaces. Cells of *E. coli* were exposed to moist (A and B) or dry (C and D) metallic copper surfaces for the indicated times, removed, washed, and plated on solidified growth media. Survivors were counted as CFU (■) (A and C). Parallel samples were mineralized and subjected to ICP-MS analysis for determination of cellular copper content (▲) (A and C) or were stained with the Cu(I)-specific fluorescent dye Coppersensor-1 and subjected to fluorescence microscopy (B and D). Shown are averages and standard deviations (error bars) from triplicate experiments (A and C) and representative phase-contrast (right) and fluorescence (left) microscopy images (B and D).

Copper ion uptake into exposed cells was also followed using the copper-specific dye Coppersensor-1 (CS1), which fluoresces red upon binding to Cu(I) (Zeng et al., 2006). Cells stained with CS1 after 1 h of exposure to moist copper were red, indicating high concentrations of intracellular cuprous copper ions. In contrast, copper-exposed cells at t_0 (Figure 1B), stainless steel-exposed cells, or

untreated cells (data not shown) fluoresced only weakly, confirming copper ion uptake from surfaces into exposed cells over the time of the experiment.

Thus, after 3 h on copper surfaces, the concentration of copper in cells was higher than that of the surrounding media. Also, buffer without cells accumulated less copper from the surface than buffer with cells. These results (Figure 1A and B) indicate that *E. coli* actively dissolved and accumulated copper ions from moist copper surfaces. Under the conditions tested, cells were inactivated during a 3 h time period, and no live cells could be recovered after 3 h (Figure 1A). Combining copper accumulation data and killing kinetics clearly demonstrated a correlation between release of copper from the copper coupons and its accumulation by the cells, with lethal consequences. *E. coli* cells accumulate large intracellular amounts of copper ions from dry metallic copper surfaces. Dry copper touch surfaces are more commonly employed to support hygiene in public and health care-related settings than moist copper touch surfaces. Therefore, we also tested copper accumulation in *E. coli* cells exposed to dry copper coupons. *E. coli* cells were killed after 1 min on dry copper (Espírito Santo et al., 2008). We repeated earlier experiments (Espírito Santo et al., 2008) to correlate cell inactivation with copper accumulation. However, since EDTA was added to cells after the copper challenge for preparation for ICP-MS analysis, complete killing was not achieved after 1 min. Instead, a 4-log reduction in numbers of live cells was observed (Figure 1C). This also suggests that addition of a copper chelator post exposure increased the survival rate of cells subjected to contact killing. The intracellular copper content at t0 (which equaled 5s of exposure, the time needed for the sample to dry) in copper surface exposed cells was $(3.8 \pm 1.3) \times 10^9$ copper atoms/cell (Figure 1C), which

was 200,000 times higher than that in unexposed cells (1.8×10^4 atoms/cell). Remarkably, the intracellular copper content after 1 min still remained at (4.1 ± 2.9) $\times 10^9$ copper atoms/cell. By this time, under our test conditions, 99.99% of all cells were lethally damaged. At 30 s, a small but reproducible increase in accumulated copper was observed, probably indicating maximal copper accumulation before cell damage after 1 min countered further accumulation.

To corroborate these findings, cells were stained with CS1 dye after t0 or 1 min of exposure to dry metallic copper. Even the shortest possible exposure time, t_0, resulted in bright red cells, indicating very large amounts of intracellular Cu(I) (Figure 1D). Copper accumulation was also investigated with cells exposed to dry copper for time periods longer than needed for killing (2 min). At that time, cells did not contain more copper than at the time of inactivation (at approximately 1 to 2 min) (data not shown), suggesting that exposure beyond death did not lead to a further increase in intracellular copper concentrations but probably resulted in compromised cell integrity and leakage of previously accumulated copper. Not only did these results demonstrate that contact killing on dry metallic copper was much faster than that on moist copper, but they also showed that copper ion accumulation was more rapid and resulted in highly elevated intracellular copper concentrations. Unexpectedly, copper accumulation from dry copper surfaces was extremely fast, probably due to the absence of buffering medium, which was present on the moist copper surfaces.

Prolonged exposure to copper surfaces leads to cell disintegration. Copper accumulation declined when cells were exposed to dry metallic copper for periods exceeding the time needed for killing (Figure 1C). Thus, we also

investigated the structural integrity of copper-surface-exposed cells. After 1 minute of exposure, *E. coli* and *B. cereus* cells removed from the copper coupons and observed by microscopy had started to disintegrate, and cell debris was detected (Figure 2). Thus, the contact killing process led to severe structural damage in both Gram-negative and Gram-positive cells. However, *Bacillus* endospores appeared intact after exposure to copper surfaces and were clearly visible after staining (Figure 2).

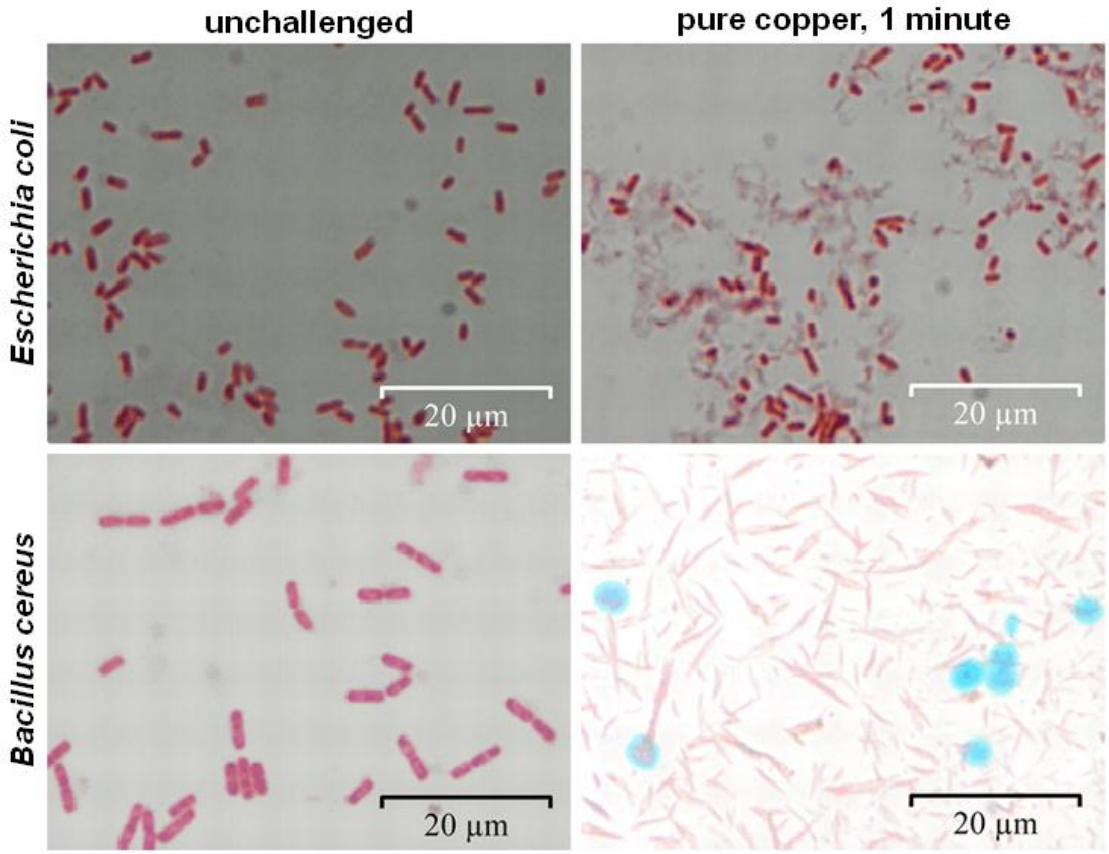

Figure 2 - Prolonged contact with metallic copper results in cell disintegration. Cells of Gram-negative *E. coli* and Gram-positive *B. cereus* were exposed to pure copper for 1 min (right) or unexposed (left), removed, washed, and stained. *E. coli* was stained red with safranin, and *B. cereus* was visualized by endospore staining. This process colors endospores green and vegetative cells red after safranin counterstaining. Shown are representative light microscopy images.

Cells exposed to dry copper surfaces have damaged membranes. Since cells exposed to copper surfaces for longer time periods acquired structural damage (Figure 2), it is likely that membrane damage contributes to the mechanism of action of contact killing. Cytoplasmic membrane damage can be assessed using the Live/Dead® staining technique, which makes use of a dye, propidium iodide, that enters cells and stains cellular DNA (and thus the cells) only if the membranes are damaged and permeable. To test the extent and rate at which *E. coli* cells suffer membrane damage, cells were exposed to dry copper coupons for either t_0 or 1 min, removed from the coupon surface with PBS buffer, Live/Dead® stained, and immediately subjected to fluorescence microscopy. Figure 3 shows that under these conditions most cells turned red, indicating membrane damage. Unchallenged cells and cells exposed to stainless steel for the same time periods remained largely green and thus undamaged (Figure 3), suggesting that contact with metallic copper and not general desiccation caused the rapid onset of membrane damage. Extended exposure of cells beyond the time needed for killing eventually led to cell disintegration, when cells were removed from surfaces and examined by microscopy (Figure 2). In contrast, observation of cells directly on copper surfaces by atomic force and scanning electron microscopy did not reveal extensive structural damage, except for a few cells that appeared to have become leaky (data not shown). Thus, it is likely that contact with metallic copper did not puncture cells and result in leakage but rather that cells were permeabilized and destabilized, rendering them more susceptible for subsequent rupture by physical forces.

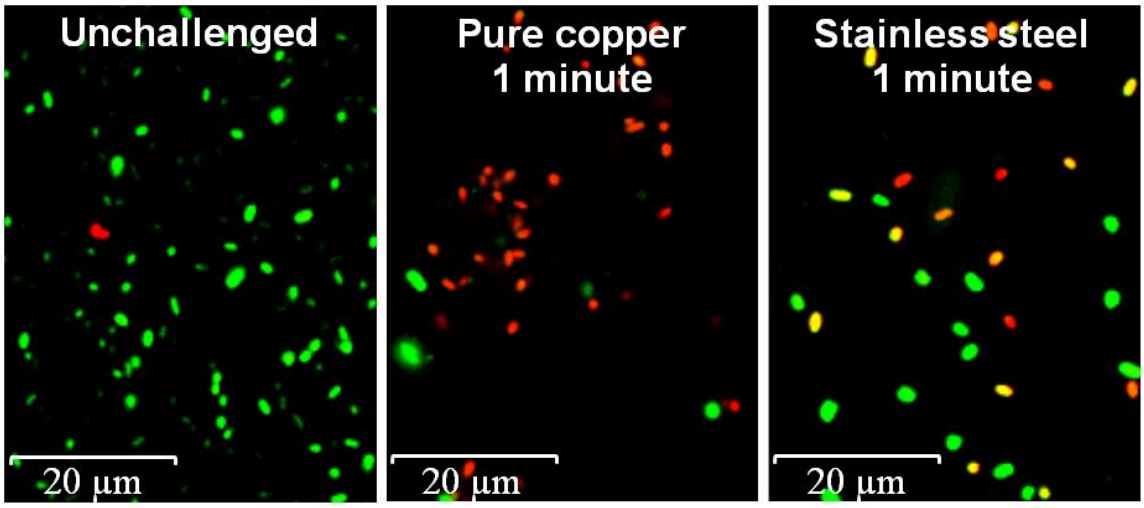

Figure 3 - Cells exposed to copper surfaces suffer membrane damage. Cells of *E. coli* were exposed for 1 min to copper or control surfaces or unchallenged, removed, stained (Live/Dead® BacLight™ bacterial viability kit; Invitrogen), and visualized by fluorescence microscopy. Live bacteria with intact membranes fluoresce green, while those with damaged membranes fluoresce red.

