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MICROREVIEW

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Copper tolerance in bacteria requires the activation of multiple accessory pathways

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Abstract

Copper is a required micronutrient for bacteria and an essential cofactor for redox-active cuproenzymes. Yet, excess copper is extremely toxic, and is exploited as a bacteriocide in medical and biotechnological applications and also by the mammalian immune system. To evade copper toxicity, bacteria not only control intracellular copper homeostasis, but they must also repair the damage caused by excess copper. In this review, we summarize the bacterial cell-wide response to copper toxicity in Enterobacteria. Tapping into the abundant research data on two key organisms, *Escherichia coli* and *Salmonella enterica*, we show that copper resistance requires both the direct copper homeostatic response and also the indirect accessory pathways that deal with copper-induced damage. Since patterns of copper response are conserved through the Proteobacteria, we propose a cell-wide view of copper detoxification and copper tolerance that can be used to identify novel targets for copper-based antibacterial therapeutics.

KEYWORDS

copper, enterobacteria, escherichia, gram negative bacteria, homeostasis, salmonella

1 | INTRODUCTION

Copper is an essential micronutrient for bacteria, serving as an electron carrier and redox catalyst in various cuproenzymes (Rensing and Grass, 2003; Andreini *et al*., 2008; Festa and Thiele, 2011; Vest *et al*., 2013). Excess copper, however, is also cytotoxic. There is growing interest in utilizing copper for infection control owing to its antibacterial properties (Noyce *et al*., 2006; Warnes *et al*., 2012; Lemire *et al*., 2013). At the same time, copper tolerance pathways can constitute important virulence determinants, since the mammalian immune system utilizes copper excess and starvation in its arsenal against pathogenic bacteria (White *et al*., 2009; Djoko *et al*., 2015; Johnson *et al*., 2015; Ladomersky *et al*., 2017; Hyre *et al*., 2017; Stocks *et al*., 2019).

The mechanisms of intracellular copper handling, which regulate its acquisition, storage, detoxification and delivery to maturing cuproenzymes, have been studied over three decades in model organisms such as *Escherichia coli* (Grass and Rensing, 2001; Rensing and Grass, 2003; Dupont *et al*., 2011; Hodgkinson and Petris, 2012; Argüello *et al*., 2013; 2016; Nies and Herzberg, 2013). More recently, a body of evidence on copper-mediated cell death has accumulated for other Gram negative bacteria, particularly the pathogen *Salmonella enterica*. These studies suggested that the effects of copper toxicity and the general principles of copper resistance can be considered universal in the bacterial kingdom, with some copper homeostasis components being shared with archaea and eukaryotes as well (Andreini *et al*., 2008).

In this review, we describe the mechanisms of copper toxicity in bacteria and the bespoke adaptation pathways that assuage it. We show that copper excess generates distinctive pleiotropic effects in the cell, and that different stress responses complement the copper homeostasis system in defending the cell against copper toxicity. Importantly, no single pathway can account for a cell's copper resistance profile, and multiple layers of gene regulation are required for a stable adaptation to copper stress. We examine the case of two well-studied proteobacteria, *E. coli* and *S. enterica*, highlighting similarities and differences between the two species. Crucially, we comment on how pathogenic strains adapt to copper stress during infection, which can highlight future therapeutic targets. We also comment on the aspects of bacterial metal usage that require further investigation, hoping to stimulate future research.

2 | COPPER BIOCHEMISTRY IN BACTERIA

Bacterial copper homeostasis shows some distinctive features, as we have extensively reviewed elsewhere (Barwinska-Sendra and Waldron, 2017) (Figure 1). Briefly, copper exists in a chemical equilibrium between two forms, which are readily complexed by biological ligands (Beswick *et al*., 1976; Outten *et al*., 2001; Dupont *et al*., 2011): cupric ion, Cu(II), is favored in oxidizing compartments such as the Gram-negative periplasm, while cuprous ion, Cu(I), is prevalent during anoxic growth, and in reducing compartments such as the cytoplasm. Cu(II) is more biologically inert, making it a relatively "safe" species for cells compared to Cu(I) (Rensing and Grass, 2003; Andreini *et al*., 2008). Intracellular reductants, including respiratory chain complexes, enterobactin, and cysteine, contribute to the generation of Cu(I) from Cu(II) (Rodriguez-Montelongo *et al*., 1993;

