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MINI-REVIEW

Survival in amoeba—a major selection pressure on the presence of bacterial copper and zinc resistance determinants? Identification of a "copper pathogenicity island"

Xiuli Hao[1] · Freja L. Lüthje[2] · Yanan Qin[2] · Sylvia Franke McDevitt[3] · Nataliya Lutay[4] · Jon L. Hobman[5] · Karishma Asiani[5] · Fernando C. Soncini[6] · Nadezhda German[7] · Siyu Zhang[2,8] · Yong-Guan Zhu[1,8] · Christopher Rensing[1,2]

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Abstract The presence of metal resistance determinants in bacteria usually is attributed to geological or anthropogenic metal contamination in different environments or associated with the use of antimicrobial metals in human healthcare or in agriculture. While this is certainly true, we hypothesize that protozoan predation and macrophage killing are also responsible for selection of copper/zinc resistance genes in bacteria. In this review, we outline evidence supporting this hypothesis, as well as highlight the correlation between metal resistance and pathogenicity in bacteria. In addition, we introduce and characterize the "copper pathogenicity island" identified in *Escherichia coli* and *Salmonella* strains isolated from copper- and zinc-fed Danish pigs.

Keywords Copper · Pathogenicity · Amoeba · Grazing resistance

✉ Christopher Rensing
chres@life.ku.dk

[1] Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, People's Republic of China

[2] Department of Plant and Environmental Science, University of Copenhagen, 1871 Frederiksberg, Denmark

[3] Department of Biology, Skidmore College, Saratoga Springs, NY, USA

[4] Division of Dermatology and Venereology, Department of Clinical Sciences, Lund University, Lund, Sweden

[5] School of Biosciences, The University of Nottingham, Sutton Bonington Campus, Sutton Bonington LE12 5RD, UK

[6] Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET, UNR), 2000 Rosario, Argentina

[7] Department of Pharmaceutical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, Amarillo, Texas

[8] State Key Lab of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, People's Republic of China

Introduction

Essential metals such as iron and copper can cycle between different oxidation states and are used in metalloenzymes that catalyze electron transport reactions. Zinc also plays a major structural and catalytic role in metalloenzymes and has been reported to counter oxidative stress. But in excess, all of these metals are deleterious to cells. To ensure their own survival, prokaryotes have developed mechanisms of maintaining cellular Zn^{2+} and Cu^{+} homeostasis, while eukaryotes invented very original Zn- and Cu-binding structures not present in prokaryotes. Such structures allow accumulation of Zn^{2+} in the intracellular organelles followed by its utilization in biological processes specific for a given cell type. In particular, macrophages employ Zn^{2+} and Cu^{+} to attack Fe–S clusters essential for bacterial survival (Braymer and Giedroc 2014; Dupont et al. 2011; Festa and Thiele 2012; Kashyap et al. 2014; Macomber and Imlay 2009; Neyrolles et al. 2015; Subashchandrabose et al. 2014; Xu and Imlay 2012). We hypothesize that such a mechanism, where bacterial killing occurs through accumulation of Zn^{2+} and Cu^{+} in the phagosome/vacuole, originated in protozoa long before multicellular life arose and that it later evolved in eukaryotic phagocytes. Our hypothesis is supported by the presence of the homologous copper transporter 1 (Ctr1) in macrophages and P80 in *Dictyostelium discoideum* and *Acanthamoeba*

castellanii—proteins, both of which are involved in Cu^{+} uptake upon phagocytosis. In addition, amoebae are known to contain P-type ATPases (German et al. 2013; Burlando et al. 2002), and similar to macrophages, at least one of these P-type ATPases in *A. castellanii* could be pumping Zn^{2+} or Cu^{+} into the phagosome of amoeba (Fig. 1). Importantly, our hypothesis explains selection of genes involved in conferring copper and zinc resistance not only by the presence of these metals in the environment but also by protozoan predation as well. Since these determinants would aid survival in both protozoans and macrophages, one could expect a higher occurrence of additional copper and zinc resistance determinants in virulent bacteria.

A copper/silver resistance cluster or an ancestral defense to phagosomal killing using copper?