Genomic DNA is not a target of copper-surface-mediated toxicity. Previously, we showed that bacteria exposed to dry metallic copper surfaces were efficiently killed (Espírito Santo et al., 2008). The molecular cellular targets of metallic copper toxicity, however, are currently not known. We used selection for D-cycloserine resistance to investigate the potential mutagenicity of metallic copper on *E. coli* cells. Exposure to metallic copper did not increase the mutation rate in *E. coli* (Figure 4). Approximately the same percentages of D-cycloserine-resistant mutants arose from sensitive cells when *E. coli* was exposed to copper and stainless steel surfaces. There was, however, a significant increase in the number of mutants when cells on control surfaces were additionally treated with formaldehyde, a known mutagen. This is the first strong indication that metallic copper is not genotoxic and does not kill exposed cells by generation and accumulation of lethal mutations in the cell DNA.

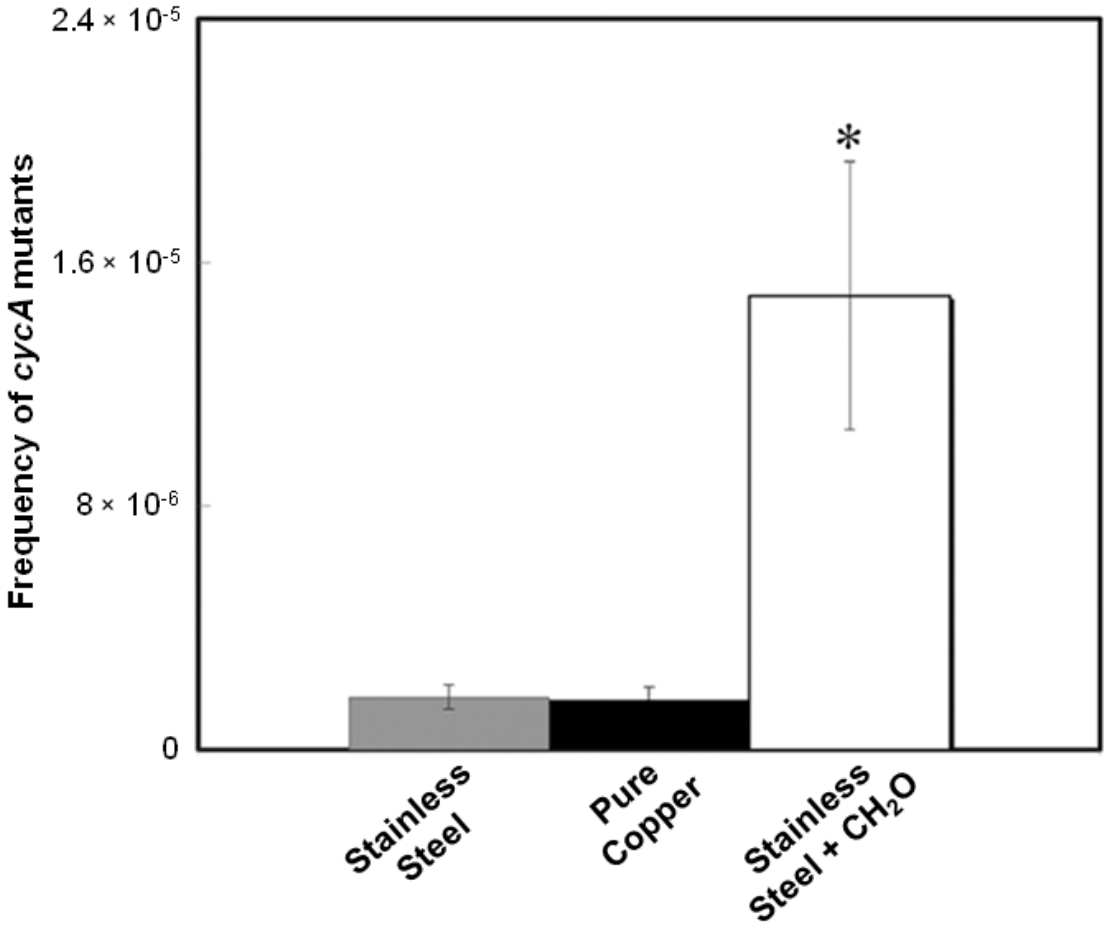

Figure 4 - Exposure to metallic copper surfaces does not lead to increased mutations in *E. coli*. A total of 10^8 *E. coli* cells were exposed for 5 seconds to copper surfaces, stainless steel surfaces, or surfaces containing 0.25% (wt/vol) of the mutagen formaldehyde (CH_2O) plus stainless steel, removed, concentrated, and spread on solid medium containing 20 $\mu g \cdot mL^{-1}$ of the bacteriostatic compound D-cycloserine. After 24 h of incubation at 37°C, colonies were counted as originating from mutation events leading to resistance via inactivation of CycA, a D-cycloserine uptake permease. Shown are averages from triplicate experiments, with standard deviations (error bars). The asterisk denotes significantly different values ($P \leq 0.05$) for formaldehyde-challenged cells.

An alternative mode of DNA damage is the generation of double-strand breaks. Recently, it was shown that this mode of action of copper ion toxicity did not apply to *E. coli* (Macomber et al., 2007). However, it is still possible that the acute toxicity exerted by metallic copper surfaces targets the DNA by this mechanism. To test this, we employed the comet assay (Singh et al., 1988), in which DNA breaks in genomes are visualized by a trail of stained DNA

fragments after gel electrophoresis. *E. coli* cells exposed to copper or stainless steel surfaces for 1 min, a time needed to kill all cells (10^6 cells) on copper, were removed and assayed for comets. Unchallenged cells and cells exposed to stainless steel served as negative controls, and cells exposed to ciprofloxacin, a known inducer of DNA breakage, served as a positive control. No comets were observed in cells from challenged or unchallenged cells (Figure 5). Comets were visible when cells stayed on copper for an extended time, 5 min, a time period 5 times longer than that sufficient to kill all cells. However, picture quality was highly diminished due to the presence of extensive cell debris (data not shown). This indicates that exposure to metallic copper did not cause extensive DNA damage, either through mutation or by fragmentation of double-stranded genomic DNA.

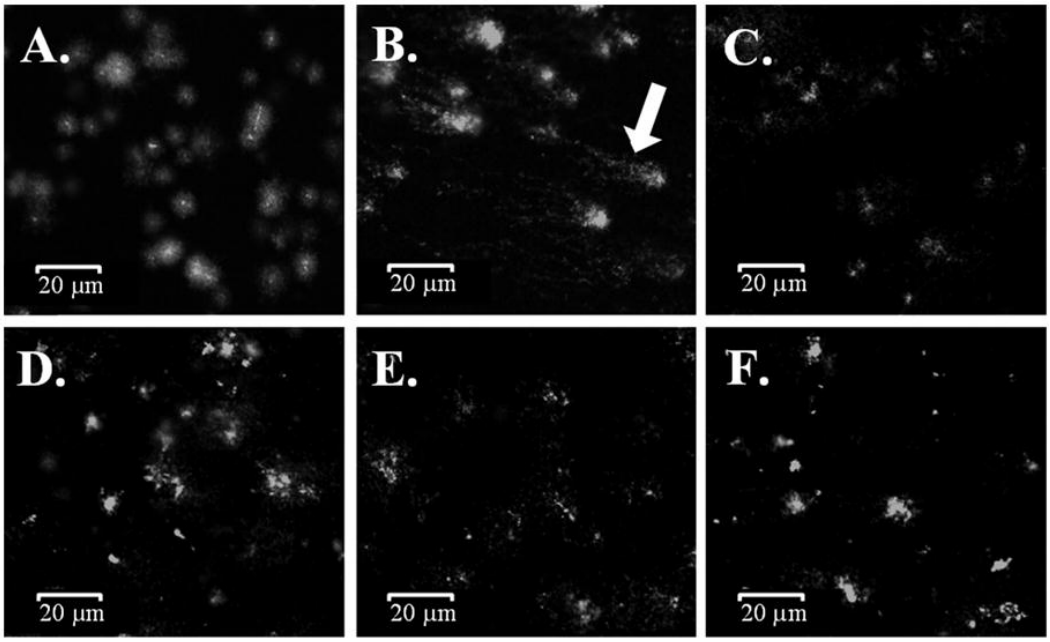

Figure 5 - Exposure to metallic copper does not cause extensive DNA breakage in *E. coli*. *E. coli* cells (10^6 cells) were challenged on copper (D and F) or stainless steel (C and E) surfaces for t_0 (C and D) or 1 min (E and F) and investigated for DNA double-strand breakage by the comet assay (Singh et al., 1988). Unchallenged (A) and ciprofloxacin-treated (B) cells indicate intact and fragmented DNA, respectively. The arrow indicates characteristic comets, highly fragmented DNA resulting from gyrase inhibition by ciprofloxacin. Images shown are representatives from three independent experiments with similar results.

To corroborate these findings, the bacterium *Deinococcus radiodurans* was also exposed to dry copper surfaces. *D. radiodurans* possesses sophisticated and very effective DNA repair systems enabling cells to recover from stresses resulting in highly fragmented genomes, damage that is lethal to most microbes (Cox et al., 2010). Nonetheless, stationary-growth-phase cells of *D. radiodurans* were inactivated after 1 min of exposure (Figure 6). This is the same time needed to kill *E. coli*. We also quantified the killing of exponential-growth-phase cells of *D. radiodurans*, because cells grown under these conditions were reported to have maximum DNA repair capabilities (Sukhi et al., 2009). Here, *D. radiodurans* cells were completely inactivated after 30 s, 50% faster than stationary-phase cells. Exponential-growth-phase *E. coli* cells were also tested for comparison. These cells too were 50% more sensitive to metallic copper than the cells in stationary phase. Finally, we tested killing of *D. radiodurans* on moist copper surfaces. Stationary-phase cells (1.1×10^7) were completely killed after 1h (*E. coli* after 3 h) (data not shown). Thus, stationary- and exponential-growth-phase cells from these two species responded similarly to exposure to dry copper surfaces, with exponentially growing cells being more prone to contact killing, but *D. radiodurans* was even more sensitive to moist copper than *E. coli*. Stainless steel control surfaces had no antimicrobial activity, but a number of the exposed cells succumbed to desiccation (Figure 6).

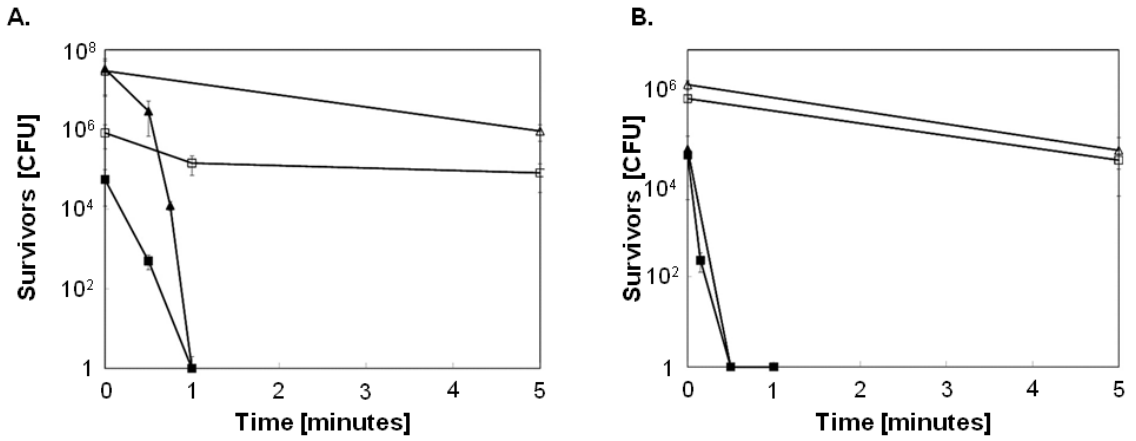

Figure 6 - Efficient DNA repair provides no protection against toxicity exerted by metallic copper. Contact killing of stationary-phase (A) and exponential-phase (B) cultures of *D. radiodurans* (squares) or *E. coli* (triangles) on stainless steel (open symbols) or copper (filled symbols) surfaces. Shown are averages and standard deviations (error bars) from three independent experiments.