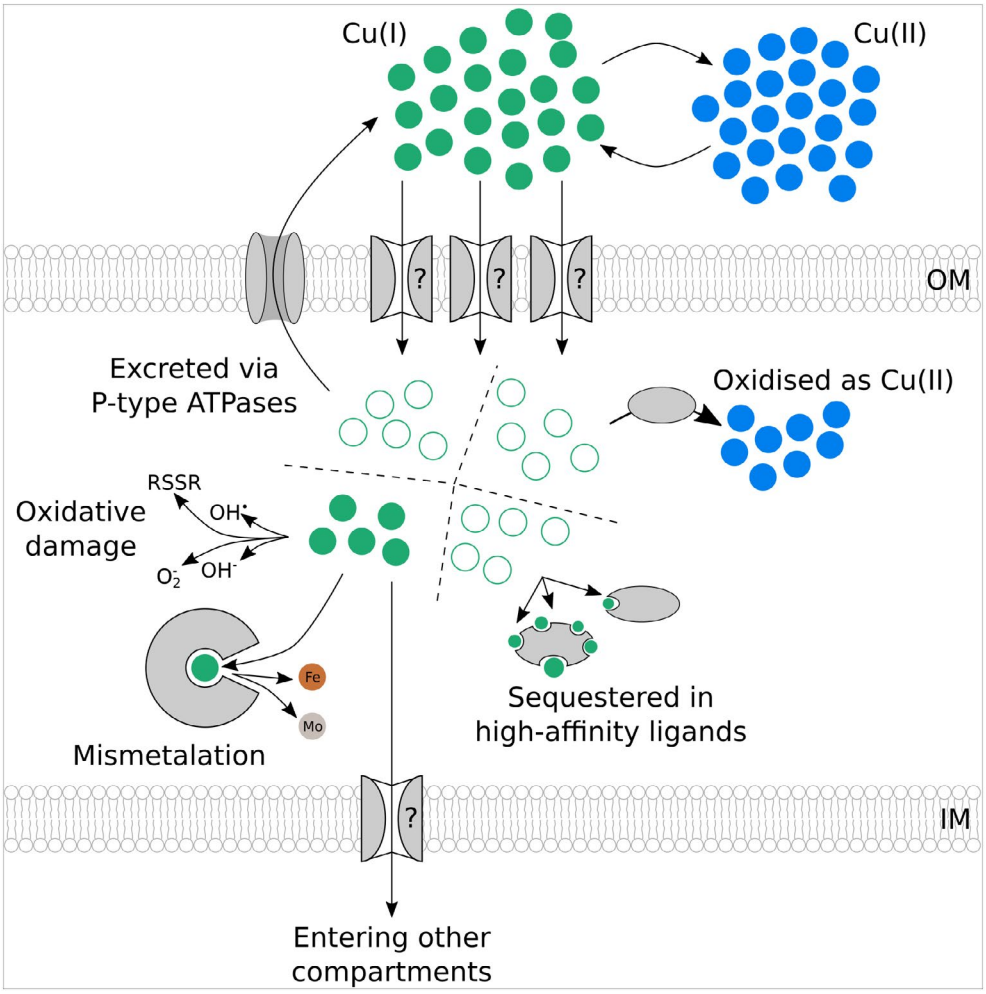

FIGURE 1 Copper fluxes in bacteria. In the extracellular environment, copper exists as a dynamic equilibrium between reduced Cu(I) (green) and oxidized Cu(II) (blue). The reduced species, Cu(I), can cross the bacterial outer membrane, although the entry route is unclear. Inside the bacterial periplasm, Cu(I) availability can be reduced by active efflux, oxidation to Cu(II), or sequestration by high-affinity ligands. Thus, the quota of bioavailable Cu(I) is lower than the total copper content. Bioavailable copper can be toxic to cells by catalyzing oxidative damage, displacing native metal cofactors in metalloenzymes, and can also enter other compartments. Similar mechanisms (efflux, oxidation, sequestration) can be employed in each bacterial compartment reducing the quota of bioavailable copper is reduced after crossing each membrane

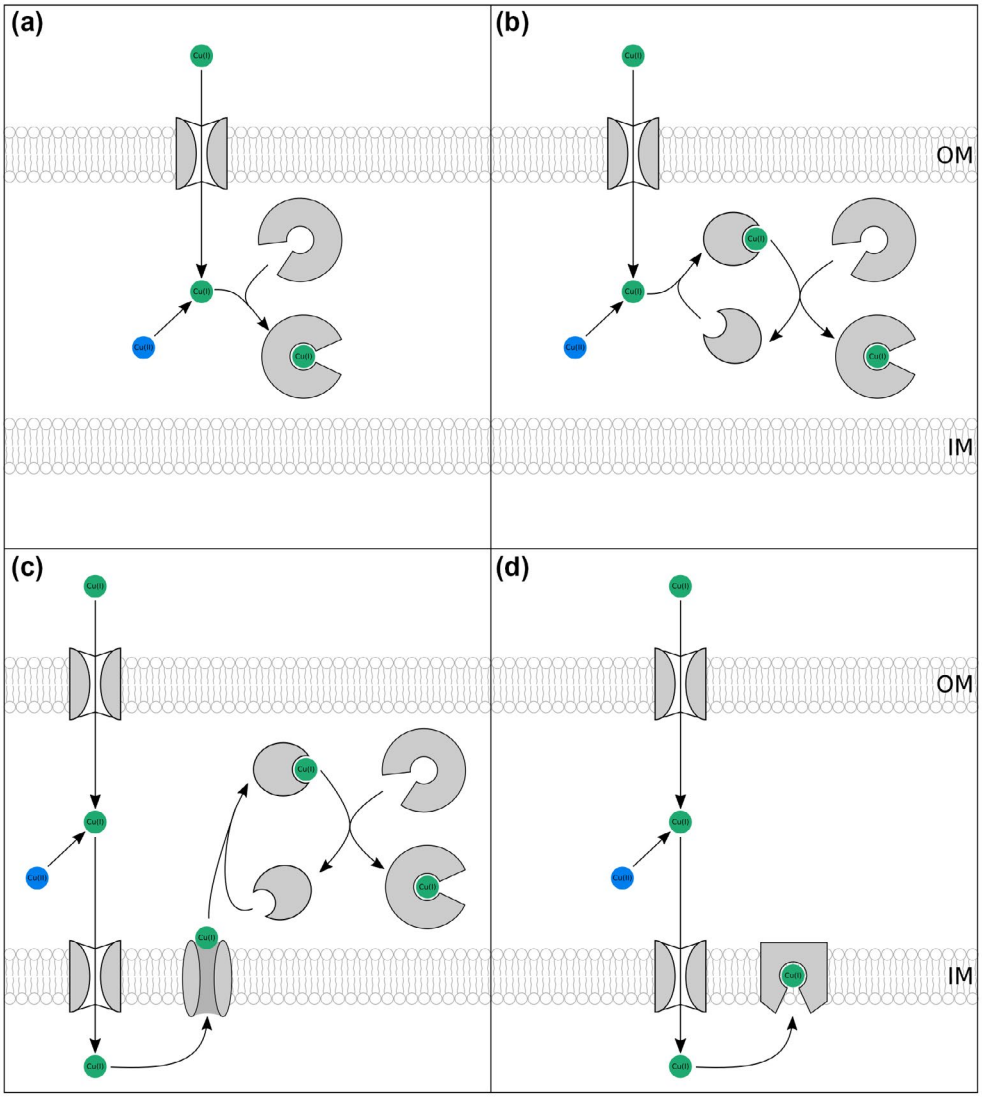

FIGURE 2 Copper supply to periplasmic cuproproteins. Most periplasmic cuproproteins utilize Cu(I) (green) as a cofactor to catalyze a range of redox reactions. After Cu(I) enters the periplasm or is generated from the reduction of Cu(II) (blue), it can be inserted in periplasmic cuproprotein in three different ways: (a) The apo-protein captures bioavailable Cu(I) from the periplasmic milieu; (b) a cuprochaperone binds Cu(I) and delivers it to the target enzyme via protein–protein interactions; or (c) Cu(I) enters the cytosol and is trafficked through a P-type ATPase before binding the cuprochaperone. (d) Inner-membrane cuproproteins, such as cytochrome oxidases, are known to acquire their copper cofactor from the cytoplasm, even though the mechanism of insertion is unclear

Rigo *et al*., 2004; Grass *et al*., 2004; Volentini *et al*., 2011), and more intracellular Cu(I) is generated enzymatically for incorporation into copper-containing cytochrome oxidases, at least in some organisms (Marckmann *et al*., 2019). Copper toxicity is enhanced during anaerobic growth (Outten *et al*., 2001; Espariz *et al*., 2007; Tan *et al*., 2017) and cells accumulate more copper under those conditions (Outten *et al*., 2001; Macomber *et al*., 2007).

