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REVIEW ARTICLE

Contact killing and antimicrobial properties of copper

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Abstract

With the emergence of antibiotic resistance, the interest for antimicrobial agents has recently increased again in public health. Copper was recognized in 2008 by the United States Environmental Protection Agency (EPA) as the first metallic antimicrobial agent. This led to many investigations of the various properties of copper as an antibacterial, antifungal and antiviral agent. This review summarizes the latest findings about 'contact killing', the mechanism of action of copper nanoparticles and the different ways micro-organisms develop resistance to copper.

Medical use of copper

Copper and its compounds have been used as disinfectant agents for many centuries (Dollwet and Sorenson 1985; Huang *et al.* 2005). Since the nineteenth century, the discovery of a causal link between diseases and pathogens has revolutionized modern medicine. Research turned to the development of antimicrobial agents, especially antibacterial ones and many studies have been carried out on the antibacterial effects of metals such as copper (Grass *et al.* 2011). The advent of antibiotics in the 1930s considerably hampered research on antimicrobial agents, but the emergence of multidrug-resistant bacteria and the spread of antibiotic resistance at the end of the 1980–1990s forced the research community to take a new approach to the use of iodine, silver and copper (Landeen *et al.* 1989; Pyle *et al.* 1992). In 2008, the EPA officially recognized copper and its alloys as the first effective metallic antimicrobial agent (http://www.epa.gov/pesticides/factsheets/copper-alloy-products.htm). They recognized its ability to kill 99·9% of pathogenic bacteria within 2 h. Since then, quick progress has been made on the bactericidal properties of copper surface, known as 'contact killing', allowing to rapidly eliminate pathogenic bacteria. This killing activity takes place at a rate of at least 7–8 logs per hour and no micro-organism survives after prolonged incubation on copper surfaces (Grass *et al.* 2011; Prado *et al.* 2012). However, the exact mechanisms involved in the so-called 'contact killing' are still not fully understood.

Current research is focused on the antimicrobial properties of copper to demonstrate its benefit and elucidate its mechanisms of action. Current applications of copper use are extensive and range from construction sites to healthcare infection prevention in hospitals (Vincent *et al.* 2016) despite the lack of understanding of the exact antimicrobial mode(s) of action and possible limitations.

This review focuses on current data on antibacterial, antifungal and antiviral properties of copper. Hypotheses concerning the antimicrobial mechanism of copper surface as well as the mechanism of copper resistance in different micro-organisms (bacteria, fungi and viruses) are

presented. The antimicrobial properties depending on the conditioning copper form, solid or ionic, and the antimicrobial activity of copper in particle form are also discussed.

Antibacterial activity of copper

Copper as an antibacterial surface agent

Copper can easily be used in public health in its solid form in hospitals and medical environments, for example, doors, knobs, piping material or other inanimate surfaces in different facilities (Casey *et al.* 2010; Mikolay *et al.* 2010; Karpanen *et al.* 2012; Schmidt *et al.* 2012; Inkinen *et al.* 2017) or for medical devices (Goudarzi *et al.* 2017; Schmidt *et al.* 2017). To test its antimicrobial surface activity, researchers mostly use coupons with varying concentrations of copper.

Noyce *et al.* (2006) tested copper alloys (61–95% Cu) for their antibacterial activity on *E. coli* O157 at different temperatures (22°C and 4°C). They demonstrated an antibacterial effect in all conditions, especially at 22°C, but only the high copper alloys (95% Cu) completely killed *E. coli* (Noyce *et al.* 2006). Similarly, Wilks *et al.* (2005) demonstrated copper antibacterial activities on *E. coli* O157 at different temperatures (20°C and 4°C). They also showed antibacterial activities in all conditions, especially at 20°C and when copper concentration in alloys was superior to 85% (Wilks *et al.* 2005).

Additionally, the effect of copper alloy surfaces (65–100% Cu) against vegetative and sporal *Clostridium difficile* was studied and it was demonstrated that copper alloys (> 70% Cu) provide a significant reduction in survival of *C. difficile* vegetative cells and spores with a complete killing after 24–48 h (Weaver *et al.* 2008). The antibacterial effect of copper coupons (C19700, 99% Cu) on *C. difficile* was also highlighted and a complete killing of *C. difficile* vegetative cells within 30 min and a 99·8% reduction in the viability of *C. difficile* spores after 3 h was observed (Wheeldon *et al.* 2008).

A study investigated the antibacterial activity of copper coupons (99% Cu and 63% Cu) on clinical isolates of *E. coli*, *Enterobacter spp.*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* all being potent multidrug-resistant Gram-negative pathogens responsible for nosocomial infections. Copper surfaces showed antimicrobial activity in all tested strains, especially with copper coupons containing 99% Cu which had a bactericidal effect within 2, 3, 5 and 6 h for *A. baumannii*, *Enterobacter spp.*, *K. pneumoniae* and *P. aeruginosa* and *E. coli* respectively (Souli *et al.* 2013).