Taken together, these results suggest that DNA is not a major target of metallic copper toxicity in Gram-negative *E. coli* and Gram-positive *D. radiodurans*. Conversely, cells exhibited vast membrane and envelope damage, which is likely linked to subsequent cell death, and dividing cells are more prone to copper surface toxicity than resting, stationary-growth-phase cells.

Discussion

Cell membranes are primary targets of contact killing through surface-released copper ions. Significant differences exist between exposure of bacteria to toxic concentrations of copper ions and exposure to metallic copper surfaces. Exposure during growth in media containing copper ions or biofilm growth in copper plumbing systems and colonized medical copper implants is chronic, whereas contact with dry metallic copper is acute. Cells on dry metallic copper surfaces are not in an environment that promotes growth. Thus, these cells face challenges that are different from those of chronically copper ion-challenged cells. Only recently was a major mode of action for the toxicity of elevated intracellular cuprous copper ion concentrations elucidated. In *E. coli*, this stress causes the inactivation of hydratases, which are necessary for normal cell function. Specifically, cuprous copper ions can damage exposed Iron-Sulfur clusters in these proteins, resulting in growth defects of challenged cells (Macomber & Imlay, 2009). Since cells exposed to dry copper surfaces do not proliferate, these sensitive Iron-Sulfur clusters within proteins needed for general cellular metabolism do not constitute a likely target of toxicity.

Previously, most studies on the antimicrobial properties of metallic copper touch surfaces described the differences in efficacy caused by various copper contents, temperatures, and other parameters (Michel et al., 2009; Wilks et al., 2005). In these studies, the killing kinetics for a wide variety of microbes on copper surfaces were described (Espírito Santo et al., 2010; Mehtar et al., 2008; Weaver et al., 2010). However, insights into the mechanisms of antimicrobial action are rare. Not surprisingly, copper ions and oxidative stress play a role during contact killing (Elguindi et al., 2009; Espírito Santo et al., 2008; Molteni et

al., 2010), even though contact killing remains efficient and rapid under anaerobiosis (Espírito Santo et al., 2008). The deletion of genes related to cellular copper ion defense was shown to speed up contact killing in Gram-negative *E. coli* (Espírito Santo et al., 2008) and *Pseudomonas aeruginosa* (Elguindi et al., 2009) and in Gram-positive *Enterococcus hirae* (Molteni et al., 2010). In our study, Gram-negative *E. coli* and Gram-positive *B. cereus* were both damaged after very similar exposure times (Figure 2), as was *D. radiodurans* (Figure 6), with an outer membrane that is different from that of proteobacteria. This suggests that differences in cell wall structure *per se* are a bad predictor of metallic copper sensitivity. In contrast, it was recently demonstrated that different buffers vary in ability to release copper ions from metallic copper, but the contribution of cells to copper solubilization and uptake was not investigated (Molteni et al., 2010). Here, we show that *E. coli* cells strongly increased the amount of copper being released from copper surfaces.

Copper accumulation within cells on dry copper was extensive and very rapid. However, it could not be determined if this accumulation is the primary cause of lethality or a secondary result caused by compromised membranes, as observed by membrane integrity staining. Clearly, membranes are damaged in cells exposed to copper surfaces. Membrane damage has been observed before (Airey & Verran, 2007), but the authors used differential staining techniques only to differentiate live from dead (lethally damaged) cells and did not make the connection to the possibility of membrane damage as the underlying mode of action. Others have used indicators for respiration, such as the fluorescent redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), to differentiate live (metabolic active) from dead (metabolic inactive) cells (Noyce et al., 2006; Wilks et al.,

2005). However, this is an indirect approach, as it measures cell activity but does not specifically indicate membrane damage. Likewise, an earlier study (Borkow & Gabbay, 2005) suggested that *E. coli* cells embedded in agar overlaid on copper and brass but not stainless steel surfaces were completely disrupted after 24 h as indicated by scanning electron microscopy. During the time periods required for contact killing, we did not observe any widespread gross damage, such as blebs or leakage, to cells exposed to metallic copper, as indicated by high-resolution microscopy (data not shown). Nevertheless, physical cell wall stability after contact killing was compromised in Gram-negative and -positive models (Figure 2) when cells were examined after contact with copper but not with stainless steel.

In our current working model, the mode of action of antimicrobial copper surfaces comprises cytoplasmic membrane damage and weakening of the cell wall. If free copper ions lead to the damage observed, it is likely that they cause a selective change in membranes, as has been described for *Saccharomyces cerevisiae* (Ohsumi et al., 1988). In that study, copper ions were shown to release amino acids, mostly glutamate, and much of the cellular K^+, suggesting copper ion-mediated selective lesions of the plasma membrane. Similarly, a study investigating oral streptococci indicated that cytoplasmic membrane-bound F_1F_0-ATPase was damaged by Cu(I) and Cu(II) ions anaerobically (Dunning et al., 1998). Together with the findings of Keevil and coworkers (Noyce et al., 2006; Wilks et al., 2005), determined using a respiration indicator, these results demonstrate that molecular targets within membranes might be related to respiration and oxidative energy conservation. Nevertheless, this does not explain why a short, 1 minute exposure completely kills cells, preventing them

from resynthesizing or repairing damaged target proteins after removal from the surfaces and recovering from this stress.

Lack of a role for DNA damage during copper-surface-mediated contact killing. Previous work has dismissed the possibility that contact killing on metallic copper surfaces causes cellular DNA damage (Espírito Santo et al., 2008; Michels et al., 2009), and for cells challenged with copper ions, DNA damage was disproved (Macomber et al., 2007). However, Warnes et al. recently suggested genotoxicity as a mode of action of copper surfaces (Warnes et al., 2010). In this study, we have provided strong evidence that genotoxicity through mutations and DNA lesions is not the underlying cause for the antimicrobial properties of dry metallic copper, and we offer an alternative explanation for the contact killing mechanism: membrane and envelope damage coupled with extensive copper ion accumulation. In contrast, Warnes et al. observed DNA damage after cells were inactivated on copper (Warnes et al., 2010). It is thus likely that the authors did not identify the primary cause of killing but describe a secondary phenomenon that occurred after the onset of cell death or after lethal damage had accumulated. Further, during the course of our experiments we noticed that fluorescence indicator dyes, such as SYBR® Gold, lose their fluorescence upon contact with metallic copper (data not shown). As a consequence, we now routinely remove cells from surfaces prior to staining and fluorescence microscopy.

The genome of *D. radiodurans*, like those of other bacteria, including *E. coli*, is highly fragmented after exposure to kGy doses of ionizing radiation (Gérard et al., 2001). However, in contrast to sensitive bacteria, *D. radiodurans* is able to

rejoin these overlapping fragments into complete genomes over a period of 3 to 4 h. After repair, cell division commences normally (Cox et al., 2010). Thus, radioresistance of *D. radiodurans* is not due to prevention; instead, this organism relies greatly on a variety of efficient DNA repair functions that have less efficient equivalents in almost all species (Gérard et al., 2001). If contact with metallic copper caused destructive DNA damage in cells, then *D. radiodurans* would be expected to recover from this stress. The opposite was observed. Cells of *D. radiodurans* were virtually as sensitive to contact killing as *E. coli* cells on dry copper surfaces, both in exponential and in stationary growth phase (Figure 6), and were even more sensitive when exposed to moist copper (data not shown). This and the observation that exposure to copper surfaces did not increase the mutation rate in *E. coli* make DNA very unlikely as a major target of acute lethal metallic copper stress. Similarly, it was found earlier that chronic, long-term exposure to ionic copper (i.e., growth in media supplemented with toxic concentrations of copper salts) neither increased the mutation rate in *E. coli* nor increased the numbers of DNA lesions. Conversely, copper ions actually protected the DNA from hydrogen peroxide-mediated oxidative damage (Macomber et al., 2007).

In conclusion, this study proposes cell envelope damage as the mode of action of contact killing mediated by dry metallic copper surfaces. The toxicity exerted does not target the genomic DNA in Gram-negative and Gram-positive organisms tested, even though cells are overloaded with copper ions. Further research will be directed at identifying the molecular targets through which membranes are damaged upon contact with metallic copper. It will be interesting to test whether specific membrane proteins or the lipids themselves constitute

the weakest link in cells exposed to this lethal challenge.

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Chapter 4

Antimicrobial metallic copper surfaces kill *Staphylococcus haemolyticus* via membrane damage

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Abstract

Recently, copper in its metallic form has regained interest for its antimicrobial properties. Use of metallic copper surfaces in worldwide hospital trials resulted in remarkable reductions in surface contaminations. Yet, our understanding of why microbes are killed upon contact to the metal is still limited and different modes of action have been proposed. This knowledge, however, is crucial for sustained use of such surfaces in hospitals and other hygiene-sensitive areas. Here, we report on the molecular mechanisms by which the Gram-positive *Staphylococcus haemolyticus* is inactivated by metallic copper. *Staphylococcus haemolyticus* was killed within minutes on copper but not on stainless steel demonstrating the antimicrobial efficacy of metallic copper. Inductively coupled plasma mass spectroscopy (ICP-MS) analysis and *in vivo* staining with Coppersensor-1 indicated that cells accumulated large amounts of copper ions from metallic copper surfaces contributing to lethal damage. Mutation rates of copper- or steel-exposed cells were similarly low. Instead, live/dead staining indicated cell membrane damage in copper- but not steel-exposed cells. These findings support a model of the cellular targets of metallic copper toxicity in bacteria, which suggests that metallic copper is not genotoxic and does not kill via DNA damage. In contrast, membranes constitute the likely Achilles' heel of copper surface-exposed cells.

Keywords: Genotoxicity, membrane damage, metallic copper toxicity, *Staphylococcus haemolyticus*.

Introduction

Metallic copper surfaces have excellent antimicrobial properties against a variety of different microorganisms from different domains of life (Grass et al., 2011). As such, copper touch surfaces can be expected to support existing hygiene-increasing procedures in public places including hospitals. Indeed, in worldwide hospital trials non-copper surfaces in frequent contact with patients and staff were replaced with their copper counterparts. This novel use of metallic copper resulted in diminishing bacterial surface-loads up to 90% as compared to controls (Casey et al., 2010; Mikolay et al., 2010). Recently, molecular mechanisms that result in rapid killing of copper surface-exposed bacteria and yeasts were studied. Both groups of organisms are killed by a sharp shock of extreme and immediate copper ion overload combined with extensive membrane and envelope damage. Importantly, exposure to metallic copper did not result in genotoxicity. Actually, similar low mutation rates were observed in cells from copper and control surfaces (Espírito Santo et al., 2008; Quaranta et al., 2011).

While it was previously reported that Staphylococci were inactivated by both moist and dry copper surfaces (Espírito Santo et al., 2010; Mehtar et al., 2008; Michels et al., 2009), the molecular mode-of-action leading to complete kill remained controversial. An alternative model that differs from the mode-of-action model involving membrane damage as outline above and in Airey and Verran (2007) predicts that the thick Gram-positive cell walls of Staphylococci were significantly different from that of *Escherichia coli*, other Gram-negative bacteria and yeasts requiring a different mechanism of kill. Indeed, Keevil and coworkers reported that DNA inside copper-exposed *Staphylococcus aureus* cells was

degraded causing cell death. Yet, the authors observed only little negative effect on cytoplasmic membrane integrity (Weaver et al., 2010).

Here, we demonstrated that killing on metallic copper of *S. haemolyticus*, as a model organism from the staphylococcal group of notorious pathogens, follows the same rules of inactivation by antimicrobial copper surfaces as observed for other microbial species.

Materials and methods

Bacterial strains and growth media. The strain used in this study was *S. haemolyticus* NRRL B-14755 (Schleifer & Kloos, 1975). It was grown in R2A broth (Difco BD, Franklin Lakes, NJ USA), at 30˚C with rotary shaking (250 rotation per minute [RPM]) until stationary growth phase (approximately 16 h of incubation). Bacto Agar (Difco BD, Franklin Lakes, NJ USA) was added at 15 g × L^{-1} for solid media.