For most bacteria, copper enters the cell *via* passive diffusion along its chemical gradient. Even though a handful of candidate bacterial copper importers have been suggested to date (Chillappagari *et al*., 2009; Balasubramanian *et al*., 2011; Ekici *et al*., 2014; Khalfaoui-Hassani *et al*., 2018; Han *et al*., 2019; Zhang *et al*., 2019), none of them seems to play this role in *E. coli* or *S. enterica*. In these organisms, a bespoke copper acquisition pathway has only been found in some pathogenic strains, which use the siderophore yersiniabactin to combat copper starvation at the host-pathogen interface (Oelschlaeger *et al*., 2003; Henderson *et al*., 2009; Koh *et al*., 2017). The *E. coli* zinc importer ZupT was considered for some time to be a candidate for a copper importer, since ZupT-overexpressing cells display a slight copper phenotype (Lee *et al*., 2002). However, subsequent studies showed that this phenotype is likely attributed to imbalances in the iron supply chain and not increased copper import (Xu *et al*., 2019). Whether *E. coli* and *S. enterica* acquire enough copper to metalate their cuproenzymes *via* passive diffusion, or an unknown copper importer exists in these organisms, is currently speculative.

Since biological membranes are relatively impermeable to copper, the cytosol of most bacterial species constitutes a

copper-poor environment. In Gram negative bacteria, copper homeostasis is mainly played out in the periplasm, where most of the intracellular copper is located (Changela *et al*., 2003; Macomber *et al*., 2007; Parmar *et al*., 2018). Copper complexes enter the periplasm through porins, whose permeability may influence the rate of its acquisition (Egler *et al*., 2005). Notably, all known copper-requiring enzymes in Enterobacteria (copper-dependent superoxide dismutases, SODs, multi-copper oxidases, amine oxidases and terminal respiratory oxidases) are localized in the cell envelope (Figure 2). Periplasmic enzymes acquire their copper cofactor in the periplasm, from either a bioavailable pool or metallochaperones (Stolle *et al*., 2016; Stewart *et al*., 2019). Copper-containing respiratory oxidases can acquire their copper from the cytoplasm, as shown in other model organisms (Ekici *et al*., 2014; Khalfaoui-Hassani *et al*., 2018; Llases *et al*., 2019; Marckmann *et al*., 2019; Zhang *et al*., 2019).

Regardless of the bacterial compartment, cells restrict the amount of copper that is available to interact with intracellular targets (Rensing and Grass, 2003). The possible strategies include controlling copper import, secreting excess copper, increasing the cell's buffering capacity and oxidizing Cu(I) to Cu(II) (Figure 1). Additional pathways are typically mobilized under stress conditions, to address the downstream effects of copper toxicity. In order to shed light on the different bacterial adaptations to copper, therefore, it is essential to understand the complex interplay between copper-induced damage and cellular responses.

3 | COPPER HOMEOSTASIS: THE EXAMPLE OF ENTEROBACTERIA

In *E. coli* and *S. enterica* (Figure 3), copper bioavailability in the cytoplasm is buffered to an exquisitely low concentration, equivalent to less than one atom per cell (Outten *et al*., 2000). Cytosolic copper concentration is under the control of the MerR-family transcriptional regulator, CueR (Outten *et al*., 2000), which has zeptomolar affinity for copper (Changela *et al*., 2003). The *cue* regulon includes the P-type ATPase CopA (Rensing *et al*., 2000), and the cuprochaperone CopZ (Meydan *et al*., 2017), both of which bind Cu(I) with attomolar affinity (Drees *et al*., 2015). The periplasmic multicopper oxidase CueO (CuiD in *S. enterica*) reduces the Cu(I) flux across the inner membrane by converting periplasmic Cu(I) to Cu(II) (Outten *et al*., 2001). The bidirectional metal-polyphosphate transporter, PitA, may also contribute to copper efflux (Keasling, 1997; Grillo-Puertas *et al*., 2014; Solioz, 2018).

The core *cue* regulon is functionally conserved in all proteobacteria (Andreini *et al*., 2008; Hernandez-Montes *et al*., 2012), and additional niche-specific adaptations can be seen in different species. For example, the anti-copper arsenal of *S. enterica* has expanded, likely as an adaptation to the host-pathogen interface in order to escape intracellular killing by macrophages (Achard *et al*., 2010; Ladomersky *et al*., 2017). In this organism, an additional periplasmic chaperone, CueP, acts as a copper sink (Pontel and Soncini, 2009; Osman *et al*., 2010) and supplies copper to