In summary, the copper antimicrobial activity increases proportionally to its concentration, in accordance with several studies (Mehtar *et al.* 2008; Elguindi *et al.* 2009; Zhu *et al.* 2012; Souli *et al.* 2013) and pure copper coupons present higher antibacterial efficacy. These studies also highlight that the experimental temperature directly impacts the 'contact killing' process, in agreement with other previously published studies (Faúndez *et al.* 2004; Elguindi *et al.* 2009; Michels *et al.* 2009). Furthermore, it was shown that copper ions released from coupons were media dependent (Molteni *et al.* 2010). The authors compared four different media: 0·1 mol l^{-1} Tris-Cl pH 7, M17 (Terzaghi and Sandine 1975), water and 100 mmol l^{-1} phosphate-buffered saline pH 7 (NaP_i), and demonstrated that in Tris-Cl and M17 media complete killing of *E. hirae* was obtained in 12 and 90 min, respectively, *vs* 6 h using the other media. They concluded that the application of bacteria to copper surfaces in Tris-Cl buffers enhanced 'contact killing' through a higher release of copper ion and established a causal link between these dissolved copper concentrations and the rates of killing. In the same way, a phosphate buffer with addition of HEPES has been previously shown to increase dramatically the bactericidal effect of the Cu-H_2O_2 mixture on *E. coli* (Hartemann *et al.* 1995).

Copper as an antibacterial particle agent

Copper is also recognized as a potent antibacterial particle agent too. Its use in particle form (especially nanoparticles) shows very interesting properties (Halbus *et al.* 2017). For example, a high antibacterial activity of chitosan-copper nanoparticles on many bacterial strains including MRSA, *Bacillus subtilis*, *P. aeruginosa* and *Salmonella choleraesuis* has been shown (Usman *et al.* 2013). Giannousi *et al.* (2014) also demonstrated the antibacterial activity of copper-based nanoparticles and showed that copper nanoparticles (Cu-NPs), as well as cuprous oxide (Cu_2O) induced plasmid DNA degradation in a dose-dependent manner in Gram-positive and Gram-negative strains. Moreover, they showed that the concentration of released ions was below the level of inhibiting bacterial growth (minimum inhibitory concentration). Based on these results, the authors concluded that the concentration of released ions was less important than the nanoparticle size for the antibacterial activity. In addition, and as for copper surface, it appears that the outer membrane would be the front line of bacterial defence against copper ions (Speer *et al.* 2013). Therefore, the size of Cu-NPs turned out to be a major contributing factor for copper antimicrobial activity. Other authors synthesized CuO nanoparticles (CuO-NPs) which were purified and dried into different CuO-NPs sizes (Thekkae Padil and Černík 2013). Small CuO-NPs (4·8 ± 1·6 nm) were found to have significantly better antibacterial activity than larger particles (7·8 ± 2·3 nm). Azam *et al.*

(2012) also confirmed these results and Applerot *et al.* (2012) highlighted that the smaller particles have a better antimicrobial activity due to their greater capacity to penetrate cells (Applerot *et al.* 2012; Azam *et al.* 2012). However, their nano size is also responsible for their cyto- and genotoxicity and must be taken into account when considering their use (Karlsson *et al.* 2009; Midander *et al.* 2009; Wang *et al.* 2012).

Many other recent studies have confirmed the antimicrobial activity of copper in nanoparticle forms and their potential usefulness against infections (Pramanik *et al.* 2012; Pinto *et al.* 2013; Shankar *et al.* 2014; Wei *et al.* 2014; Bogdanović *et al.* 2015; Kruk *et al.* 2015; Gutiérrez *et al.* 2017; Maqbool *et al.* 2017; Qadri *et al.* 2017). However, their rapid oxidation upon air exposure limits their antimicrobial use in aerobic conditions (Usman *et al.* 2013).

Antifungal activity of copper

Copper as an antifungal surface agent

Although less research has been carried out on antifungal activity of copper it is unanimously accepted that an activity similar to that described for bacterial species exists in fungus.

Quaranta *et al.* (2011) investigated the mechanism of 'contact killing' in *Candida albicans* and yeast *Saccharomyces cerevisiae* cells in contact with copper coupons (C11000 99·9% Cu and C75200 62% Cu) (Quaranta *et al.* 2011). The modification of copper homeostasis caused four to six times faster killing of *C. albicans* lacking copper-ATPase export and *S. cerevisiae* deficient for copper uptake transporters, both involved in the intracellular regulation of copper, than of wild-type cells due to an intracellular accumulation of copper. The authors showed that the first damages were localized on membranes, similar to the bacterial mechanism of 'contact killing'. In addition, mutation detection assays showed the complete absence of DNA damage. The Live/Dead staining assay confirmed this hypothesis by showing rapid and extensive cytoplasmic membrane damage after yeast exposure to copper surfaces.

However, in *C. albicans* strains, which express higher levels of the *CRP1* P1-type ATPase copper transporter gene, the resistance against copper is greater by regulating the intracellular uptake of copper. *ALS1* and *ALS3,* a cluster of genes encoding cell surface-associated glycoproteins, could regulate *CRP1* and *de facto* suggest a different resistance mechanism from 'contact killing' (Zheng *et al.* 2016).

Copper as an antifungal particle agent

As in bacteria, the Cu-NPs have a potent antifungal activity. Ghasemian *et al.* (2012) tested antifungal activity against filamentous fungi such as *Alternaria alternata*, *Aspergillus flavus*, *Fusarium solani* and *Penicillium chrysogenum*. They synthesized 8 nm Cu-NPs and showed an important antifungal activity of these particles with minimal inhibitory concentrations (MIC) ranging from 40 to 80 mg l^{-1} (Ghasemian *et al.* 2012) and that particle size is an important factor in the antimicrobial activity of copper. Other studies have reported the antifungal activity of Cu-NPs against *Candida* species (Usman *et al.* 2013; Kruk *et al.* 2015).