Contact killing assay on metal surfaces. Metal surfaces used in this study were 2.5 × 2.5 cm copper coupons (C11000, 99.9% Cu) or stainless steel control coupons (AISI 304, approximately 67–72% Fe, 17–19.5% Cr, 8–10.5% Ni). Coupons were provided by the International Copper association (New York City, NY USA). All copper-alloy coupons were treated prior to each experiment to standardize the surface properties. Coupons were incubated for 30 sec in 3% (w/v) NaOH solution at 70˚C and rinsed in distilled water. After transfer into 10% (v/v) sulfuric acid solution for 5 sec at room temperature (23˚C) coupons were immediately washed with distilled water. All coupons, copper and stainless steel, were disinfected and cleaned by immersion in ethanol and kept in a sterile container. To prevent surface reoxidation cleaned coupons were not flamed after immersion in 95% ethanol. For determination of the survival of cells on dry metal surfaces, cultures were concentrated 10-fold and tested as described in Espírito Santo et al. (2011) with minor changes. Aliquots of 10^6 cells were streaked out on coupons using sterile cotton swabs. All samples dried completely within 5 sec after contact with the surfaces. Unless indicated otherwise, this time point is considered “0” or t0 throughout this study. Cell-laden coupons were incubated in

sterile Petri dishes at 23˚C for different times to avoid contamination from the laboratory environment. Coupons were transferred into 10-mL ice-cold phosphate-buffered saline (PBS) with approximately 20 glass beads (2 mm, Sigma-Aldrich, St. Louis, MO USA) (PBSG buffer). Samples were vortexed for 1 min, diluted in PBS buffer and plated on LB agar. Surviving bacteria were counted as colony forming units (CFU) using an automatic counter (Acolyte, Synbiosis, Cambridge UK) and the associated software (Version 2.0.8).

Mutagenicity assay. The occurrence of mutations as the emergence of D-cycloserine resistant clones in copper surface-exposed cells and controls was tested as described previously (Espírito Santo et al., 2011). In short, cells were applied for 5 sec to the surface of the metal coupons (a time period of exposure shorter than required for killing), removed with PBS as described above and concentrated. Cells were spread on solidified minimal medium with glycerol as sole carbon source for determination of total colony forming units (CFU) and on minimal media containing glycerol and 80 µg × mL^{-1} D-cycloserine (Sigma-Aldrich, St. Louis, MO USA) to select for D-cycloserine resistant mutants. Colonies assumed to have originated from mutations in the *aapA* gene inactivating D-cycloserine uptake, were counted after 24 h of incubation. The percentage of *aapA* mutants was calculated by dividing the number of CFU of *aapA* mutants by the total number of CFU. For comparison, cells were exposed for the same period of time on stainless steel or on stainless steel with 0.9% (w/v) formaldehyde as a known mutagen. To assess if groups of data were statistically different from each other, t-test was performed with data of copper-, stainless

steel-, or formaldehyde-exposed cells on stainless steel (positive control). The two-tailed probability values (P) were ≤ 0.05.

Inductively coupled plasma mass spectroscopy (ICP-MS) analysis. The uptake of solubilized copper ions from metallic surfaces was determined as described by Espirito Santo et al. (2011). For this, cells were spread directly on surfaces of copper coupons as described above. At various time points cells were removed from surfaces and excess copper was removed by washing with ice-cold PBS-buffer containing 20 µM EDTA for chelating externally bound copper. Acid-mineralized samples were diluted to adjust to a final concentration to 5% v/v of nitric acid. As internal standard Gallium ($Ga(NO_3)_3$) was added at a final concentration of 50 ppb. Element analysis was performed using an Agilent ICP-MS model 7500cx (Agilent, Santa Clara, CA USA) operating with a collision cell with a flow of 3.5 mL × min^{-1} of H_2 and 1.5 mL × min^{-1} of He. Data for each sample were accumulated in triplicate for 100 msec. For quantification an external calibration curve was recorded with Gallium in 5% nitric acid. Initial cell numbers were determined by plating as described above.

Live/Dead® staining to evaluate membrane damage. A live/dead staining technique was employed to differentiate cells on copper and control surfaces with undamaged and damaged, permeable membranes (Live/Dead® BacLight™ Bacterial Viability Kit, Invitrogen, Grand Island, NY USA) as described earlier (Espírito Santo et al., 2011). Stained cell samples were examined by fluorescence microscopy (λ_{Ex}= 488/543 nm, λ_{Em}= 522/590 nm) under oil immersion using an inverted confocal microscope (Olympus, IX 81, Olympus

America, Center Valley, PA USA). For the dye SYTO® 9, the laser used was Argon 488 nm and for propidium iodide HeNe G 543 nm. Image capture software was Fluoview 500 (Olympus America, Center Valley, PA USA).

Visualization of labile intracellular Cu(I) pools. Coppersensor-1 (CS1, 8-[N,N-Bis (3,6-dithiaoctyl)-aminomethyl]-2,6-diethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4-a-diaza-s-indacene) is a membrane permeable fluorescent dye, which after selectively binding to Cu(I) increases its red fluorescence by 10-fold. CS-1 was synthesized (Miller et al., 2006) and employed to quantify changing intracellular Cu(I) concentrations as described in Espírito Santo et al. (2011). copper accumulation within cells was examined under oil-immersion (λ_{Ex}= 543 nm, λ_{Em}= 555–600 nm) with an upright fluorescence microscope (Olympus AX70, Olympus America, Center Valley, PA USA). Image capture software was Fluoview 500 (Olympus America, Center Valley, PA USA) and the laser used was HeNe G 543.

Results

***Staphylococcus haemolyticus* is quickly killed on dry metallic copper.** Previous studies tested Staphylococci on moist copper (Airey & Verran, 2007; Weaver et al., 2010) or investigated long-term survival on dry copper surfaces (Espírito Santo et al., 2010). Here we tested in a time course exposure experiment the killing kinetics of *S. haemolyticus* on dry copper. Cells were grown, exposed to copper or stainless steel control surfaces, removed, and survivors counted. Cells were largely unaffected by contact to stainless steel for the duration of the experiment. However, on copper all 10^6 cells were killed after 7 min (Figure 1A) demonstrating that *S. haemolyticus* can be inactivated within minutes on dry copper.

Cells rapidly accumulate large amounts of dissolved copper from surfaces. We employed the qualitative copper-specific fluorescent dye Coppersensor-1 and quantitative ICP-MS to follow the degree and kinetics of copper ion uptake from the surfaces into cells. Cells even immediately removed from copper (t_0) had accumulated about 10 billion copper atoms (Figure 1B, upper panel). After 5 min, maximum concentrations of copper were reached and at 7 min, the time when all cells were killed, the concentrations declined again. In contrast copper concentrations in cells from stainless steel remained constant at low levels throughout (at about 2×10^8). Concentrations of other metals were also measured by ICP-MS (data not shown). For instance, concentrations of zinc or iron remained very similar in cells exposed to stainless steel or copper, respectively.

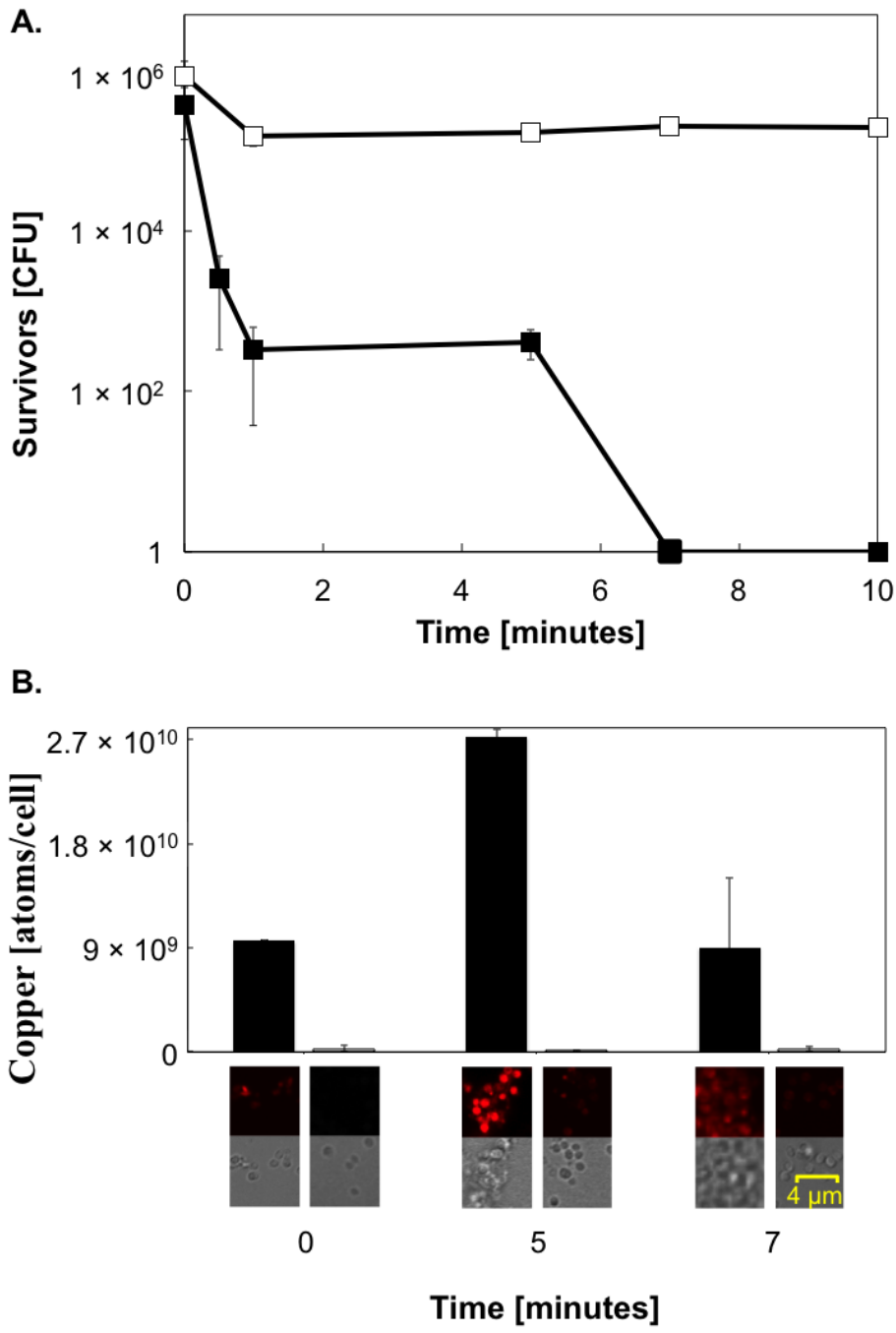

Figure 1 - *Staphylococcus haemolyticus* is rapidly killed on dry metallic copper surfaces and cells accumulate large amounts of copper. Cells of *S. haemolyticus* were exposed to dry metallic copper surfaces (■) or stainless steel (□) for the indicated times, removed, washed, and plated on solidified growth media. Survivors were counted as colony forming units (CFU) (A). Parallel samples (black bars, from copper; white bars, from stainless steel) were mineralized and subjected to ICP-MS analysis for determination of cellular copper content (B, upper panel) or were stained with the Cu(I)-specific fluorescent dye coppersensor-1 and subjected to fluorescence microscopy (B, lower panel). Shown are averages of triplicate experiments with standard deviations (error bars) and representative phase contrast and fluorescence microscopy images, respectively.

Cells stained with Coppersensor-1 fluoresced brightly red when exposed to copper surfaces for 5 min, at time by which about 99.9% of the cells have succumbed to copper toxicity (Figure 1B, lower panel). In contrast, cells immediately removed from copper (t0) or from stainless steel fluoresced weakly indicative of low copper (Figure 1B, lower panel). The apparent conflicting data (Coppersensor-1/ICP-MS) for copper exposed cells at t0 can easily be explained by the thick peptidoglycan of the cells. This polymer likely accumulated and slowed down the copper ions diffusing toward the cytoplasm, where Coppersensor-1 was located.