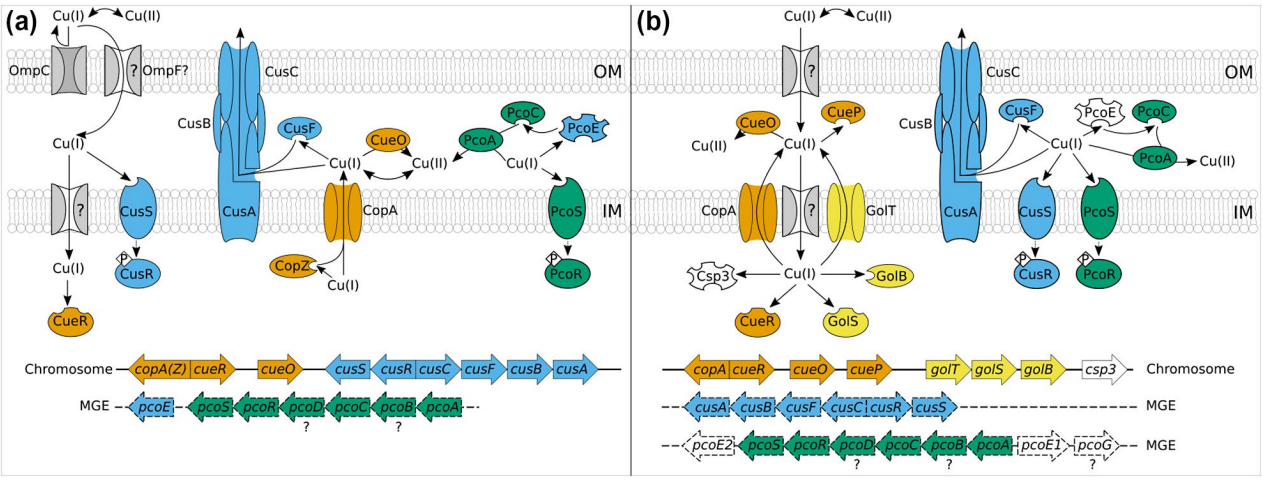

FIGURE 3 Copper homeostasis in *Escherichia coli* and *Salmonella enterica*. (a) In *Escherichia coli*, three sensory systems, CueR, CusSR, and the plasmid-borne PcoSR, monitor Cu(I) concentration in the two cellular compartments (cytoplasm and periplasm). Two efflux pumps, CopA and CusCBA, actively secrete copper from the cytoplasm and periplasm, respectively. Each ATPase is associated with one high-affinity cuprochaperone that scavenges copper in the respective compartment (CopZ in the cytoplasm; CusF in the periplasm). A multi-copper oxidase, CueO, carries out copper detoxification in the periplasm. The plasmid-borne Pco system provides two additional periplasmic chelators, PcoC and PcoE, and one additional multicopper oxidase, PcoA. Although the copper entry route across biological membranes is unknown, the outer membrane porin, OmpC, may contribute to copper resistance by selecting against copper. (b) In *Salmonella enterica*, the *cue* regulon is duplicated. The homologous *gol* regulon includes the transcriptional regulator GolS, the efflux pump GolT, and the cytoplasmic chaperone GolB. Moreover, the cytosolic copper-storage protein Csp3 is present. The *cus* system is absent from the chromosome but can be provided by mobile genetic elements. MGE = Mobile Genetic Element. Color codes correspond to different regulons: orange = CueR, blue = CusRS, green = PcoRS, yellow = GolS, white = unknown. The function of PcoB, PcoD and PcoG are only putative

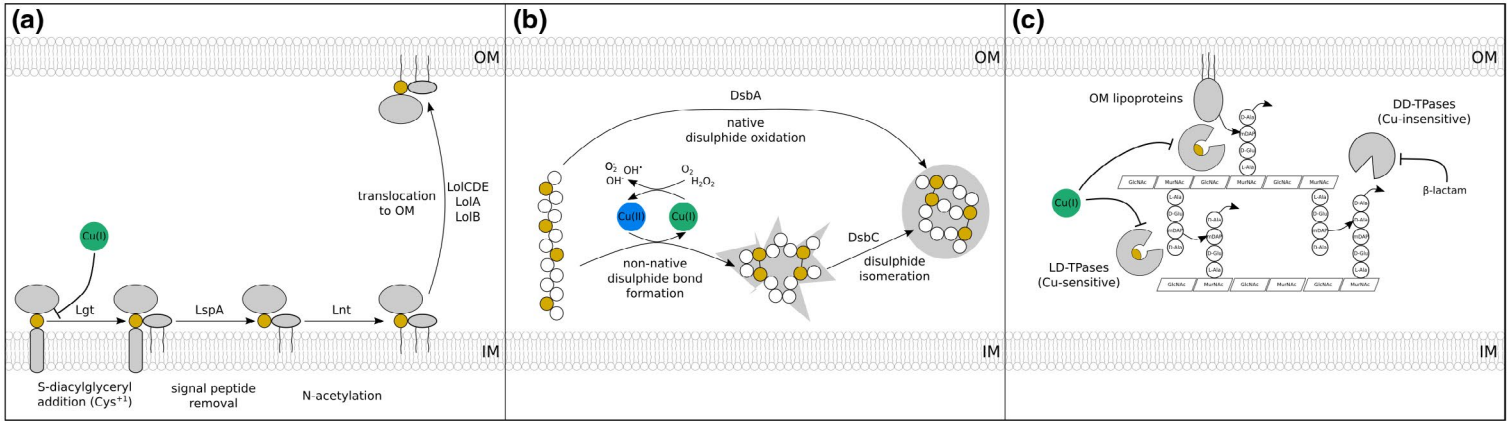

FIGURE 4 Copper toxicity in the cell envelope. (a) Lipoprotein maturation is a complex process requiring the attachment of a lipid moiety to the maturing polypeptide and the removal of the signal peptide, which occurs on the periplasmic side of the inner membrane; outer membrane lipoproteins are further translocated to the outer membrane after maturation. Periplasmic Cu(I) (green) inhibits protein maturation by binding the conserved cysteine-1 residue (amber), where lipid modifications occur. Cells can reduce the effect of copper toxicity by overexpressing lipoprotein-maturation enzymes, which compete with Cu(I) for access to cysteine-1. (b) Periplasmic protein maturation often involves covalent linking of cysteine residues through disulfide bonds, chaperoned by disulfide oxidases. Copper can also catalyze disulfide bond formation, which often occur in a non-native fashion. Misfolded products must be resolved by a disulfide isomerase to avoid cell toxicity. (c) Peptidoglycan maturation is catalyzed by *DD*-transpeptidases (TPases), which cross-link different peptidoglycan layers, and *LD*-transpeptidases, which cross-link peptidoglycan and anchor it to outer membrane lipoproteins. *LD*-TPases have a cysteine residue in the active site (amber), which can be inhibited by Cu(I). During copper toxicity, Gram negative bacteria can still cross-link peptidoglycan using *DD*-TPases, but cannot attach it to the outer membrane, resulting in increased cell fragility. Moreover, *DD*-TPases can be inhibited by β-lactam antibiotics, which show a potent synergistic effect with copper. Protein names correspond to the *Escherichia coli* enzymes

periplasmic SODs in conjunction with the P-type ATPases (Nies and Herzberg, 2013; Osman *et al*., 2013; Fenlon and Slauch, 2017). Moreover, most *S. enterica* serotypes (with the exception of the human-adapted serovars Typhi and Paratyphi A), carry a duplication of the entire *cue* system in the form of the partially redundant *gol* regulon (Osman *et al*., 2010; 2013). In these strains, the CueR homologue, GolS, regulates the expression of an extra CopA homologue, GolT (Espariz *et al*., 2007), and a putative cytosolic CopZ-like cuprochaperone, GolB (Checa *et al*., 2007). The pathogenic serovars take copper homeostasis a step further through the cytosolic copper storage protein, Csp3 (Vita *et al*., 2015), possibly as an adaptation to the intraphagosomal phase.

In contrast to *S. enterica*, *E. coli* takes a different approach to copper homeostasis to suit its surface- and biofilm-associated lifestyle. In *E. coli*, no additional cytosolic proteins are used on top of CopA and CopZ. Periplasmic copper homeostasis is controlled by the two-component sensory system CusSR (Gudipaty and McEvoy, 2014), which regulates the expression of the resistance, nodulation and division (RND) proton-cation antiporter CusCBA (Grass and Rensing, 2001), and the periplasmic cuprochaperone CusF (Franke *et al*., 2003). The *cus* regulon plays a role during anaerobic growth, when cells are especially susceptible to Cu(I) (Outten *et al*., 2001), and is tuned to maintain periplasmic copper concentration at micromolar levels (Bagai *et al*., 2007; Yun *et al*., 2010; Gudipaty and McEvoy, 2014).