Antiviral activity of copper

Copper as an antiviral surface agent

Copper also has the ability to destroy viruses, such as influenza viruses, noroviruses or human immunodeficiency virus (HIV).

Warnes and Keevil (2013) showed a rapid inactivation of murine norovirus (MNV-1) on copper alloy dry surfaces (65% to 99·9% Cu) at room temperature (Warnes and Keevil 2013). Similar to 'contact killing' of bacterial and fungal species, they demonstrated that Cu(II) and especially Cu(I) were the primary effectors of MNV-1 inactivation. Quenching superoxide and hydroxyl radicals did not provide better protection suggesting ROS (reactive oxygen species) had little effect on norovirus inactivation in this situation. Finally, they showed that the viral genome is targeted by copper, especially a gene encoding VPg (viral-genome-protein-linked, a viral protein essential for viral infectivity), by gene copy number reduction. Warnes *et al.* (2015) confirmed these results and reported that MNV-1 was inactivated more rapidly on alloys containing 79–89% copper than on alloys containing 70% copper (Warnes *et al.* 2015). These results confirm that copper concentration affects the antiviral efficiency of copper.

In addition, the inactivation of monkeypox and vaccinia viruses have also been demonstrated on copper surfaces (Bleichert *et al.* 2014).

Copper as an antiviral particle agent

Noyce *et al.* (2007) tested the antiviral property of copper against influenza A virus (Noyce *et al.* 2007). They inoculated $2{\cdot}10^6$ virus particles onto a copper surface at 22°C and 50–60% relative humidity. They demonstrated that only 500 viral particles were active after an incubation of 6 h on copper surface while up to 500·000 viral particles still remained infectious after 24 h of incubation on stainless steel.

Moreover, Fujimori *et al.* (2012) showed that Cu-NPs (nano-sized 160 nm copper iodide CuI) exert antiviral activity on influenza A virus by degradation of viral

proteins (Fujimori *et al.* 2012). They also demonstrated that antiviral activity was closely related to the generation of hydroxyl radicals derived from Cu(I).

Other studies have looked at the usefulness of these antiviral properties for public health. Borkow *et al.* (2010) have demonstrated that oxide copper impregnation in respiratory protective face masks confers biocidal properties against human and avian influenza A virus without altering their physical barrier properties (Borkow *et al.* 2010). Tested in simulated breathing conditions (28·3 l min^{-1}), no infectious viral particle was recovered from the copper oxide containing masks after 30 min, unlike with the control masks. In the same way, Borkow *et al.* (2008) demonstrated the effectiveness of copper during HIV-1 passage through filters containing copper oxide powder or copper oxide-impregnated fibres (Borkow *et al.* 2008). HIV-1 is inactivated when exposed to copper oxide in a dose-dependent manner, without neither cytotoxicity nor strain specificity. It is also assumed that the mechanism of HIV-1 inactivation targets the viral genome, and more particularly the HIV viral protease, which is essential for the replication of the virus. Karlström and Levine (1991) showed that an approximate stoichiometric concentration of Cu(II) ions could inhibit this viral protease in a rapid and irreversible manner (Karlström and Levine 1991).

More recently, Hang *et al.* (2015) showed antiviral activity of Cu-NPs against hepatitis C virus (HCV) and that Cu-NPs inhibit HCV infection by targeting the binding of infectious HCV particles to hepatic cells and the virus entry into the cells (Hang *et al.* 2015).

Mechanisms of antimicrobial action of copper surface

Although most of the antibacterial mechanisms of copper surface are well known, some points still need clarification. For example, there is no consensus among scientists as to the exact sequential events of 'contact killing' on various cellular elements but all researches, in accordance with EPA criteria, show a potent antibacterial action of copper (Figure 1). This lack of consensus could be explained by (i) the differences between strains tested and the surface components (Liu and Zhang 2016), (ii) the different protocols and tests conducted, (iii) the various experimental conditions (Vincent *et al.* 2016), or (iv) the different forms of copper tested (e.g. coupons and particles). Despite these differences, it appears that the antimicrobial activity of copper is directly linked to the oxidative behaviour of copper, coupled with the solubility properties of copper oxides (Hans *et al.* 2016; Vincent *et al.* 2016).

Membrane damage

Many studies suggest that 'contact killing' is initiated by the dissolved copper ions released from the copper surfaces by the culture medium and causing cell alterations (Grass *et al.* 2011). Espírito Santo *et al.* (2012) studied 'contact killing' on *Staphylococcus haemolyticus* using copper (C11000 99·9% Cu) and stainless steel coupons (Espírito Santo *et al.* 2012). They showed a great intracellular accumulation of copper inducing cell death by