Exposure to metallic copper is not genotoxic to *Staphylococcus.* Because genotoxicity caused by metallic copper is controversial in Staphylococci, we next investigated if exposure to metallic copper caused an increase in mutations in S. haemolyticus. For this, cells were exposed to copper or stainless steel for 5 sec (before onset of massive cell death), washed, and plated onto solid media containing 80 µg/mL D-cycloserine. D-cycloserine interferes with cell wall biosynthesis and cells can only grow in its presence when a mutation event in the *aapA* gene has occurred, inactivating the D-serine/D-alanine/glycine transporter AapA by which D-cycloserine is likely taken up. Exposure to both copper and stainless steel resulted in very similar numbers of resistant mutants, clearly indicating that metallic copper did not increase mutation events in exposed cells (Figure 2A). In contrast, when the known mutagen formaldehyde was added to cells before exposure to stainless steel, significant higher mutant numbers (t-test, $P \leq 0.05$) were observed.

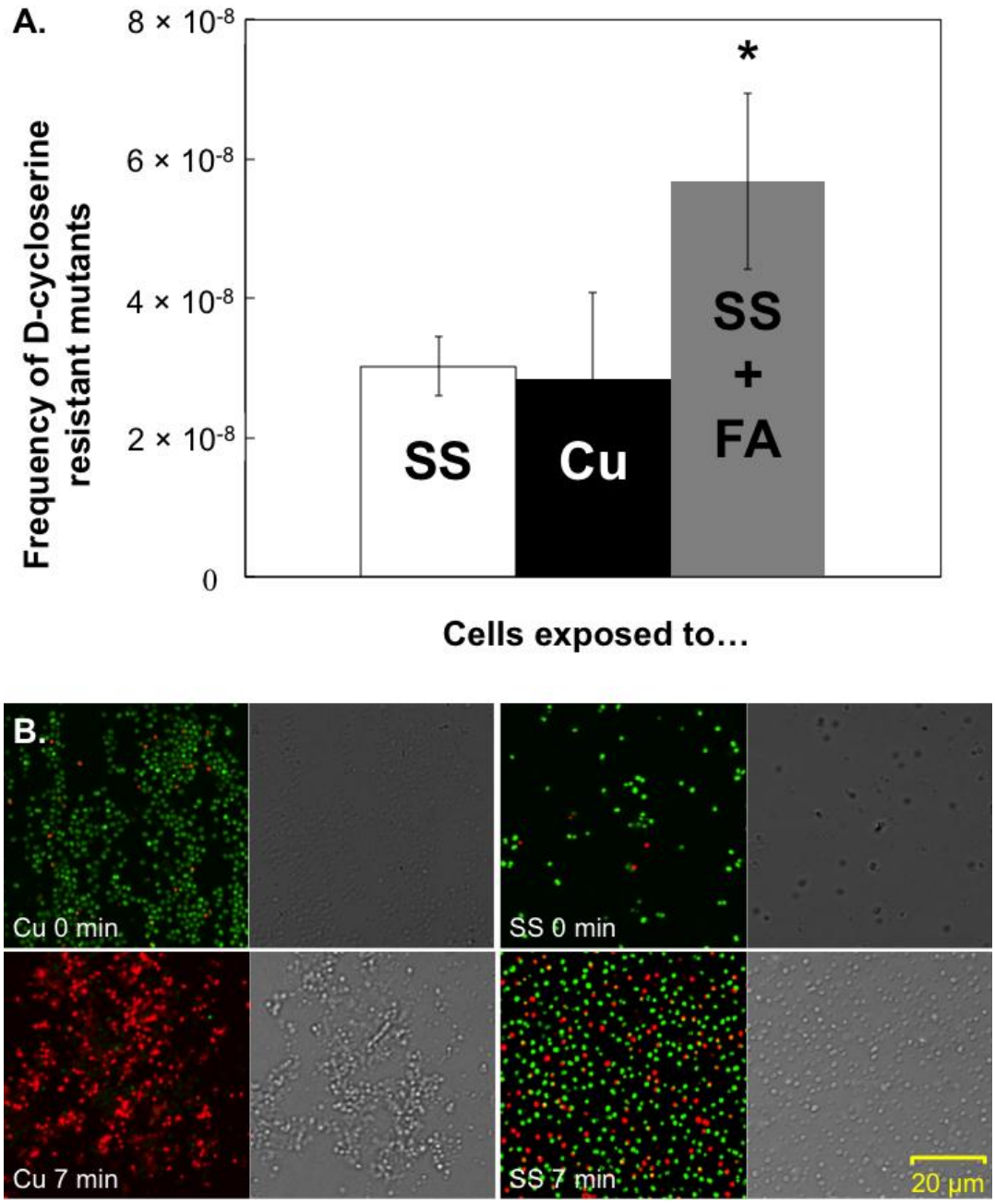

Figure 2 - Exposure to metallic copper surfaces does not promote mutations but causes membrane damage. Cells of *Staphylococcus haemolyticus* (1010 cells per sample) were exposed for 5 seconds to copper, stainless steel, or 0.25% (wt/vol) of the mutagen formaldehyde (CH_2O) + stainless steel surfaces. Cells were washed from surfaces, concentrated, and spread on solid media containing 80 µg × mL^{-1} D-cycloserine. D-cycloserine is bacteriostatic and colonies arise from inactivating mutations in the gene of the D-cycloserine uptake-permease AapA (A). Cells were exposed to metal surfaces for 0 or 7 minutes, removed, washed, subjected to Live/Dead® staining, and observed by fluorescence microscopy (B). Live bacteria with undamaged membranes fluoresce green, cells with damaged membranes fluoresce red. Shown are averages of triplicate experiments with standard deviations (error bars, A) or representative micrographs from three independent experiments with similar results (B). The asterisk denotes significantly ($P \leq 0.05$, t -test) different values in the mutagen formaldehyde-treated controls.

Contact to metallic copper damages *Staphylococcus* membranes. Dry copper surfaces did not cause mutation damage to the DNA (Figure 2A). An alternative explanation for cell death after contact to metallic copper might be lethal membrane damage. We investigated membrane damage using viability staining (Live/Dead® BacLight™ Bacterial Viability Kit, Invitrogen, Grand Island, NY USA). One dye (SYTO® 9) stains DNA in all cells, those with intact and those with compromised membranes, green. The other dye (propidium iodide) can only enter cells with damaged membranes and stains DNA red. Cells in contact with copper at t_0 had largely undamaged membranes and stained green (Figure 2B) but virtually all cells had membrane damage (red) after 7 min. Conversely, the majority of cells on stainless steel remained green, that is, had undamaged membranes throughout the experiment. The increase in numbers of damaged (red) cells correlated well with the killing kinetics (Figure 1A) in which also some death on stainless steel was observed. This background damage and lethality is likely owed to desiccation events occurring on these dry surfaces. However, the stainless steel controls clearly indicate that the killing on copper is not due to simple desiccation but rather mediated by contact with the copper surfaces.

Discussion

Overall, our results suggested that death in *S. haemolyticus* after contact to antimicrobial metallic copper coincided with membrane damage and that lethality was not caused by genotoxicity. As such, the Gram-positive Staphylococci were not very different in the events leading to killing from the Gram-negative *E. coli*, *Deinococcus radiodurans* from the bacterial Deinococcus–Thermus phylum (Espírito Santo et al., 2011) or the yeast *Candida albicans* (Quaranta et al., 2011). All these organisms suffered extensive membrane damage by metallic copper but their genetic materials were unaffected during the stress event prior to death. That copper, both in its ionic and its metallic form, is not genotoxic is probably best documented by two observations. First, copper ion stress did not cause mutations in *E. coli* (Macomber et al., 2007). Second, an organism with exceptional DNA-repair capabilities, such as *D. radiodurans*, was as efficiently inactivated by metallic copper as *E. coli* (Espírito Santo et al., 2011) further disfavoring the DNA-damage hypothesis of copper-mediated cell death. Furthermore, care has to be taken not to confuse the *in vitro* redox-activities of copper with what is happening inside the cell. For example copper had strong mutagenic properties when phage-DNA was in contact with copper ions *in vitro* and the DNA was then transfected into *E. coli* (Tkeshelashvili et al, 1992). In contrast, when the toxic properties of copper ions on living cells were studied *in vivo* recently, copper damaged catalytic iron–sulfur clusters in essential proteins rather than DNA (Macomber & Imlay, 2009).

Previous studies have demonstrated both the antimicrobial properties of ionic (e.g. Borkow & Gabbay, 2004; De Muynck et al., 2010; Nie et al., 2010) and also metallic copper surfaces against Staphylococci (Noyce et al., 2006; Airey et al.,

2007; Tolba et al., 2007) but did not offer a conclusive explanation for the mechanism of action of metallic copper surfaces. Only recently an effort was made to elucidate the underlying reasons why copper surfaces efficiently kill Staphylococci (Weaver et al., 2010). The authors claimed to have found two independent cellular targets of metallic copper toxicity, DNA, and respiration. Conversely, little damaging effect on cell membrane integrity was observed. This is remarkable, because respiration is a process tied to the cytoplasmic membrane that depends on intact membranes for buildup and use of a proton-motive force across the membrane for ATP biosynthesis. It is hard to consolidate inhibition of respiration with little membrane damage. Certainly, it is possible but unlikely that the observed damage accrued only in the respiratory proteins embedded within the membrane but not in the membrane itself.

Along this line of argumentation it is noteworthy that *D. radiodurans* was killed on copper surfaces as quickly as *E. coli* (Espírito Santo et al., 2011). *Deinococcus radiodurans* is resistant to oxidative protein carboxylation and can reconstitute genomes fragmented from exposure to ionizing radiation (Daly et al., 2007). Because *D. radiodurans* is nevertheless rapidly inactivated by metallic copper, makes it unlikely that DNA-genotoxicity and lethal protein damage are the major mechanism-of-action of contact killing by copper surfaces.

In one aspect, staphylococcal cells were clearly different from those of other bacteria tested previously on dry copper. It took about seven times longer to kill *Staphylococcus* compared to *E. coli* or *D. radiodurans* (Espírito Santo et al., 2011). A prolonged killing-process was certainly due to the thick peptidoglycan of staphylococcal cell walls. This strong diffusion barrier might also account for the

poor propidium iodide staining seen in Weaver et al. (Weaver et al., 2010) though in our hands we had little difficulties staining with this dye.

Our findings that *Staphylococcus* membranes were severely damaged upon contact with metallic copper, propose the membrane as primary target of copper surface-induced lethality. This notion is supported by our ICP-MS analysis. After 7 min of exposure to copper, when the cells were completely killed, the intracellular copper concentration had reached lower levels than at 5 min (Figure 1B) indicative of membrane leakage. Also, because the membrane had become permeable to the dye propidium iodide, the membrane potential had dissipated and so too had respiration ceased. Previously, we had noticed that fluorescent dye staining gave nonreproducible results when performed directly on metallic copper (Espírito Santo et al., 2011). Now we routinely remove cells from surfaces before staining. It might be that negative staining-artifacts accounted for the contradictory results reported in Weaver et al. (Weaver et al., 2010) and the patchy appearance of live and dead *S. aureus* cells in Airey and Verran (Airey & Verran, 2007). However, it should be noted that these studies investigated moist copper surfaces. Our study was concerned with dry copper surfaces because such dry touch-surfaces may be encountered in public and clinical environments where copper has recently been put to use (Casey et al., 2010; Mikolay et al., 2010). Nevertheless, contradictory results were presented for Enterococci on dry copper surfaces recently (Warnes & Keevil, 2011). While that study partially confirmed earlier work from our laboratory (Espírito Santo et al., 2011; Espírito Santo et al., 2008) the authors suggest DNA damage was among the first events of copper surface mediated killing. In this competing model, membranes were not compromised at an initial early stage but only after cells were inactivated.

This study at hand suggests that killing of Staphylococci on dry metallic copper surfaces follows the same principles as inactivation of other bacteria and yeasts. These results thus offer an alternative on the molecular mechanisms leading to cell death in these thick-cell-walled coccoid bacteria: genotoxicity may not be responsible for killing of the cells but rather a compromised cytoplasmic membrane leads to cessation of life processes.