The principles exemplified in Enterobacteria are conserved throughout the Proteobacteria clade: the copper-secreting ATPase CopA is the core component of copper homeostasis in most species, and additional systems, both chromosome- and plasmid-encoded, are apparently interchangeable based on the biological niche (Andreini *et al*., 2008; Hernandez-Montes *et al*., 2012). The copper-storage proteins, including metallothioneins, constitute an interesting example of niche-specific adaptation: these are primarily found in organisms that rely on cuproproteins for core metabolic processes, such as methanotrophs (Vita *et al*., 2015), but they may also contribute to the survival of intracellular pathogens within macrophages (Gold *et al*., 2008; Wolschendorf *et al*., 2011).

The evolutionary dynamism of copper homeostasis is especially evident in copper-rich environments, where horizontal gene transfer can blur the differences between species. This has particular relevance when considering copper as an antimicrobial, since copper-resistant strains may arise in a similar way as multidrug-resistant ones do. For example, some antibiotic-resistant strains of *S. enterica* have acquired a functional *cus* locus (Arai *et al*., 2019). The use of copper as a food supplement in the animal industry is associated with the spread of the *pco* locus in both *E. coli* and *S. enterica* (Tetaz and Luke, 1983; Brown *et al*., 1995; Qin *et al*., 2014; Fang *et al*., 2016; Billman-Jacobe *et al*., 2018; Chalmers *et al*., 2018). This genomic island provides enhanced copper oxidation through the multicopper oxidase PcoA (Lee *et al*., 2002), increased copper chelation through the periplasmic chaperones PcoC and PcoE (Djoko *et al*., 2008; Zimmermann *et al*., 2012), as well as an additional copper-sensing system, PcoSR (Rouch and Brown, 1997). Furthermore, many pathogenic *E. coli* and *S. enterica* strains have also acquired pathogenicity islands for the biosynthesis of yersiniabactin (Oelschlaeger *et al*., 2003; Henderson *et al*., 2009), which sequesters Cu(II) in the extracellular space (Chaturvedi *et al*., 2012) and scavenges nutrient copper during starvation (Koh *et al*., 2017).

4 | COPPER PREVENTS LIPOPROTEIN MATURATION IN THE CELL ENVELOPE

One important route of copper toxicity is the inhibition of lipoprotein maturation, leading to the accumulation of toxic precursors in the inner membrane (Figure 4a). Outer membrane lipoprotein maturation (reviewed in Okuda and Tokuda, 2011) involves the sequential acylation of the polypeptide chain, removal of the signal peptide, and translocation to the outer membrane. Copper binds to the acyl-accepting N-terminal cysteine residue, making it inaccessible to the enzymes in the pathway (Yakushi *et al*., 1997; May *et al*., 2019).

As a consequence, the adaptation to copper stress requires the activation of the envelope stress response (reviewed in Raivio, 2014), which in *E. coli* and *S. enterica* is coordinated by the two-component regulator CpxAR (Egler *et al*., 2005; Pontel *et al*., 2014). In *E. coli*, where this system has been characterized, CpxA senses the accumulation of the outer membrane lipoprotein NlpE (Yamamoto and Ishihama, 2005; 2006; Price and Raivio, 2009; May *et al*., 2019). Activated CpxR induces the overexpression of the two lipoprotein-acylating enzymes, Lgt and Lnt, and the inhibition of lipoprotein production through the small inhibitory RNA, MicL. All of these components contribute to copper tolerance (Rogers *et al*., 1991; Gupta *et al*., 1997; Egler *et al*., 2005; Yamamoto and Ishihama, 2005; Yamamoto and Ishihama, 2006; Riley *et al*., 2006; Price and Raivio, 2009; Guo *et al*., 2014; May *et al*., 2019). In fact, some of these genes are so important for the copper response that they were initially identified as copper tolerance determinants (*nlpE* as *cutF*, *lnt* as *cutE*, and *micL* as *cutC*) (Rouch *et al*., 1989; Rogers *et al*., 1991; Gupta *et al*., 1995; Arnesano *et al*., 2003; Riley *et al*., 2006; Guo *et al*., 2014). In *S. enterica*, CpxR also contributes to copper homeostasis by regulating the expression of *cueP* (Pezza *et al*., 2016).

Overall, the *cpx* regulon represses the biosynthesis of copper-sensitive lipoproteins, and induces the production of maturation enzymes to remove the damage that was already dealt (Raivio, 2014; Mitchell and Silhavy, 2019). The *cpx* response also inhibits cell growth and proliferation, and promotes biofilm formation and cellular persistence. As a consequence, care must be taken to interpret experimental data on copper toxicity, since sub-toxic concentrations can lead to the formation of long-term persister cells (Grey and Steck, 2001).

5 | COPPER CAUSES PROTEIN MISFOLDING BY CATALYZING THE FORMATION OF NON-NATIVE DISULFIDE BONDS

The second route of copper toxicity in the bacterial envelope targets maturing polypeptides. Under aerobic conditions, copper can

oxidize exposed thiols catalyzing the formation of disulfide bonds (Figure 4b). In Enterobacteria, native folding of periplasmic polypeptides is normally mediated by two oxidoreductases, DsbA and DsbB (DsbL and DsbI in *S. enterica*) (Kadokura *et al*., 2003; Lin *et al*., 2009). DsbA/DsbL transfers its disulfide bond to maturing polypeptides, and is regenerated by DsbB/DsbI using reducing equivalents from the cytoplasm. When incorrect disulfide bonds are inserted by copper, however, an additional oxidoreductase pair is required to rearrange the misfolded peptides; this role is fulfilled by the *E. coli* DsbC and DsbD (also known as CutA) (Ito and Inaba, 2008), and the *S. enterica* ScsC and ScsD (Subedi *et al*., 2019). Expression of these proteins is induced by CpxR in response to envelope stress, and is required for copper tolerance in both organisms (Rietsch *et al*., 1996; Hiniker *et al*., 2005; Lopez *et al*., 2018). Importantly, the *S. enterica* Scs system is required for intraphagosomal survival (Yucel *et al*., 2020) and was initially identified as a copper tolerance determinant (suppressor of copper sensitivity).