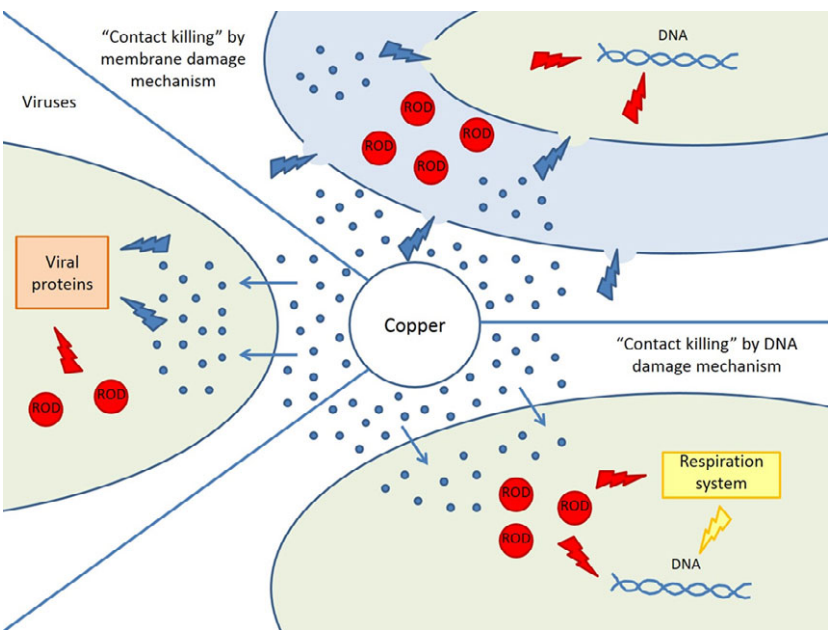

Figure 1 'Contact killing' mechanisms. Current studies tend to be in agreement on the viral 'contact killing' mechanism but few questions remain to be clarified, including the involvement or not of ROS. In contrast, bacterium 'contact killing' mechanism is much discussed in the literature. Two hypotheses compete, the first showing bacterial death by membrane degradation and the second by genotoxicity of copper. Finally, fungal 'contact killing' mechanism has been very little studied in the literature and the results suggest a mechanism by membrane damage. Abbreviations: VRE, vancomycin-resistant *Enterococcus faecalis*; MRSA, methicillin-resistant *Staphylococcus aureus*; ROS, reactive oxygen species. [Colour figure can be viewed at wileyonlinelibrary.com]

membrane damage. In addition, cells exposed to copper did not present more DNA mutation than those exposed to steel. These data suggest that copper is not genotoxic and does not kill by DNA damage but rather by damaging the cell membrane. Many other studies on *Escherichia coli* have also shown that copper exposure rapidly leads to membrane alterations before DNA degradation (Espírito Santo *et al.* 2011; Warnes *et al.* 2012). Hong *et al.* 2012 studied more specifically the mechanism of membrane degradation in *E. coli* exposed to different copper alloy surfaces (70–99·9% Cu) or in medium containing $CuSO_4$. They showed that 'contact killing' of *E. coli* is triggered by nonenzymatic oxidative damage of membrane phospholipids, resulting in the loss of membrane integrity and cell death.

All these studies concluded that the antibacterial effect of copper was related to its ability to release copper ions and their damaging effect on cell membrane. In addition, numerous recent studies on new copper-based material confirm this theory (Zhang *et al.* 2013; Liu *et al.* 2014; Mathews *et al.* 2015; Emam *et al.* 2017).

Oxidative stress and DNA degradation

Many studies attribute the antibacterial activity of copper to its capacity to release ions that cause oxidative stress by production of ROS in aerobic conditions (Espírito Santo *et al.* 2008, 2011). Following membrane degradation, copper-released ions can penetrate into the cell. The increase in the intrinsic amount of copper rapidly causes an important oxidative stress demonstrated by redox cycling between the different forms of copper: native Cu, Cu(I) and Cu(II) (Espírito Santo *et al.* 2011). Under aerobic conditions, redox potential enables copper to produce hydroxyl radicals according to the Haber–Weiss and Fenton reactions (Liochev and Fridovich 2002). The release of ROS leads to damage of lipids, proteins and nucleic acids, and eventually to the destruction of all genetic material (Dalecki *et al.* 2017).

Other studies tend to demonstrate that the membrane is the first target to undergo damage, allowing copper ion penetration into the cell, followed by oxidative stress and DNA degradation (Espírito Santo *et al.* 2012; Tian *et al.* 2012; Cui *et al.* 2014; San *et al.* 2015). All these data are consistent with the review of Grass *et al.* (2011) and Prado *et al.* (2012) explaining the same sequence of events for the 'contact killing' mechanism (Grass *et al.* 2011; Prado *et al.* 2012).

Despite these mechanisms, the exact molecular mechanism of 'contact killing' remains controversial. Weaver *et al.* (2010) investigated the "contact killing" mechanism of copper surface on methicillin-resistant *S. aureus* (MRSA) (Weaver *et al.* 2010). They inoculated MRSA onto copper coupons (C11000 99·9% Cu) and showed a rapid killing of MSRA by compromising cellular respiration and inducing DNA damage. However, little effect on cell membrane integrity was found. The same results were obtained by other authors with vancomycin-resistant *E. faecalis* (VRE) and *Enterococcus faecium*, under similar experimental conditions (Warnes *et al.* 2010; Warnes and Keevil 2011). They established that enterococci exposed to a copper surface for at least 2 h showed no detectable viable cell using cell culture and plate counts. They demonstrated an inhibition of cell respiration and cell death and therefore suggested that membranes could not be damaged after cell death. Hence, they concluded that membrane damage could not be assumed to be a universal and unique 'contact killing' starting point. They proposed an alternative model where copper antimicrobial efficacy is linked to the release of copper ionic species that trigger ROS production and lead to cell respiration arrest and DNA breakdown at the first stages of cell death. Recently, the same authors confirmed this hypothesis on MRSA and methicillin-sensitive *S. aureus* strains and confirmed the role of ROS in 'contact killing' but not *via* Fenton chemistry (Warnes and Keevil 2016). In addition, Mathews *et al.* (2015) suspected that ROS action was probably not the primary cause of cell death but an accompanying effect (Mathews *et al.* 2015).