Molecular knowledge of the mode-of-action exerted by metallic copper on microbes is certainly not strictly necessary for widespread application of antimicrobial surfaces in hygiene-sensitive areas. Currently, it is agreed-upon that genomic material will eventually degrade on metallic copper (Warnes et al., 2010; Warnes et al., 2011; Espírito Santo and Grass, unpublished observations) but it is controversial if this process is causative for or subsequent to cell death (Espírito Santo et al., 2011; Weaver et al., 2010). We propose that current data favor the model that membranes are damaged first, causing lethality, followed by protein oxidation (Nandakumar et al., 2011) and DNA-degradation. In depth understanding of the sensitive cellular targets of copper toxicity and the order of events leading to death, however, can be expected to provide new opportunities for improving the efficacy of copper surfaces against microbes.

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Chapter 5

General Discussion

Metallic copper is an effective antimicrobial against a variety of microorganisms. Strongly contributed by the work at hand, the antimicrobial efficacy of metallic copper surfaces has now been well established. These "self-sanitizing" surfaces have proven to be extremely effective in laboratory conditions and in hospital trials (Chapters 2, 3, 4 and Casey et al., 2010; Elguindi et al., 2009; Elguindi et al., 2011; Espírito Santo et al., 2008; Faúndez et al., 2004; Gould et al., 2009; Karpanen et al., 2012; Marais et al., 2010; Mehtar et al., 2008; Michels et al., 2009; Michels et al., 2008; Mikolay et al., 2010; Molteni et al., 2010; Noyce et al., 2006a, Noyce et al., 2006b, Noyce et al., 2007; Quaranta et al., 2011; Warnes et al., 2010; Warnes & Keevil, 2011; Weaver et al., 2008, Weaver et al., 2010; Weaver et al., 2010; Wheeldon et al., 2008; Wilks et al., 2005; Wilks et al., 2006). A variety of microorganisms, including viruses, bacteria, and fungi, were inactivated by metallic copper toxicity as shown by killing kinetics in Chapter 2 and, e.g., Mehtar et al., (2008); Noyce et al., (2007), Quaranta et al., (2011). Correspondingly, hospital trials using copper surfaces provide promising data: lower surface burden (Casey et al., 2010; Karpanen et al., 2012; Marais et al., 2010; Mikolay et al., 2010) and consequently lower infection rates (Schmidt, personal communication). In addition to strict hygiene policies and proper medical practices, metallic copper surfaces can help reduce hospital acquired infections (HAI). Surfaces can be contaminated by droplets (fomite) or by touch (direct contact). When people pick-up microbes from surfaces, the probability of being infected decreases with copper surfaces when compared to non-copper containing surfaces, due to a lower surface burden and to a possible inactivation of germs (Casey et al., 2010; Marais et al., 2010; Mikolay et al., 2010; Karpanen et al., 2012; Schmidt, personal communication). But metallic copper cannot

prevent all the HAI ways of transmission (Chapter 1). Hospitals still need to reinforce strict hygiene conditions, proper medical practices and manage ordinarily patients and staff to ensure that all measures are taken to reduce HAI (Ducel et al., 2002). Certainly, all these measures are not easy to apply and are prone to user error, being another reason why it is necessary to have additional tools to help reduce HAI. Metallic copper can be an important improvement for the healthcare environment, but additional studies are needed to determine which way metallic copper should be applied in order to be the most cost-effective (Grass, Rensing, & Solioz, 2011). This means that many diverse aspects need to be developed: (1) usage of a copper alloy that is the most active and able to keep its activity in the longer-term, along with a good esthetic appearance; (2) integration with other disinfection utensils in order to reach maximum efficacy; (3) use of suitable spore germinants that help eliminate spore contamination.

Antimicrobial activity of metallic copper surfaces is emphasized in this work: Chapter 2, shows that a variety of Gram-positive and -negative bacteria are effectively killed by dry exposure to copper surfaces; in Chapter 3, *Escherichia coli* is inactivated by dry (in one minute) and wet (three hours) exposure, *Deinococcus radiodurans* is killed in one minute by dry exposure; in Chapter 4, *Staphyloccoccus haemolyticus* is completely inactivated by dry exposure within seven minutes. Despite of these results, it is important to mention that this work does not focus exclusively on testing a variety of microorganisms on copper surfaces, instead this present work explores the factors and targets involved in the mechanism by which bacteria are inactivated by contact with copper surfaces.

The rise of metallic copper surface bacterial resistance is a risk factor for HAI control. Are there innate resistant bacteria to metallic copper? In Chapter 2, bacteria were isolated from copper coins (European 50-cent). A total of 294 isolates were investigated for metallic copper resistance (Chapter 2). Some isolates showed prolonged survival on dry copper surfaces (up to 3 days), but not on wet copper surfaces (Chapter 2). Additionally, these strains were not copper ion resistant compared to their respective type strains (Chapter 2). The observed survival on dry copper surfaces appeared to be due to persistence possibly mediated by protection via associated dirt particles, endospore formation, or alternatively may be caused by an innate ability to tolerate copper surface toxicity (Chapter 2). This latter kind of resistance would be novel and yet not understood. However the rise and more so, spread of dry copper surface resistance seems to be unlikely because: transfer of resistance determinants is prevented, given that plasmid DNA is completely degraded after cell death by metallic copper (Chapter 3 and by Hong et al., 2012; Warnes et al., 2010; Warnes & Keevil, 2011); contact killing is very fast and cells are not dividing on surfaces as shown in Chapter 2, 3, 4 and by Quaranta et al. (2011); finally, copper alloys have been used for thousands of years (Dollwet & Sorenson, 1985; Fields, 1947; Yu et al., 1995), and until now we do not experience wide-spread resistance to metallic copper toxicity.

Studies on antimicrobial properties of metallic copper surfaces. Toxic properties of wet and dry exposure to copper surfaces. There are two major ways employed to study metallic copper antimicrobial properties: wet and dry methods. The first method used was the wet method and it was developed by

Wilks et al. (2005). This method consists on applying a droplet of cells suspended in a buffer onto a surface. The dry method entails applying microbes directly on a surface with a very small amount of buffer that dries within seconds (Espírito Santo et al., 2008). This method was intended to simulate touch surfaces. These two methods have different toxicity mechanisms that result in unique killing kinetics as observed in Chapter 2 and 3 and by, e.g., Elguindi et al., (2009), Espírito Santo et al., (2008), Molteni et al., (2010), Wilks et al., (2005). The difference can be explained because, in the wet method, cells are not directly in contact with the surface. In order to be active, surfaces need to release ionic copper into the buffer suspension (Chapter 3 and Molteni et al., 2010). In other words, copper ion concentration needs to reach a certain level within the droplet in order to be toxic (Chapter 3 and Elguindi et al., 2011; Molteni et al., 2010). Consequently, cells will be inactivated as a result of deadly concentrations of copper ions and copper-induced stress (Chapter 3 and Molteni et al., 2010). Typically, cells are thus killed within hours (Chapter 3 and Elguindi et al., 2009; Mehtar et al., 2008; Molteni et al., 2010; Noyce et al., 2006b, 2007; Warnes et al., 2010; Weaver et al., 2008; Weaver, et al., 2010; Wilks et al., 2005; Wilks et al., 2006). Composition of the buffer in which cells are suspended is important for copper ion release and, consequently, killing efficacy differs in different buffer systems (Molteni et al., 2010). Tris-buffer e. g., oxidized and solubilized copper ions efficiently from the surfaces (Molteni et al., 2010). Hence, cells become more sensitive to copper surface toxicity when Tris-buffer, instead of water and phosphate-based buffer, was used. Complementary results were noticed with inhibition of surface corrosion. The presence of corrosion inhibitors enhances survival on wet copper surfaces (Elguindi, et al., 2011). With those results, new

disinfection and cleaning materials need to be developed to aid maintaining the antimicrobial activity of copper surfaces.

For the dry method, mainly used during this work described in Chapters 2, 3 and 4, the small amount of buffer that avoids direct contact of cells with the surface, dries quickly (Espírito Santo et al., 2008). Thus, surfaces undergo oxidation and cells accumulate copper almost instantaneously and are killed within minutes of exposure (Chapter 3, 4 and by Quaranta et al., 2011). Even at the shortest time (few seconds) of exposure cells accumulate high copper amounts that reach 10^9 atoms/cell (Chapter 3, 4 and by Quaranta et al., 2011). As a result, cells experience a short sharp shock by contact with copper surfaces (Grass et al., 2011).

Toxic properties of dry exposure to metallic copper surfaces are complex and diverse. Here, cells undergo multiple stresses: osmotic stress (Espírito Santo et al., 2008), surface oxidation (release of reactive oxygen species (ROS) and copper ions) and copper accumulation (Chapter 3, 4 and Quaranta et al., 2011). Studies on metallic copper toxicity cannot be directly compared to studies on ionic copper toxicity. This statement is based on the fact that ionic copper toxicity studies are based on microbial cultures that suffer a continuous exposure to copper while dividing and growing. And, on the other hand, metallic copper surfaces studies (wet and dry exposure) expose a non-dividing culture on a surface (Chapters 2, 3 and 4 and e.g. Elguindi et al., 2009; Molteni et al., 2010; Wilks et al., 2005). Therefore, there is no growth and thus, e.g., also no protective biofilm formation with dry surface exposure as described in Chapters 2, 3 and 4.

Distinctive cellular characteristics result in different killing efficacy. Results obtained here and in other work showed that the antimicrobial activity of metallic copper differs for Gram -positive and -negative bacteria (Chapter 2, 3 and 4). In general, for both methods (wet and dry) Gram-positive bacteria are able to survive longer on metallic copper surfaces when compared to Gram-negative bacteria as outlined by the killing experiments in Chapters 2, 4 and by Elguindi, et al. (2011); Molteni et al. (2010); Noyce et al. (2007). Interestingly, both Gram-positive and -negative bacteria show similarly high copper accumulation, as shown by copper accumulated by cells in Chapters 3 and 4. Differences in survival coupled with similar copper accumulation, might be due to an intrinsic feature of the Gram-positives: a thicker cell-wall peptidoglycan layer which functions as a buffer and diffusion barrier for copper ions. Additionally, these cells may also have an innate ability to resist desiccation (in the case of dry-exposure) playing a role on the survival on copper surfaces.

Bacterial inactivation mechanism upon dry exposure to copper surfaces. There are multiple interrelated molecular factors that play a role during bacterial killing by dry exposure to copper surfaces. When cells get in contact with copper surfaces, copper ions are dissolved from the surface leading to the first steps of cell damage (Chapter 3 and Espírito Santo et al., 2008; Molteni et al., 2010; Quaranta et al., 2011). First of all, the presence of copper ions and ROS stress induces toxicity to the membranes leading to loss of membrane structure (Chapter 3, 4, Espírito Santo, Bleichert and Grass, unpublished results and Hong et al., 2012; Quaranta et al., 2011). This is the major step that causes cellular inactivation. Further cell damage is induced by copper ions and ROS generation

affecting other cellular biomolecules such as proteins (Nandakumar et al., 2011). After cell death, genomic and plasmid DNA become degraded (Chapter 3 and Hong et al., 2012). This mechanism supports the view of the proposed chain of events that lead to cell inactivation by copper surfaces by Grass, Rensing and Solioz (2011) (Figure 1).