In *E. coli*, the accumulation of misfolded proteins in the periplasm induces the RpoE (σ^E) regulon (reviewed in Barchinger and Ades, 2013), which cooperates with CpxAR to fine-tune the envelope stress response. Interestingly, copper stress induces the *rpoE* response in *E. coli* but not *S. enterica* (Egler *et al*., 2005; Yamamoto and Ishihama, 2006; Pontel *et al*., 2014), suggesting that *S. enterica* responds to copper-induced envelope damage exclusively through *cpx*.

6 | COPPER IMPAIRS PEPTIDOGLYCAN MATURATION

The last envelope-specific effect of copper toxicity is the weakening of peptidoglycan (Figure 4c). In Enterobacteria, peptidoglycan is assembled by two classes of transpeptidases (TPases): *LD*-TPases and *DD*-TPases. Cu(I) binds to the catalytic cysteine of *LD*-TPases, preventing them from cross-linking peptidoglycan and attach it to outer membrane lipoproteins (Mainardi *et al*., 2005; Peters *et al*., 2018). As a consequence, copper-stressed cells have a weaker cell envelope and are more sensitive to detergents and other membrane-destabilizing compounds (Peters *et al*., 2018).

DD-TPases are copper-insensitive, however, they are inhibited by β-lactam and carbapenem antibiotics, resulting in a synergistic effect of these compounds with copper. The synergy with carbapenems is especially relevant, since copper inhibits metallo-β-lactamases with carbapenemase activity (Djoko *et al*., 2018).

7 | SOME ENVELOPE COMPONENTS CONTRIBUTE TO COPPER TOLERANCE IN UNKNOWN WAYS

In addition to the copper tolerance systems outlined above, additional envelope proteins have been implicated in the copper response. In *E. coli*, the porin OmpC and the outer membrane protein ComC (BshA) are induced upon copper stress (Kershaw *et al*., 2005; Mermod *et al*., 2012) and are required for full copper tolerance (Egler *et al*., 2005). Copper-dependent induction of *ompC* is mediated by RpoE, while that of *comC* occurs through a dedicated TetR-like transcriptional regulator, ComR (Mermod *et al*., 2012). The mechanism by which these proteins contribute to copper tolerance has not been elucidated, but they are known to respond to a number of envelope-targeting stressors (Richmond *et al*., 1999; Zheng *et al*., 2001; Egler *et al*., 2005; Maurer *et al*., 2005; Zhang *et al*., 2007). It has been proposed that outer membrane proteins may reduce copper entry to the periplasm by selecting against this ion on the cell surface (Egler *et al*., 2005), although such a model has not been thoroughly investigated to the best of our knowledge. These findings show that our knowledge of copper tolerance is not yet complete, and further studies are required to advance our understanding.

8 | COPPER DISPLACES OTHER METALS FROM PROTEIN BINDING SITES

Because of its strong affinity for thiol ligands, copper can mismetalate maturing proteins that require a molybdenum or iron cofactor. Mismetalation not only can occur on exposed metal-binding pockets, but also during protein maturation, as copper can mismetalate the metallochaperones supplying the metal cofactor to nascent polypeptides (Neumann and Leimkühler, 2008; Macomber and Imlay, 2009; Iobbi-Nivol and Leimkühler, 2013; Tan *et al*., 2014; 2017; Djoko *et al*., 2017). In some cases, cells can prevent mismetalation by expressing metallochaperones that have a higher specificity for their cognate partners. This is the case in *E. coli*, which switches from the copper-sensitive chaperone IscA to the copper-resistant SufA to survive copper stress (Macomber and Imlay, 2009; Fung *et al*., 2013; Tan *et al*., 2014).

Copper mismetalation affects cell metabolism in pleiotropic ways, since many biosynthetic enzymes are affected (Jo *et al*., 2008; Neumann and Leimkühler, 2008; Macomber and Imlay, 2009; Djoko *et al*., 2017; Tan *et al*., 2017). This can generate functional auxotrophies (Fung *et al*., 2013; Djoko *et al*., 2017), which the cell can adapt to by inducing alternative biosynthetic pathways. For example, *E. coli* responds to copper mismetalation of glutamate synthase by inducing an alternative route for glutamate biosynthesis from glutamine (Djoko *et al*., 2017). A better understanding of the biosynthetic alterations induced by copper, therefore, can inform future synergic therapies. Recently, it was proposed that a glutamate- and glutamine-deficient phenotype may make *E. coli* more sensitive to low pH under copper stress, although this has only been shown in a *copA* background (Djoko *et al*., 2017).

9 | COPPER, IRON AND ZINC ACT SYNERGISTICALLY IN CELLULAR KILLING

In addition to bypassing copper-sensitive enzymes, copper-stressed cells face the double challenge of supplying enough native cofactor