Interestingly, another primary mechanism for copper toxicity in bacteria has been shown by non-Fenton destruction of iron–sulphur cluster enzymes in *E. coli*. Toxicity of copper ions is due to the displacement of iron atoms from iron–sulphur clusters and a copper binding to the corresponding sulphur atoms (Macomber and Imlay 2009). The rapid inactivation of iron–sulphur cluster enzymes by copper results in the damage of the central catabolic and biosynthetic pathways. Therefore, copper ions may compete with other metal ions for important protein binding sites.

Mechanisms of microbial copper resistance

To become resistant, pathogens must be able to adapt quickly to their environment. Exposed to micro-organisms, the human body rapidly defends itself by using mechanisms of the innate immune system, like ROS production. Several studies suggest that our innate immunity uses high copper concentrations as a toxic agent against bacteria and, in response, that bacteria could induce copper resistance as a way to increase their potential virulence (Djoko *et al.* 2015; Besold *et al.* 2016; German *et al.* 2016). This control of copper homeostasis essentially acts as a defence system for pathogenic bacteria. The three most common regulator mechanisms of copper homeostasis are suggested: (i) export of copper in the

extracellular environment with copper-ATPase export pumps (Solioz and Odermatt 1995; Ladomersky and Petris 2015; Dietl *et al.* 2016); (ii) sequestration of copper in the cytoplasm by copper-binding metal chaperones (Mealman *et al.* 2012; Zimmermann *et al.* 2012), by metallothionein in the periplasm (Gold *et al.* 2008; Festa *et al.* 2011) or outside the cell (Chaturvedi *et al.* 2012); and (iii) generation of less toxic form, for example, Cu(II) by oxidation (Macomber and Imlay 2009; Abicht *et al.* 2013; Chaturvedi and Henderson 2014; Mathews *et al.* 2015; Inesi 2017). These mechanisms of defence, mediated through ATPase pumps, are found mostly in microorganisms living in copper-rich environments (Besaury *et al.* 2013), and represent an adaptation behaviour.

Most copper-sensing regulators are intracytoplasmic and periplasmic transcriptional regulators (Inesi 2017), but it was discovered that a post-transcriptional copper response mechanism also exists (Rademacher *et al.* 2012).

Copper resistance in Gram-negative bacteria

Gram-negative bacteria activate copper-ATPase export and chaperone gene transcription *via* an 'activator-type' (CueR-like and CusRS-like) when cytoplasmic and periplasmic concentrations of copper increase. The CueR-like are one-component regulators that respond to cytoplasmic copper concentration while the CusRS-like are two-component regulators that respond to periplasmic copper concentration. In addition, many Gram-negative species synthesize multicopper oxidases (MCO), by the same 'activator-type', to allow the transformation of Cu (I) into the less toxic Cu(II) form. Likewise, Gram-negative species synthesize resistance nodulation cell division (RND)-type multicomponent copper efflux systems that export copper to the periplasmic space when a high concentration of copper is detected (Rademacher and Masepohl 2012). When copper concentration increases, the CusRS-like two-component system detects the high periplasmic copper amounts, triggers transcriptional activation of *cusRS* gene, which subsequently leads to the export of copper to the extracellular compartment. In parallel, the one-component CueR-like system recognizes high cytoplasmic copper concentrations and triggers transcriptional activation of copper resistance genes, with or without operon organization, for example, MCO, chaperone and copper-ATPase export (Figure 2).

Osman *et al.* (2013) demonstrated how these mechanisms are taking place in *Salmonella enterica* sv. Typhimurium (Osman *et al.* 2013). When copper cytoplasmic concentration increases, copper is transported from the cytoplasm to the periplasm by the CopA and GolT—two copper-ATPases export—and transferred to SodCII—a superoxide dismutase—and CueO, a MCO, with a periplasmic copper chaperone CueP. It has been shown that CueP expression is controlled by a CueR-like 'activator-type' which can detect an increase in cytoplasmic concentration of copper using the two-component system CpxR/CpxA when stress is detected at the bacterial membrane level (Pezza *et al.* 2016).

Recently, a CopM Cu-binding protein was found both in the periplasm and in the extracellular space of the cyanobacterium *Synechocystis*. CopM could bind copper in the extracellular compartment, preventing copper accumulation in the periplasm, but its role outside the cell is not clearly known (Giner-Lamia *et al.* 2015, 2016).

These observations raise many questions regarding the exact mechanism of copper homeostasis and underline the need to carry out further investigations (Rademacher and Masepohl 2012).

Copper resistance in Gram-positive bacteria

Contrary to Gram-negative bacteria, Gram-positive bacteria repress copper-ATPase export and chaperone gene transcription by a 'repressor-type' (CopY-like and CsoR-like) under copper deficiency conditions (Rademacher and Masepohl 2012). When the amount of copper increases, the CopY-like and CsoR-like repressors are released from the DNA to allow expression of the downstream genes, enabling transcription of copper-ATPase and chaperone for export and sequestration of free copper ion respectively (Figure 3).