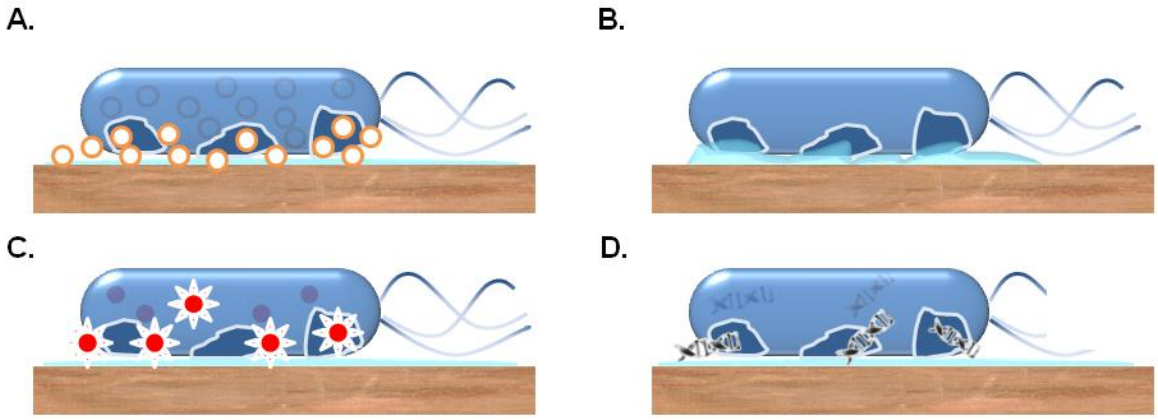

Figure 1 – Schematic that represents chain of events in contact killing. (A) Cells enter in contact with the surface, copper is released causing cellular damage. (B) Cell membrane becomes permeable due to copper and other stress, leading to loss of membrane potential and cytoplasmic content. (C) Generation of reactive oxygen species is provoked by copper ions, which cause further cell damage. (D) cellular DNA becomes degraded (adapted from Grass et al., 2011).

This chain of events can be explained in more detail. When cells are applied on a dry metallic copper surface, copper ions are rapidly released and high quantities are quickly accumulated by cells, as outlined by the copper quantification assays in the Chapters 3 and 4 and by Quaranta et al. (2011). Simultaneously, ROS are generated as evidenced by protective effects of ROS quenchers which help prolong survival on copper surfaces (Espírito Santo et al., 2008; Warnes & Keevil, 2011) and by ROS fluorescent indicators (Quaranta et al., 2011). As a consequence, these two related events (copper and ROS generation) induce toxicity and damage cellular components. Indeed, membranes are the first component to be damaged by copper surface toxicity as observed by the

Live/Dead experiments in Chapters 3, 4 and Quaranta et al. (2011). Present evidences indicates that membranes are damaged due to lipid peroxidation (Hong, et al. 2012). Consequently, when lipid oxidation reaches an overwhelming level, the process becomes lethal for the cells. Thus, cells become inactivated by the damage inflicted on the membranes, which then leads to loss of membrane potential (Quaranta et al., 2011; Warnes & Keevil, 2011) and likely release of cytoplasmic contents. Finally, continuous presence of copper leads to further ROS production which induces further damage to various biomolecules, including proteins and cellular DNA (Chapter 3 and Hong et al., 2012).

Details of the proposed chain of events are still under discussion, in particular their exact temporal sequence. Keevil and co-workers (Warnes et al., 2010; Warnes & Keevil, 2011) suggested an alternative chain of events, authors argue that Cu(I) and Cu(II) and superoxide are responsible for killing under wet and dry exposure; and the first event that leads to cell death is DNA damage followed by cessation of bacterial respiration and membrane depolarization, with no observed membrane damage (Warnes & Keevil, 2011). However, it is clear by the killing experiments done with *D. radiodurans* (Chapter 3) and by the mutation rate experiment with *E. coli* (Chapter 3), *S. haemolyticus* (Chapter 4) and *S. cerevisae* (Quaranta et al., 2011) that upon contact to metallic copper, DNA is not the first target of copper surface-induced toxicity. Eventually, when cells are dead and with no further protection against toxicity, DNA becomes degraded, as demonstrated by the comet assay in Chapter 3. Additionally, freshly surface-released copper and ROS will induce toxicity to the closest biomolecules available, the lipids. Indeed, recent experimental data suggest that lipids are damaged first (Hong, et al. 2012) followed by protein oxidation (Nandakumar, et

al., 2011). Lipids were found to be affected by peroxidation (Hong, et al. 2012). Copper and ROS are known contributors for initiation of lipid oxidation processes (Chapter 1). In fact, cells accumulate such a high quantity of copper ions that copper-induced lipid peroxidation seems more than likely. Considering the presence of ROS, in particular the highly reactive hydroxyl radical ($HO^{\bullet}$), lipid peroxidation appears to be inevitable. Lipid peroxidation is an autocatalytic and self-propagating mechanism (Schaich, 2005) and is then boosted by the continuous presence of high copper-levels and further ROS production. Additionally, oxidation is rapid, and it propagates into many different reactions, which further enhances other reactions leading to further lipid degradation. This fits with the observed fast killing kinetics of cells exposed to copper surfaces. Preliminary data from fatty acid methyl esters (FAME) analysis revealed that the most predominant fatty acids were affected by metallic copper exposure compared with stainless steel surfaces (personal unpublished observations). Further analysis is needed to determine which lipids are mainly targeted by the toxicity and by which reactions occur during oxidation.

Damage to the membranes also can explain the loss of respiration observed by Keevil and co-workers (Noyce et al., 2006a, 2006b; Warnes et al., 2010; Warnes & Keevil, 2011; Weaver et al., 2008; Sandra A. Wilks et al., 2006) likely via loss of the proton motive force. Also, respiration can be inactivated by protein oxidation (Nandakumar et al., 2011). Alternatively, as suggested by Warnes & Keevil (2011), some cytochromes are inhibited by copper binding, through a change in their conformation. However, this alternative seems untimely: the first damage that causes lethality, occurs on the membranes, making the membrane permeable (Chapters 3, 4 and Hong et al., 2012) and uncoupling the respiratory

chain. Additionally, due to the fast killing kinetics (Chapters 3 and 4), high copper accumulation and high ROS generation (Chapters 3 and 4 and Espírito Santo et al., 2008; Quaranta et al., 2011), toxicity should not be focused just on cytochromes but on all components of the membrane (including the complete respiratory chain).

Closing remarks. Despite all the differences suggested on the mechanism by which copper surfaces inactivate cells, it is clear that metallic copper is an important tool that surely can help reduce HAI, when applied correctly. Hospitals and other public places are expected to benefit from these natural antimicrobial surfaces, which quickly inactivate microbes with a low probability of rise of resistance (DNA is degraded in the killing process). Additionally, these surfaces are easy to apply or refit. History has shown us the benefits of copper usage for thousands of years with little toxicity observed, making metallic copper surfaces irrevocably safe for human usage.

Main conclusions:

- Cells are inactivated both by wet and dry exposure to metallic copper (Chapter 2, 3 and 4). Contact-killing is rapid (within minutes) and complete; cells are incapable of developing resistance. On moist copper surfaces, due to the dependence of dissolved copper ions and the corresponding concentration in the droplet, the killing is slower than by contact-killing but still fast (within hours) (Chapter 3). Development of resistances is also unlikely if the droplet dries up. Otherwise, the liquid might have a chance of repopulation and, consequently, may lead to biofilm formation (e.g. biofilms in used copper pipes).
- Since bacterial DNA is degraded in the process of cell death, the emergence and the spread of resistance is unlikely (Chapter 3).
- Classical antibiotics hit only one target, copper toxicity hits several targets (Chapter 3 and 4).
- Historical usage of copper has proven to be safe for human health. Additionally, wide-spread emergence of microbes fully resistant to metallic copper toxicity has not yet been observed (Chapter 2).

Potential expectations for safe and proficient appliance of metallic copper surfaces*:* There are still unanswered questions to be addressed that are essential for a safe and efficient application of metallic copper as an additional tool for fighting HAI:

- It should be determined if copper surfaces have long-term (years, not months) antimicrobial properties in public places.

- Which alloys are the most effective in the longer-term, and able to keep the surface' esthetic looks.
- Improved disinfection regiments need to be developed for the surfaces to reach maximum efficacy.
- Ultimately, metallurgic chemistry or surface micro-architecture and disinfection chemistry need to find alternatives to completely eliminate endospore contamination.

In order to complete the elucidation of the molecular mechanism by which cells are inactivated by metallic copper there are still unexplored fields to explore:

- Further research on the lipid oxidation is required, e.g., which lipids are mainly targeted by the toxicity.
- Additionally, protein oxidation needs to be examined, e.g., determine which proteins are mainly affected and by which process proteins suffer oxidation.
- What is the role (if any) of osmoregulated periplasmic glucans (OPG) in the temporary protection of cells against metallic copper surface exposure.
- Determine if metallic copper induces the so-called viable but nonculturable state (VBNC) in which bacteria become dormant but may retain their virulence and pathogenicity (Baffone et al., 2003; Du et al., 2007; Rahman, Shahamat, Chowdhury, & Colwell, 1996; Sun et al., 2008).

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Annex A

Isolation and Characterization of Bacteria Resistant to Metallic Copper Surfaces

Evaluation of antibiotic resistance levels amongst Staphylococci.

Table A1 - Antibiotics used for the BioMérieux antibiotic disk assay.

Antibiotic class	***Antibiotic name***	***Quantity (µg)***
Rifamycin	Rifampicin (RA)	30
Aminoglycoside	Gentamicin (GM)	10
	Kanamycin (K)	30
	Streptomycin (S)	10
Glycopeptide	Vancomycin (VA)	30
Penicillin A	Ampicillin (AM)	10
Amphenicol	Chloramphenicol (C)	30
Quinolone	Nalidixic Acid (NA)	30
Cephalosporin	Ceftriaxone (CRO)	30
	Cefalotin (CF)	30
	Ceftazidime (CAZ)	30
Macrolides (MLS)	Erytromycin (E)	15
Lincosamides (MLS)	Lincomycin (L)	2
Polypeptides	Colistin (CL)	50
	Polymyxin B (PB)	300
Tetracycline	Tetracycline (TE)	20
	Doxycycline (D)	30

Table A2 - Resistances of staphylococcal copper coin isolates to antibiotics in the BioMérieux antibiotic disk assay.

A.	AMINOGLYCO-SIDE			RIF-AMYCIN	TETRA-CYCLINE		GLYCO-PEPTIDE	PENI-CILLIN A	AMPHE-NICOL	QUINOLONE
Isolate	K 30	GM 10	S 10	RA 30	TE 30	D 30	VA 30	AM 10	C 30	NA 30
N7	S	S	S	S	S	S	R	S	S	R
L69	S	S	R	S	S	S	S	S	S	S
N19	S	S	S	S	S	S	S	R	S	R
R10P	R	R	R	R	R	R	R	R	R	R
N63	S	S	S	S	S	S	S	R	S	S
R28P	S	S	R	S	S	S	R	R	S	R
N51	S	S	S	S	S	S	R	S	S	S
N32	S	S	S	S	S	S	S	R	S	S
L44	S	S	S	R	S	S	R	S	S	R
N93*	S	S	S	S	S	S	S	S	S	S
N78	S	S	S	S	S	S	R	R	S	S
L46	S	S	S	S	S	S	S	R	S	R
N42	S	S	S	S	S	S	S	S	S	S
N16	S	S	R	I	S	S	R	I	I	S
N91	S	S	S	S	S	S	S	I	S	S
R77	R	I	S	R	S	R	R	I	I	S
N22	S	S	S	S	S	S	R	S	I	S
N60	S	S	I	S	S	S	S	R	R	S
R4P	S	S	R	S	S	S	R	I	S	S
L47*	S	S	S	S	S	S	R	R	I	S
N44*	S	I	S	S	S	S	R	I	S	R
N70	R	R	R	S	S	I	R	R	I	S
N56*	S	S	S	S	S	S	R	I	S	S
N85	S	S	R	S	S	S	R	I	S	R
R52	S	S	R	S	S	S	R	S	S	R
R40	I	S	S	S	R	S	S	S	S	R
N23	R	S	S	S	S	S	R	S	S	I
L77*	R	I	R	S	S	S	R	S	S	R
N49	S	S	S	I	S	S	S	R	R	S
R8P	R	S	S	I	I	R	R	R	R	R
N40	S	S	S	S	S	S	S	I	S	S
N22	S	S	I	S	S	S	R	I	R	R
N78	S	S	S	S	S	S	S	R	S	S
R7P	S	S	S	S	S	S	S	R	S	R
L62	S	S	S	S	S	S	S	S	S	S
N28	S	S	S	S	S	S	S	S	S	R
L63	S	S	S	S	S	S	S	I	S	R
L42	S	S	S	S	S	S	R	S	S	S
R13P	S	S	S	S	S	S	S	S	S	R
N5	S	S	S	S	S	S	S	I	I	R
N27	S	S	S	S	S	S	S	I	S	S
N46*	S	S	S	S	S	S	R	I	S	R
N10*	S	S	S	S	S	S	R	I	S	S
N99	S	S	S	S	S	S	S	S	S	R
N73	S	S	S	S	S	S	R	S	I	R
N22	S	S	S	S	S	S	S	S	S	S
N16	S	S	S	S	S	S	R	I	S	S
N42	S	S	S	S	S	S	S	S	S	S
N4*	S	R	I	I	R	S	R	R	I	S
R32P	S	S	S	S	S	S	S	I	S	S
R42	I	I	S	I	S	I	R	S	I	S
N65	S	S	S	S	S	S	R	R	R	R
R42P	S	S	S	S	S	S	R	R	R	R
N101*	S	S	R	S	I	R	R	R	R	R
N97	R	R	S	S	R	R	R	I	R	R
R77	R	R	S	I	S	R	R	I	S	S