Genome sequencing projects have revealed that several strains of *Salmonella enterica* subspecies *enterica* harbor a ca. 12-kb copper resistance locus. This cluster is shown to either form part of a Tn*7*-like transposon inserted at the 3′ end of the gene that encodes a NAD-utilizing dehydrogenase on the chromosome (Peters et al. 2014), as found in isolates from serovars Heidelberg, Montevideo, Senftenberg, or Tennessee, or to be a part of a larger integrating conjugative element inserted at the *pheV* phenylalanine tRNA, as present in strains of the serovars Senftenberg, Ohio, or Cubana. Although there is a history of copper- and zinc-resistant bacteria being isolated from feces of animals fed with metal-supplement-containing diets, the presence of this resistance cluster has just recently been recognized.

Recently, we have sequenced the genomes of two *Escherichia coli* and three *S. enterica* serovar Typhimurium strains isolated from copper- and zinc-fed Danish pigs, hence displaying high-level copper resistance (Lüthje et al. 2014; Qin et al. 2014). One of the *E. coli* strains and all three of the *Salmonella* strains contained a specific 19-gene mobile genetic element that we have named as the "copper pathogenicity island." In the *E. coli* isolate, we have identified this island as a part of aTn7-like transposon, while in the *S.* Typhimurium strains, it forms part of an approximately 80-kbp chromosomal element inserted at the *pheU* phenylalanine tRNA, similar to that identified in Heidelberg, Montevideo,

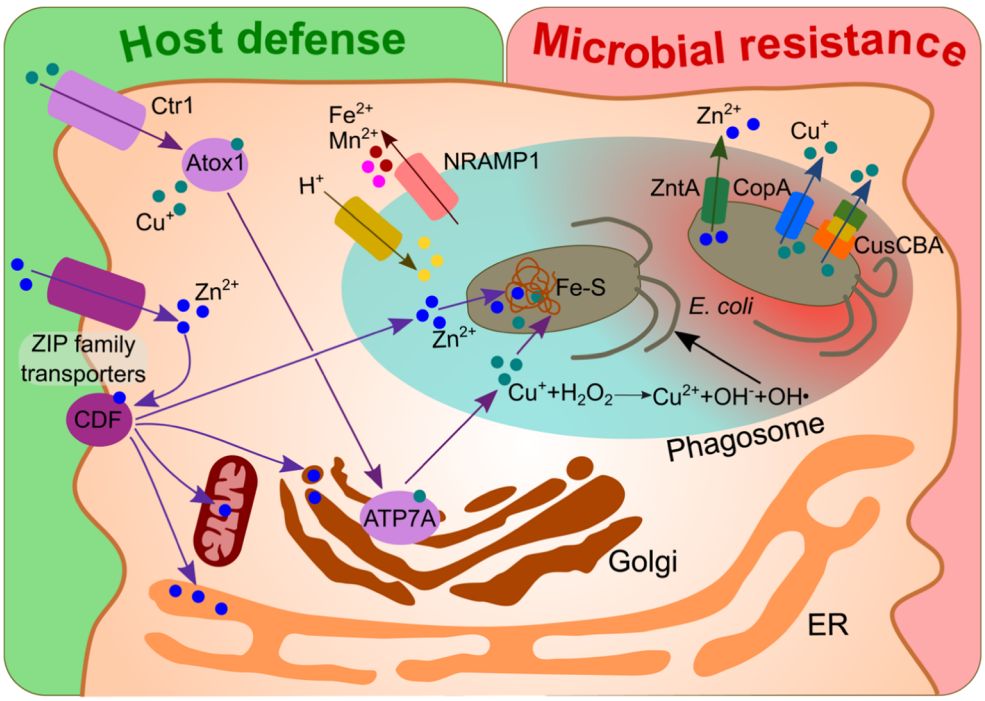

Fig. 1 A schematic overview of Zn and Cu involvement in phagosomal killing of bacteria. Macrophages and amoeba can exploit similar molecules for Zn^{2+} and Cu^{+} trafficking. ZIP family transporters allow Zn^{2+} uptake into the cytoplasm, and cation diffusion facilitator proteins (*CDF*) could deliver Zn^{2+} to the phagosome and other organelles, like mitochondria, Golgi, and endoplasmic reticulum (*ER*). Cu^{+} uptake and delivery to phagosomes occur due to copper transporter 1 (Ctr1, in amoeba known as P80), antioxidant 1 copper chaperone (Atox1), and in human macrophages, the P-type ATPase ATP7A. H^{+}–ATPase causes acidification of the phagosomal milieu, while natural resistance-associated macrophage protein 1 (NRAMP1) removes Fe^{2+} and Mn^{2+}, which are needed to protect (Mn^{2+}) and rebuild degraded Fe–S clusters of bacteria. In addition, Cu^{+} amplifies toxicity of reactive oxygen species (hydroxyl radical (·OH) and hydroxide anion (OH^{-})). *E. coli* express genes encoding ZntA for Zn^{2+} efflux, CopA for Cu^{+} efflux, and the CusCBA complex for periplasmic Cu^{+} efflux, but virulent strains have additional copper resistance systems