Cobine *et al.* (1999) demonstrated, in *E. hirae*, that copper chaperones CopZ deliver Cu(I) to the Zn(II)CopY complex resulting in displacement of Zn(II) (Cobine *et al.* 1999). This direct interaction leads to the release of the CopY repressor from the promoter and allow the transcription of *copYZAB* operon when copper is in excess. However, direct copper transfer between CopZ and CopA is strongly suspected *via* specific interactions but further studies are needed to confirm this hypothesis (Multhaup *et al.* 2001). A similar hypothesis was made for CopB (Solioz and Stoyanov 2003).

Beside these transcriptional regulators, a study in *E. hirae* has demonstrated that higher copper concentrations (>0·5 mmol l^{-1}) down-regulate CopZ by inducing its proteolysis by a copper-dependent protease (Lu and Solioz 2001; Solioz 2002). This study reports a post-translational mechanism responsible for the control of the intracellular amount of CopZ. Authors concluded that this copper-stimulated proteolysis of CopZ could play an important role in *E. hirae* copper homeostasis. Further studies are needed to confirm whether the post-translational proteolysis of copper regulators occurs in other bacterial species and is a regulatory mechanism of CopZ-like proteins and possibly also copper-ATPases.

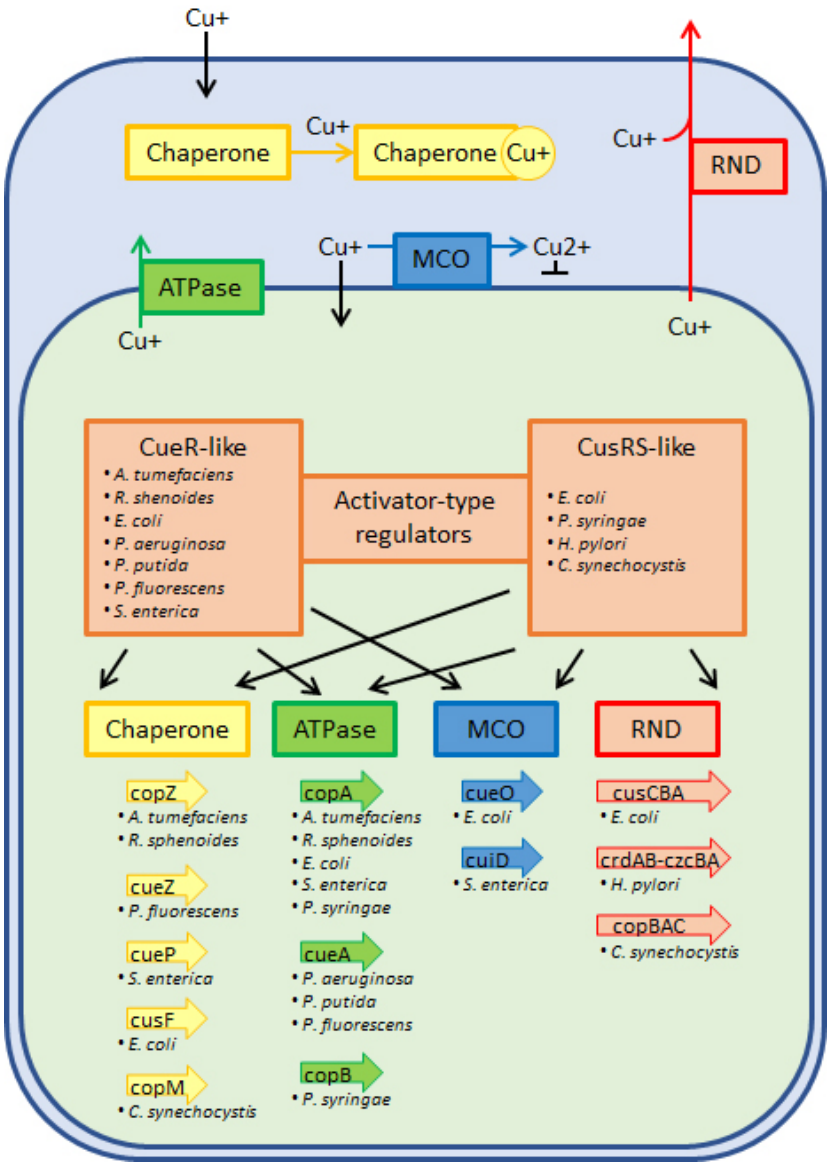

Figure 2 The different common copper homeostasis systems involved in the copper resistance of Gram-negative bacteria. When copper concentration increases, the activator regulates the transcription of the genes involved in copper resistance. All studies cited show that deletion of these genes reduce copper resistance (Mills *et al.* 1993; Outten *et al.* 2000; Adaikkalam and Swarup 2002; Schwan *et al.* 2005; Waidner *et al.* 2005; Espírito Santo *et al.* 2008; Zhang and Rainey 2008; Nawapan *et al.* 2009; Pontel and Soncini 2009; Thaden *et al.* 2010; Peuser *et al.* 2011; Giner-Lamia *et al.* 2012, 2015; Osman *et al.* 2013; Pezza *et al.* 2016). Abbreviations: MCO, multicopper oxidases; RND, resistance nodulation cell division. [Colour figure can be viewed at wileyonlinelibrary.com]

Other aspects of bacterial copper resistance

It is generally recognized that Gram-positive species express 'repressor types' while Gram-negative bacteria express 'activator types' ones. However, these mechanisms are not systematically used. For example, the Gram-positive bacillus, *Corynebacterium glutamicum*, expresses the two-component system CopRS, a copper-ATPase CopB and the extracellular MCO CopO following exposure to an increase in copper concentration. Furthermore, *C. glutamicum* also has a copper-ATPase *ctpV* gene and a repressor *csoR* gene, both repressed by CsoR during copper deficiency (Schelder *et al.* 2011). Thus, *C. glutamicum* possesses both copper resistance mechanisms, *via* activation and repression. Similarly, *Thermus thermophilus*, a Gram-negative bacterium, possesses a *copZ-csoR-copA* operon that is repressed by CsoR during copper deficiency (Sakamoto *et al.* 2010).