Table A2 continued

A.	AMINOGLYCO-SIDE			RIF-AMYCIN	TETRA-CYCLINE		GLYCO-PEPTIDE	PENI-CILLIN A	AMPHE-NICOL	QUINOLONE
Isolate	K 30	GM 10	S 10	RA 30	TE 30	D 30	VA 30	AM 10	C 30	NA 30
N91*	S	S	S	S	S	S	S	S	S	I
L78	S	S	S	S	R	I	S	S	S	S
L49	S	S	S	S	S	S	R	R	S	S
N88	R	S	S	I	S	S	R	R	R	R
N55	S	S	S	I	S	S	R	I	I	S
N76*	S	S	S	S	S	S	S	R	S	S
R33	S	S	I	S	S	R	R	S	R	I
R66P	I	S	S	R	R	I	R	R	R	R
R51*	R	R	R	R	R	R	R	R	R	R

B.	CEPHALOSPORIN			LINCOSAMIDE	MACROLIDE	POLYPEPTIDE	
Isolate	CRO 30	CF 30	CAZ 30	L 2	E 15	CL 50	PB 300
N7	S	S	I	R	R	I	
L69		S	S	I	S	S	R
N19	R	R	R	R	S	R	
R10P	R	R	R	R	R	S	
N63	R	I	R	R	S	R	
R28P	R	R	R	R	S	R	
N51		R	I	R	R	S	S
N32		S	S	S	R	S	S
L44	S	S	S	I	S	S	
N93*	S	S	I	R	S	S	
N78	S	S	I	R	S	R	
L46	R	S	R	R	S	S	
N42	S	S	S	S	S	S	
N16	S	I	S	R	R	S	
N91*	S	S	I	R	S	S	
R77	I	R	R	R	R	S	
N22	S	S		R	S	S	
N60		R	R	R	S	R	R
R4P		I	R	R	S	S	
L47*	R	R	R	R	R	I	
N44*	R	I	R	R	R	I	
N70	R	R	R	R	S	R	
N56*		I	I	R	R	S	R
N85	S	R	R	R	R	S	
R52	I	S	R	R	S	S	
R40	S	S	S	I	S	S	
N23		S	I	R	S	S	R
L77*	S	S	I	R	S	S	
N49		R	R	R	S	R	R
R8P	R	R	R	R	R	R	
N40	R	R	R	R	S	R	
N22	R	R	R	R	R	I	
N78	R	R	R	R	S	R	
R7P	S	I	I	R	S	S	
L62	S	S	S	S	R	S	
N28	S	S	S	S	R	S	
L63*	R	I	R	R	S	I	
L42	S	S	S	R	S	S	
R13P	S	S	S	R	S	S	

Table A2 continued

B.	CEPHALOSPORIN			LINCOSAMIDE	MACROLIDE	POLYPEPTIDE	
Isolate	**CRO 30**	**CF 30**	**CAZ 30**	**L 2**	**E 15**	**CL 50**	**PB 300**
N5	S	S	S	R	S	S	
N27	R	I	R	R	R	S	
N46*	R	I	R	R	R	I	
N10*		I	I	R	S	S	R
N99		S	R	I	R	S	S
N73	R	S	I	R	R	S	S
N22	S	S		I	S	S	
N16	S	I	S	R	R	S	
N42	S	S	S	S	S	S	
N4*	R	R	R	R	S	R	
R32P	R	S	R	R	S	S	
R42	S	R	S	R	R	S	
N65		I	R	R	R	S	R
R42P		S	R	R	S	S	R
N101*	R	I	R	R	R	R	
N97		I	R	R	R	R	R
R77	S	R	I	R	R	S	
N91	S	S	I	R	S	S	
L78	S	R	S	S	I	S	
L49	R	R	R	R	R	S	
N88	R	R	R	R	S	R	
N55		I	S	R	R	S	R
N76*		I	R	R	S	R	R
R33	R	S	R	R	S	S	
R66P	R	R	R	R	R	R	
R51*	R	R	R	R	R	R	

*indicate copper surface resistant strains (at least 1 day resistant)

Annex B

Draft Genome Sequence of *Pseudomonas psychrotolerans* L19, Isolated from Copper Alloy Coins

Results published in:

Espírito Santo, C., Lin, Y., Hao, X., Wei, G., Rensing, C., & Grass, G. (2012). Draft genome sequence of *Pseudomonas psychrotolerans* L19, isolated from copper alloy coins. Journal of Bacteriology, 194(6), 1623–4.

Abstract

We report the draft genome sequence of *Pseudomonas psychrotolerans* strain L19, isolated from a European 50-cent copper alloy coin. Multiple genes potentially involved in copper resistance were identified; however, it is unknown if these copper ion resistance determinants contribute to prolonged survival of this strain on dry metallic copper.

Genome Announcement

Copper is an essential trace element in most living organisms, including humans. While needed in small amounts, copper can easily become toxic when in surplus. Coins from many countries are made from copper or its alloys. In order to isolate metallic copper-resistant bacteria, European 50-cent coins from general circulation were sampled (Espírito Santo et al., 2010). Coins were kept under sterile conditions for 24 h before bacteria were isolated by plating coins on solidified medium. The Gram-negative *Pseudomonas psychrotolerans* strain L19 was isolated from an LB agar plate. Sequencing of the 16S rRNA gene was performed and the strain was also reexposed to metallic copper for 1, 2, or 7 days. *P. psychrotolerans* strain L19 (classified as *P. oleovorans* L19 in reference Espírito Santo et al., (2010)) was able to survive on copper surfaces for >48 h, which is >5,000 times longer than *Escherichia coli* under identical conditions. The MIC of strain L19 for $CuCl_2$ was 3.5 mM (Espírito Santo et al., 2010). For further characterization of strain L19, colony morphology, antibiotic resistance, utilization of carbon sources, and enzymatic characterizations were performed (Hauser, Kämpfer, & Busse, 2004) and compared with the type strains *P. psychrotolerans* C36T and its closest relative *Pseudomonas oleovorans* DSM 1045T (Espírito Santo, unpublished results).

Reads were generated by 454 GS FLX sequencing (Margulies et al., 2005), and raw data were assembled using the GS de novo assembler (“Newbler”) version 2.5.3 (Roche Diagnostics). The assembled contigs were submitted to the RAST annotation server for subsystem classification and functional annotation (Aziz et al., 2008). Coding sequences (CDSs) were assigned using BLASTp with KEGG Orthology (KO). The G+C content was calculated using an in-house Perl script.

The NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) was employed for gene annotation in preparation for submission to GenBank (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html).

The draft genome sequence of *P. psychrotolerans* strain L19 comprises 5,098,787 bases representing a 42-fold coverage of the genome. The assembled genome consists of 46 large contigs (>500 bp) with an average contig size of 110,827 bp and a G+C content of 65.68%. The genome encodes 4,660 putative CDSs, of which 4,641 CDSs have functional predictions. The draft genome sequence contains two ribosomal RNAs, three 5S rRNAs, and 54 tRNAs loci. For the CDSs, 3,887 proteins could be assigned to cluster of orthologous groups (COG) families (7). A total of 2,454 proteins have orthologs (bit score > 60), with 27 of the closest neighbors to strain L19 belonging to eight genera (Pseudomonas, Azotobacter, Chromohalobacter, Marinobacter, Shewanella, Alcanivorax, Hahella, and Methylococcus) as identified by RAST (Aziz et al., 2008).

The *P. psychrotolerans* strain L19 genome carries multiple genes and operons potentially involved in copper resistance, such as the cus operon encoding an RND-type efflux system and genes encoding multicopper oxidases typically involved in oxidizing Cu(I) to Cu(II).

Recent publications (Espírito Santo et al., 2011; Espírito Santo et al., 2008; Warnes & Keevil, 2011; Weaver et al., 2010) have focused on the antibacterial mode of action exerted by metallic copper surfaces. It is tempting to speculate that the peculiar membrane composition of *P. psychrotolerans* contributes to the extended survival of this species on metallic copper. In the type strain, diverse unidentified phospholipids, lipids, and aminophospholipids are present (Hauser et

al., 2004), Similar compounds in strain L19 constitute promising candidates for further studies.

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited in DDBJ/EMBL/GenBank under accession number AHBD00000000. The version described in this paper is the first version, AHBD01000000.

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Warnes, S. L., & Keevil, C. W. (2011). Mechanism of copper surface toxicity in vancomycin-resistant enterococci following wet or dry surface contact. *Applied and environmental microbiology*, *77*(17), 6049–59. doi:10.1128/AEM.00597-11

Weaver, L., Noyce, J. O., Michels, H. T., & Keevil, C. W. (2010). Potential action of copper surfaces on meticillin-resistant *Staphylococcus aureus*. *Journal of applied microbiology*, *109*(6), 2200–5. doi:10.1111/j.1365-2672.2010.04852.x

Errata:

1. Missing Bolds:
   Page 10: “Figure 3”;
   Page 11: “Figure 4”;
   Page 12: “Figure 5”;
   Page 14: “Figure 6”, “Figure 7”, “Figure 8”;
   Page 15: “1.4”.

2. Wrong figure numbering:
   From page 18 section 1.5 until the end of the section at the page 26:
   “Figure 8” should be stated “Figure 9”;
   “Figure 9” should be stated “Figure 10”;
   “Figure 10” should be stated “Figure 11”.

3. Reference statement mistake:
   Page 30: “Lambert, P. a.” should be stated “Lambert, P. A.”;
   Page 31 and 141: “Elguindi, Jutta” should be stated “Elguindi, J.”;
   Page 32: “Michels, C. a.” should be stated “Michels, C. A.”;
   Page 33, 102, 124: “Imlay, J. a.” should be stated “Imlay, J. A.”;
   Page 34: “Michels, Harold” should be stated “Michels, H.”;
   Page 35: “Hale, J. a.” should be stated “Hale, J. A.;
   Page 74: “Sies, Abelson, & Sino, 1990” should be stated “Sies et al., 1990);
   Page 75: “Outen, Huffman, Hale, & O’Halloran, 2001” should be stated “Outen et al., 2001” and “Macomber, Rensing, & Imlay, 2007” should be stated “Macomber et al., 2007”;
   Page 79: “Singh, McCoy, Tice, & Schneider, 1998” should be stated “Singh et al., 1998”;
   Page 80: “Sambrook, Fritsch, & Maniatis, 1989” should be “Sambrook et al., 1989”;
   Page 103: “Kumar, S. a.” should be stated “Kumar, S. A.” and “Loeb, L. a.” should be stated “Loeb, L. A.”;
   Page 121: “Weaver et al.” is repeated, should be stated “Weaver et al. (2010)”;
   Page 137: “Sandra A. Wilks et al., 2006” should be stated “Wilks et al., 2006”;
   Page 140: “Rahman, Shahamat, Chowdhury, & Colwall, 1996” should be stated “Rahman et al., 1996”;
   Page 143: “Wilks, Sandra” should be stated “Wilks, S.”

4. Missing italics on species names:
   Page 58: “Bacillus” and “Staphylococcus”
   Page 81: “B. cereus”
   Page 84: “E. coli”