Senftenberg, or Tennessee isolates (Qin et al. 2014). This genetic cluster is comprised of two previously reported metal ion resistance determinants, neither of which was realized until recently to be part of a single contiguous gene cluster (Crossman et al. 2010; Hobman and Crossman 2015). One, the *pco* determinant was first isolated from plasmid pRJ1004 from an Australian pig *E. coli* isolate (Brown et al. 1995) and confers copper resistance. The other, the *sil* determinant originally located on *Salmonella* Typhimurium plasmid pMG101, but later shown to have transferred into the chromosome of the host *E. coli* K-12 J53 strain (Randall et al. 2015), is associated with silver resistance (McHugh et al. 1975; Gupta et al. 1999). Later sequencing of pRJ1004 (NCBI accession no. KC146966) has identified two new genes among the entire 19-gene cluster—*pcoF* encoding a putative copper-binding protein and *pcoG* encoding a putative M23B metallopeptidase, an enzyme that has been implicated in pathogenicity (Bonis et al. 2010) (Fig. 2). We have identified this arrangement of *pco*/*sil* genes in a number of different genome and plasmid sequences.

Similarly, the *sil* determinant has been associated with pathogenicity in the *Enterobacter cloacae* complex, where the presence of the genes conferring silver resistance was increased in isolates from hospital settings vs. strains associated with plants (Kremer and Hoffmann 2012). Although identification of the *pco* genes was not part of that study, their presence within the isolates harboring the full *sil* determinant is very likely based on the high rate of co-representation (Mourão et al. 2015) (Table 1).

Previous studies and genomic analysis have shown that the copper pathogenicity island is often plasmid associated. Transfer of such plasmids has resulted in a nosocomial outbreak of *Klebsiella pneumoniae* (Sandegren et al. 2012). Moreover, the *pco*/*sil* cluster has been identified on pAPEC-O2-R plasmids from avian pathogenic *E. coli* (Johnson et al. 2005), R478 from *Serratia marcescens* (Gilmour et al. 2004), plasmids pK2044 and pLVPK from *K. pneumoniae* strains (Chen et al. 2004; Wu et al. 2009), and on the chromosome or plasmids of many pathogenic enteric bacteria such as ETEC H10407 (Crossman et al. 2010) and EHEC O104:H4 (Hobman and Crossman