Several other control systems of copper homeostasis have been reported. *Mycobacterium tuberculosis* has a *ctpV* gene, repressed by CsoR, responsible for the expression of copper-ATPase membrane exporter (CtpV) (Liu *et al.* 2007; Ward *et al.* 2010; Marcus *et al.* 2016). However, Wolschendorf *et al.* (2011) showed another *M. tuberculosis* defence system against copper: the loss of the *mctB* gene, encoding the copper transport protein B (MctB), causes considerable intracellular accumulation of copper (Wolschendorf *et al.* 2011). Similarly, another defence pathway has been shown in *M. tuberculosis* involving the copper repressor RicR regulon encoding for (i) MymT, a

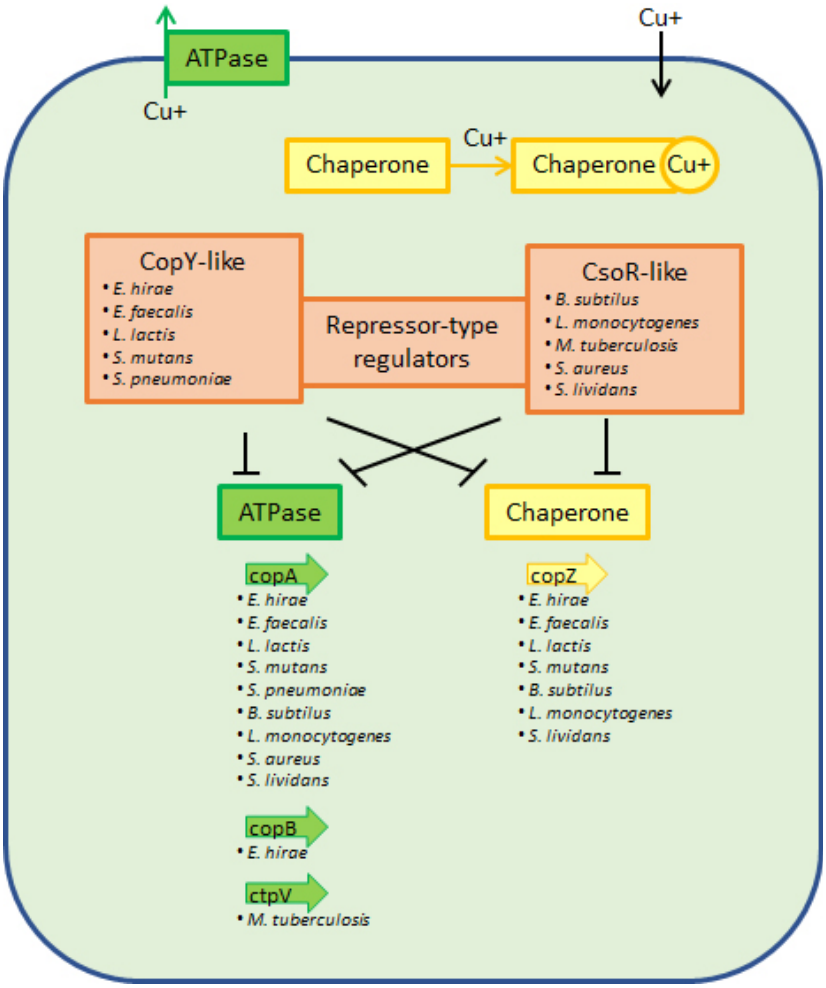

Figure 3 The different common copper homeostasis systems involved in the copper resistance of Gram-positive bacteria. When copper concentration increases, the repressor regulator is released from the DNA and the different genes involved in copper resistance are transcribed. All studies cited show that deletion of these genes reduce copper resistance (Odermatt and Solioz 1995; Vats and Lee 2001; Liu *et al.* 2007; Smaldone and Helmann 2007; Magnani *et al.* 2008; Reyes-Jara *et al.* 2010; Corbett *et al.* 2011; Grossoehme *et al.* 2011; Shafeeq *et al.* 2011; Dwarakanath *et al.* 2012; Guo *et al.* 2015; Singh *et al.* 2015; Young *et al.* 2015). [Colour figure can be viewed at wileyonlinelibrary.com]

copper-protective metallothionein causing copper sequestration (Mierek-Adamska *et al.* 2017), (ii) LpqS, a putative lipoprotein able to export copper and (iii) Rv2963, a putative permease aiding the export of copper (Festa and Thiele 2011; Shi *et al.* 2014). Finally, another study has shown that MmcO, a mycobacterial MCO, was an important mechanism of copper resistance in the same bacterium (Rowland and Niederweis 2013).