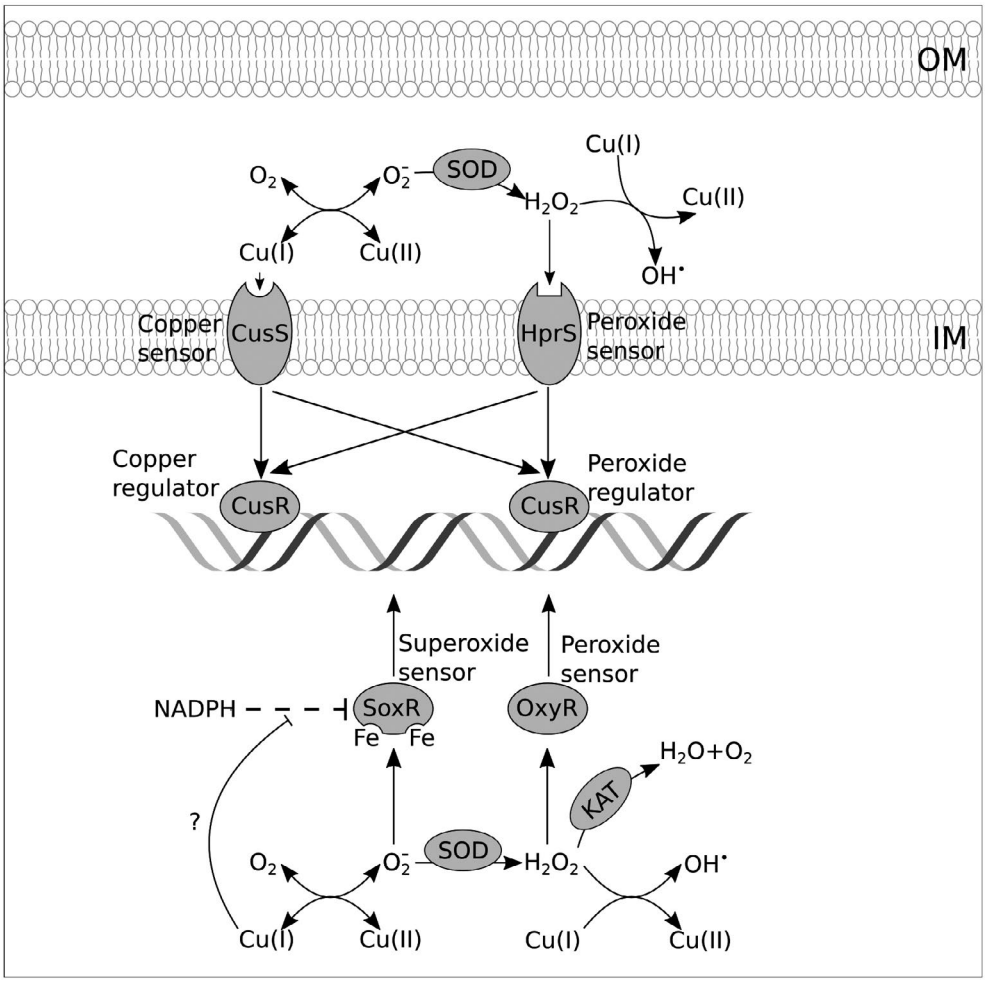

FIGURE 5 Copper-induced redox stress in Enterobacteria. Gram-negative bacteria respond to copper-catalyzed generation of reactive oxygen species (ROS) with different pathways, which monitor the state of each cellular compartment. Superoxide dismutases (SOD) convert copper-generated superoxide into peroxide. In the periplasm, peroxide is detected by two-component sensors such as HprSR. In *E. coli*, HprSR and the copper sensor CusSR can cross-talk, modulating each other's regulon. In the cytoplasm, free peroxide is scavenged by catalases (KAT). Superoxide and peroxide are sensed by SoxR and OxyR, respectively. Because SoxR inhibition depends on the cellular NADPH pool, copper may induce the *sox* response by interfering with NADPH production

to nascent metalloenzymes while scavenging "free" metal ions liberated by copper. This is especially important in the case of iron, since it can generate ROS through Fenton/Haber-Weiss chemistry (Py and Barras, 2010). The master regulator of iron metabolism, Fur, is overexpressed under copper stress in both *E. coli* and *S. enterica* (Kershaw *et al*., 2005; Yamamoto and Ishihama, 2006; Price and Raivio, 2009; Pontel *et al*., 2014) and is required for copper resistance even at low copper concentrations (Grass *et al*., 2004). Moreover, many copper-responsive genes in the *fur* regulon are also regulated by CpxR and SoxS, the master regulator of the ROS response (Zheng *et al*., 1999; Kershaw *et al*., 2005; Große *et al*., 2006; Yamamoto and Ishihama, 2006; Cao *et al*., 2007; Price and Raivio, 2009; Fung *et al*., 2013; Pontel *et al*., 2014; Tan *et al*., 2014; 2017). These multiple layers of regulation are required to fine-tune the *fur* response, since copper- and iron-derived peroxide can damage the Fur protein (Varghese *et al*., 2007; Seo *et al*., 2014). Moreover, iron-generated ROS can damage CueR, resulting in reduced levels of *copA* and *cueO* transcripts (Xu *et al*., 2019). As a consequence, iron excess can increase copper sensitivity in the same way as copper excess can increase iron sensitivity (Grass *et al*., 2004; Chaturvedi *et al*., 2012; Koh *et al*., 2017).

Another ion that displays synergistic effects with copper is zinc (Grass *et al*., 2002). Even though copper and zinc do not appear to mismetalate each other's partners, excess zinc also dysregulates Fur, increasing intracellular iron with the effects detailed above (Xu *et al*., 2019). Because the mammalian immune system exploits copper and zinc synergistically within macrophages (Kapetanovic *et al*., 2016), intraphagosomal pathogens have adapted to survive copper and zinc toxicity at the same time. For example, expression of the zinc-exporting ATPase, ZntA, is induced by copper in *S. enterica* (Kapetanovic *et al*., 2016) and strains lacking *zntA* are more sensitive to copper stress (Huang *et al*., 2017). Many other genes involved in the copper and zinc responses are activated by either ion (Pontel *et al*., 2014), which mediates pathway co-activation in a number of cases.

10 | COPPER DISRUPTS CELLULAR REDOX POTENTIAL

Because of its facile redox cycle, copper can interact with many electron carriers, including molecular oxygen (Hiniker *et al*., 2005), peroxide (Gunther *et al*., 1995) and thiols (Rigo *et al*., 2004). Similarly to iron, copper can generate reactive oxygen species (ROS) via Fenton/Haber-Weiss chemistry in vitro (Macomber *et al*., 2007). However, the extent to which ROS contribute to copper toxicity in bacteria is still unclear.

In Enterobacteria, the response to redox stress is effected by the *hpr* and *sox* regulons (Figure 5), respectively, protecting the periplasm and cytoplasm (Kimura and Nishioka, 1997; Yamamoto and Ishihama, 2005; Urano *et al*., 2015). The *sox* response, in particular, is strongly upregulated under copper stress in both *E. coli* and *S. enterica* (Egler *et al*., 2005; Kershaw *et al*., 2005; Yamamoto and Ishihama, 2005; Urano *et al*., 2015) and is required for full copper resistance (Kershaw *et al*., 2005). The *hpr* regulon (also known as *yed* in *E. coli* and *cop* in *S. enterica*) is also involved in the copper response, at least in some conditions (Yamamoto and Ishihama, 2005; Espariz *et al*., 2007). The third regulon involved in the redox response, the cytosolic *oxy* system, does not seem to respond to copper in either organism (Yamamoto and Ishihama, 2005; Pontel *et al*., 2014).

One challenge in assessing whether ROS play a role in copper toxicity is that *hpr* and *sox* monitor the general redox state of the cell, responding to non-oxygen stressors such as nitric oxide, chlorine and various redox-cycling compounds (Nunoshiba *et al*., 1993; Dukan *et al*., 1996; Gu and Imlay, 2011; Liochev and Fridovich, 2011; Gennaris *et al*., 2015; Urano *et al*., 2015; 2017) in addition to peroxide and superoxide (Chiang and Schellhorn, 2012; Urano *et al*., 2017). Moreover, the two-component sensory system HprSR is a close homologue of CusSR, and cross-talk between the regulators has been demonstrated in *E. coli* (Yamamoto and Ishihama, 2005). The depletion of cellular NADPH levels, by contrast, can adventitiously activate the *sox* response by interfering with the regulator's futile cycle (Liochev and Fridovich, 1992; Gaudu *et al*., 2000; Krapp *et al*., 2011). Therefore, the activation of *hpr* and *sox* does not necessarily demonstrate a role of ROS production in copper toxicity, as was assumed in the earliest models (Kimura and Nishioka, 1997).