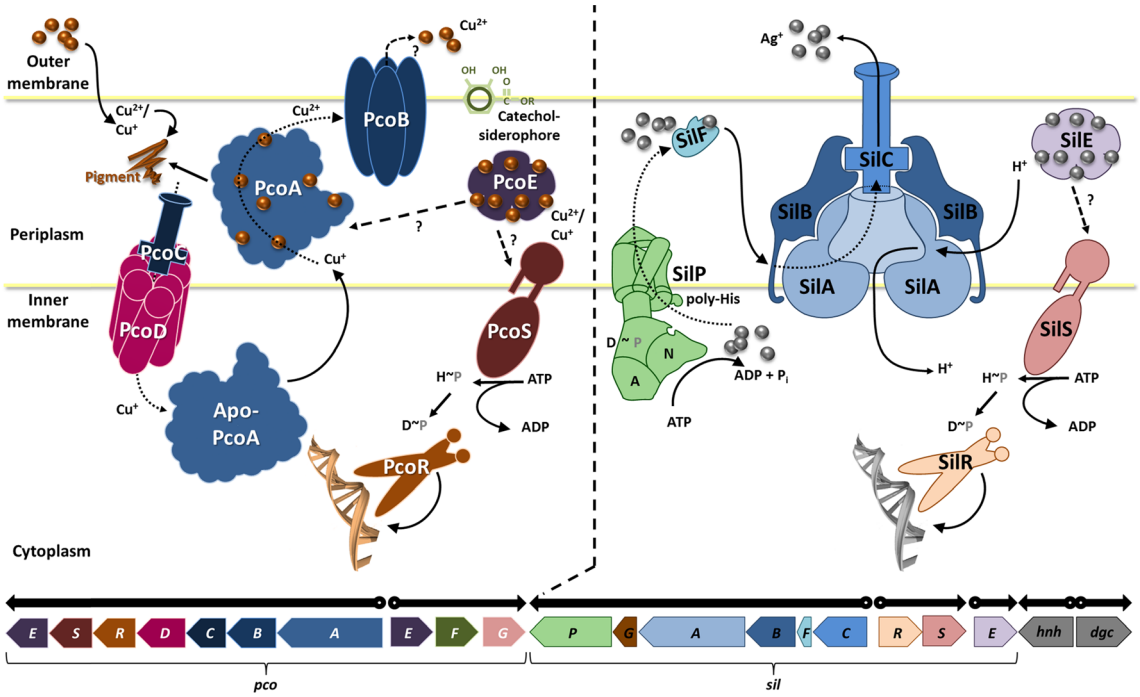

Fig. 2 Pco and Sil mechanisms in action. Proposed genes and protein products forming the molecular mechanisms of Pco- and Sil-mediated copper and silver detoxification and control in the cell. The *bottom line* indicates the genes and their transcriptional and translational directions, with the *open circles* representing potential promoter regions/transcript start sites. The illustrated function of each *sil* and *pco* gene product within the operon (Gram-negative) is deduced from homology modeling. The transcription of Pco proteins PcoABCDEFG appears to be regulated by PcoRS (*left*). The roles of PcoFG have not been elucidated. In addition to the oxidized catechol siderophores, copper may be detoxified from Cu^{+} to Cu^{2+} by the suggested multicopper oxidase PcoA. PcoB possibly functions as the outer membrane transporter, while, sitting in the inner membrane, PcoD drives the transport of Cu^{+} from the periplasm to the cytoplasm, with periplasmic PcoC chaperoning/delivering the Cu^{+} to PcoD. PcoE is an additional chaperone that binds copper in the periplasm and probably shuttles it to PcoA and/or PcoS. Similarly, the Sil system (*right*) contains a homolog to PcoE—periplasmic protein SilE. SilE is predicted to bind and chaperone Ag^{+}, Cu^{+}, and Cu^{2+} to the three-polypeptide, transmembrane, chemiosmotic RND exchange system (SilCBA), exporting the metal ions out of the cell. Likewise, SilF acts as a chaperone to SilCBA too. The other putative efflux pump mediating the mechanism is a P-type ATPase—SilP. Although conserved within the *sil* determinant, a role of SilG has not yet been determined. While the expression of *silCFBAGP* is thought to be governed by the two-component membrane sensor and transcriptional responder SilRS; the expression of *silE*, just like homolog *pcoE*, is thought to be regulated/co-regulated by the Cus system (Zimmerman et al. 2012), and therefore, SilE perhaps is involved in the activation of regulators SilRS (proposed by *dotted arrow*)

Table 1 Distribution of copper/silver resistance cluster and yersiniabactin biosynthesis among *Enterobacteriaceae*

Genus[a]	Number of sequences analyzed[b]	Occurence of copper/silver tolerance determinants				
		pco[c]	*sil*[d]	*pco*/*sil*[e]	Yersiniabactin synthesis[f]	*pco*/*silP* and yersiniabactin synthesis[g]
Citrobacter	4 genomes	2	2	2	1	0
	5 plasmids	0	0	0	0	
Cronobacter	6 genomes	1	1	1	0	0
	5 plasmids	3	3	3	0	
Enterobacter	16 genomes	5	6	5	1	0
	12 plasmids	3	3	3	0	
Escherichia	74 genomes	10	10	10	42	6
	71 plasmids	2	4	2	0	
Klebsiella	32 genomes	0	0	0	17	6
	33 plasmids	29	29	29	0	
Raoultella	2 genomes	0	0	0	1	0
	1 plasmid	0	0	0	0	
Salmonella	252 genomes	5	5	5	0	0
	77 plasmids	1	1	1	1	
Serratia	18 genomes	0	0	0	0	0
	8 plasmids	1	1	1	0	
Yersinia	33 genomes	0	0	0	27	0
	33 plasmids	0	0	0	0	