Another way of copper sequestration has been demonstrated in *E. coli*. Phenolate siderophores, such as yersiniabactin, sequester Cu(II) outside the cell and prevent its reduction to the more toxic Cu(I) form (Chaturvedi *et al.* 2012). In addition, copper-yersiniabactin complexes can catalyse superoxide dismutase, and thus reduce the concentration of superoxide (Chaturvedi *et al.* 2014). As a result, Cu(I) concentration is reduced while Cu(II) amount increases. Therefore, copper sequestration by yersiniabactin can promote bacterial survival (Chaturvedi and Henderson 2014).

Resistance in *E. hirae* and more generally enterococcus genus has also been studied. Studies showed that the *tcrYAZB* operon conferred copper resistance in *E. faecium*, *E. faecalis* and other enterococcal species (Hasman 2005; Hasman *et al.* 2006). This operon is genetically organized as the *copYZAB* operon found in *E. hirae* and presents the same mode of repressor control. However, few studies have reported the intra- and interspecies transferability potential of these genes, located on a plasmid, and their role in acquiring copper resistance (Hasman and Aarestrup 2002; Amachawadi *et al.* 2013, 2015).

Copper resistance in yeasts

In yeasts, the three most common regulator mechanisms of copper homeostasis are: (i) reducing the copper influx pump; (ii) exporting copper in the extracellular environment with copper export-ATPases and (iii) expression of metallothioneins to chelate free copper ions (Ooi *et al.* 1996; Georgatsou *et al.* 1997; Labbé *et al.* 1997; Liu and Thiele 1997; Yamaguchi-Iwai *et al.* 1997; Riggle and Kumamoto 2000; Weissman *et al.* 2000). However, the 'contact killing' mechanism in yeasts is not fully understood.

In *C. albicans* and under copper-limiting conditions, the *CTR1* and *FRE7* genes, coding respectively for the copper import protein Ctr1p and two proteins with cupric reductase activity Fre7p and Fre10p, are expressed (Marvin *et al.* 2004; Jeeves *et al.* 2011; Mackie *et al.* 2016). On the contrary, under copper-replete conditions their expression is repressed (Woodacre *et al.* 2008). Other genes were identified and linked to copper homeostasis in *C. albicans*. *CRP1* encodes copper-ATPase export pumps and *CUP1* and *CRD2* encode copper metallothioneins. A deletion of one of these genes decreases the copper resistance of *C. albicans* (Riggle and Kumamoto 2000; Weissman *et al.* 2000; Mackie *et al.* 2016). The literature reports similar mechanisms for *S. cerevisiae* (Thiele 1988; Buchman *et al.* 1989; Szczypka and Thiele 1989) and *Aspergillus nidulans* (Antsotegi-Uskola *et al.* 2017).

Other studies of copper homeostasis in fungi have shown the same mechanisms and proteins involved. Ding *et al.* (2013) showed that *Cryptococcus neoformans* neutralizes copper to promote infection by encoding copper importer (Ctr1), copper-detoxifying metallothionein (Cmt) and a transporter implicated in copper sequestration (Ding *et al.* 2013) similarly, Zhang *et al.* (2016) showed that the Ctr4 transporter is involved in copper homeostasis (Zhang *et al.* 2016).

Wiemann *et al.* (2017) reported that copper transporter proteins Ctr1 and copper-ATPase export pumps play a major role in copper resistance of *A. fumigatus* (Wiemann *et al.* 2017) and Dietl *et al.* (2016) recently showed that histidine is involved in copper detoxification in *A. fumigatus*. They demonstrated that deletion of the gene encoding imidazoleglycerol-phosphate dehydratase (HisB) reduces resistance of *A. fumigatus* to copper (Dietl *et al.* 2016).

Conclusion

Here, we described the antimicrobial property of copper by 'contact killing' that is widely accepted. Mechanisms of the antimicrobial action of copper are well known. Copper nanoparticles are very effective against bacteria, fungi and viruses infections. However, their little sizes cause cyto- and genotoxicity and must therefore be taken in account for their medical use.

All authors agree that copper homeostasis plays a major role in micro-organism resistance to copper and point to very complex copper homeostasis mechanisms in bacteria involving a large number of interrelated factors (Rademacher and Masepohl 2012; Inesi 2017).

Although the antimicrobial activity of copper is relatively clearly established, the exact mechanisms still raise many questions. On the one hand, the activation of these mechanisms seems to be directly related to the modes of experimentation (Vincent *et al.* 2016). On the other hand, although similarities in copper homeostasis exist between micro-organisms, each strain has structural and genomic features that lead to own regulation and survival mechanisms (Elguindi *et al.* 2009; Espírito Santo *et al.* 2011, 2012; Tian *et al.* 2012; Warnes *et al.* 2012; San *et al.* 2015). Finally, different antimicrobial mechanisms of action of copper have been suggested including membrane damage, inhibition of respiration, protein inactivation and DNA degradation, all leading to the antimicrobial effect.

Because of such variability, studies do not tend to use standardized protocols and it is therefore difficult to directly compare their results. Nevertheless, the most interesting common point of the literature is that all bacteria exposed to copper provide survival systems only for a few minutes before undergoing cell death. No complete resistance to survive in prolonged exposure with copper has been found (Liu and Zhang 2016).

As a result copper certainly holds potential solutions towards infection prevention in public health environment such as against nosocomial infections or the worrying rapid development of resistance to antibiotics, but further studies are needed to fully understand the mechanisms of copper cellular homeostasis, key to its antimicrobial activity.

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Conflict of Interest

No conflict of interest declared.

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