One further challenge to the model of copper-induced ROS production is that copper toxicity is maximal under anaerobic conditions in bacteria (Outten *et al*., 2001; Espariz *et al*., 2007). Moreover, although copper has been shown to generate hydroxyl radicals and damage DNA in vitro (Cai *et al*., 1995; Moriwaki *et al*., 2008), direct DNA damage upon copper exposure has not been confirmed in vivo to the best of our knowledge, even in *E. coli* strains lacking all known copper-efflux genes (Macomber *et al*., 2007). Measurements of intracellular ROS showed that copper supplementation does not increase hydroxyl radical production and may even reduce superoxide levels (Park *et al*., 2012). Finally, the *oxy* regulon, which responds to cytosolic peroxide, does not seem to be upregulated under copper stress in either *E. coli* or *S. enterica*. Taken as a whole, these results suggest that ROS production may be one of many routes of copper toxicity, but not the main one.

How exactly copper activates *hpr* and *sox*, if not through ROS, is currently unclear. One possible explanation for *hpr* activation is that the sensor HprS may monitor the redox state of the inner membrane (Urano *et al*., 2017). Since ionic copper interacts with respiratory complexes (Volentini *et al*., 2011), it is possible that HprS responds to alterations in bacterial respiration under copper stress. In organisms possessing both the *hpr* and *cus* systems, cross-talk between the regulators could be an additional source of *hpr* activation. The recruitment of the *sox* response may occur by direct copper-catalyzed reduction of the regulator, or indirectly, through alterations in the NADPH pool.

In any case, the pattern of copper damage through ROS seems to be less universal than generally assumed. It must be noted that efficient oxidation of periplasmic Cu(I) and a copper-poor cytoplasm are key features of enterobacterial copper homeostasis. In organisms lacking multicopper oxidases, or with an increased cytosolic copper quota, Fenton/Haber-Weiss chemistry may play a bigger role. Therefore, more research on the subject is required to inform possible therapeutic approaches.

11 | ADDITIONAL FACTORS CONTRIBUTE TO COPPER TOLERANCE DURING ANAEROBIC GROWTH

Anaerobic conditions pose a specific challenge to copper homeostasis, especially in Enterobacteria. On the one hand, multicopper oxidases such as CueO/CuiD are inactive without dioxygen (Outten *et al*., 2001). On the other, the lack of oxygen shifts the chemical equilibrium toward the more toxic Cu(I) (Beswick *et al*., 1976). It has also been observed that bacterial cells accumulate more copper under anoxic conditions (Outten *et al*., 2001; Macomber *et al*., 2007), although the mechanism causing this is unclear. In any case, bacterial cells are more sensitive to copper during anaerobic growth (Outten *et al*., 2001; Espariz *et al*., 2007).

Some components of the copper response are specifically mobilized during anaerobic growth. Glutathione, for example, may contribute to the *E. coli* copper tolerance by chelating copper and restoring the cellular redox balance during anaerobic (Macomber and Imlay, 2009) but not aerobic growth (Helbig *et al*., 2008). The DNA-binding mini-ferritin, Dps, also protects *E. coli* from copper damage during anerobiosis (Thieme and Grass, 2010) but is dispensable during aerobiosis (Große *et al*., 2014). Accordingly, many copper homeostasis genes are upregulated during the transition to anaerobic growth. This includes the *cus* operon as well as *copA*, which is regulated by the oxygen sensor FNR in addition to CueR (Partridge *et al*., 2007). Interestingly, it has been proposed that the *S. enterica* CueO homologue contributes to copper tolerance even under anaerobic conditions (Espariz *et al*., 2007), but the mechanism of this contribution is unclear since the lack of oxygen should preclude oxidase activity.

Taken as a whole, the available evidence suggests that strict or facultative anaerobes must face additional challenges when adapting

to copper toxicity under anoxic conditions. This shows promise for therapeutic approaches, since reducing agents should feature a powerful synergy with copper treatment.

12 | CONCLUSIONS AND PERSPECTIVES

In this review, we provided a holistic view of the bacterial response to copper toxicity, focusing on two intensively studied organisms, *E. coli* and *S. enterica*. Many studies have focused on elucidating copper homeostasis in diverse bacteria. The direct response to copper excess is highly conserved and generally involves: (a) sensing of the increased copper availability by sensors located in the different bacterial compartments; (b) activation of bespoke transcriptional networks; (c) overproduction of copper efflux pumps that secrete copper from the copper-poisoned compartment; and (d) recruitment of copper-binding and copper-oxidizing proteins, such as metallothioneins, copper oxidases, copper storage proteins, and/or cuprochaperones that prevent copper from interacting with cellular components.

The copper homeostasis system, however, is insufficient to protect cells from copper toxicity. Additional bacterial pathways are needed to minimize and repair copper-induced damage, enabling cellular survival under copper stress. The collective data on enterobacterial systems suggests that these systems are mainly responsible for: (a) protecting the integrity and function of the cell envelope; (b) maintaining the cell's redox balance and detoxifying any ROS eventually generated by copper excess; and (c) ensuring the homeostasis of other metals, especially iron. Importantly, understanding the copper response can shed light on the mechanisms of copper toxicity. For example, we notice that copper adaptation is most often explored under aerobic conditions, whereas oxygen availability should be considered an important variable in studies of copper toxicity.

A full understanding of the bacterial response to copper toxicity is not just of academic interest. Emerging evidence demonstrates that copper plays a role in the mammalian immune system, potentially making components of the bacterial copper response suitable targets for future drug development. The "contact-killing" toxicity of solid copper-alloy surfaces has also received attention in recent years, owing to their potential use to reduce the spread of surface-associated pathogens in the clinic (Noyce *et al*., 2006; Warnes *et al*., 2012). Copper-based compounds and materials have a long history of such applications in agriculture and in medicine, and are appealing due to a wide-spectrum antimicrobial activity, low cost, and what seems to be a relatively low risk of resistance emerging among bacteria (Lemire *et al*., 2013). Moreover, metal ions promise to be potent synergistic drugs to enhance or complement the effectiveness of existing and future antibacterials (Peters *et al*., 2018). As a consequence, a better understanding of bacterial copper responses can inform novel therapeutic approaches, and will be required to fully unlock the potential of copper as an antimicrobial of the future.

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AUTHOR CONTRIBUTIONS

AG conceived the concept for review and drafted the manuscript, with writing input and supervision from KJW.

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