[a] Genera of *Enterobacteriaceae* harboring *pco*, *sil*, and/or *ybt*

[b] Number of completed genomic and plasmid sequences of respective genera available for Microbial Genome BLAST® (http://blast.ncbi.nlm.nih.gov; accessed 05/18/2015)

[c] Analysis (blastn) using *pco* from pRJ1004 (accession no. X83541.1; Brown et al. 1995) as query

[d] Analysis (blastn) using *sil* from pMG101 (accession no. NG_035131.1; Gupta et al. 1999) as query

[e] Analysis (blastn) using *pco* (accession no. X83541.1; Brown et al. 1995) and *sil* (accession no. KC1469.66.1) from pRJ1004 as query

[f] Analysis (tblastn) using Ybt peptide/polyketide synthetase HMWP1 (accession no. AAC69588.1; Gehring et al. 1998) as query

[g] Number of strains harboring *pco*/*sil* and *ybt* with determinants being located on chromosome and/or plasmid, respectively

2015) and *Enterobacter cloacae* subsp. *cloacae* strain ATCC 13047 (Ren et al. 2010). For *E. coli* and *Enterobacter* strains, the copper pathogenicity island was identified in close vicinity to Tn7-like transposons with *tnsABCD* being present (Peters et al. 2014). At the same time, in *Klebsiella* strains, it was often associated with IS4-related elements and genes encoding a HNH endonuclease. These data together with the fact of a similar arrangement of a copper/silver resistance island found in *Salmonella* (Moreno Switt et al. 2012) indicate that the gene cluster behaves like a typical pathogenicity island.

Yersiniabactin—not just for iron

Our recent sequencing of *E. coli* strains isolated from copper-fed pigs allowed us to identify another determinant conferring increased copper resistance—a ten-gene yersiniabactin synthesis cluster (Lüthje et al. 2014). The yersiniabactin determinant is a well-known virulence factor responsible for copper binding (Chaturvedi et al. 2014) that can be present in pathogens such as *K. pneumoniae* (Fodah et al. 2014); *Salmonella* (Aviv et al. 2014); *E. coli* (Schubert et al. 2004), including the EHEC O104:H4 outbreak strain; and the highly virulent *Yersinia pestis* (Rakin et al. 2012). Interestingly, several strains of *Klebsiella* and *E. coli* appear to have the *sil*/*pco* determinant in addition to the yersiniabactin synthesis cluster (Table 1).

Copper and zinc resistance in Gram-positive bacteria

Pathogenicity of Gram-positive bacteria such as *Enterococcus faecium*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Staphylococcus haemolyticus* is also linked to transition metal

resistance. Currently, we have sequenced the genomes of three highly copper-resistant *E. faecium* and three *E. faecalis* strains isolated from copper-fed pigs in Denmark (Zhang et al. 2015). As a result, we have identified additional copper resistance determinants characteristic for many pathogenic enterococci, *tcrYAZB*, encoding a negative transcriptional regulator, a copper chaperone, and two P_{1B}-type ATPases flanked by mobile elements (Hasman 2005). In *E. faecalis*, this determinant has often been found in the vicinity of a gene encoding a multicopper oxidase resembling CueO, an adjacently encoded two-component system, and possibly CopY. Whether CueO is regulated by the adjacent two-component system or CopY is not known. In this genome region, there are also several putative copper chaperones and a prolipoprotein diacylglyceryl transferase, which has been associated with virulence (Cho et al. 2013; Reffuveille et al. 2012) (Fig. 3).

In addition to copper, zinc resistance has also been linked to virulence and increased survival rates of pathogens. For example, in group A, *Streptococcus czcD* and *gczA* deletion mutants characterized by higher zinc sensitivity had shown much lower survival rates in the presence of neutrophils compared to wild-type strains (Ong et al. 2014). Certain correlation between the presence of the zinc resistance gene *czrC*, methicillin resistance, and virulence has been found in many *S. aureus* strains (Slifierz et al. 2014; Aarestrup et al. 2010). The gene *crzC* encodes a Zn^{2+}-translocating P-type ATPase and is located next to a gene encoding a possible transcriptional regulator of the ArsR/SmtB family and a gene encoding a putative iron/zinc permease. According to genomic similarities, the latter might be a distant homolog of the zinc/iron importer ZupT. Sequencing of the *S. haemolyticus* SH32 clinical strain has identified two incomplete staphylococcal cassette chromosome (SCC) elements, with one of them, *ψ*SCC*mec*(*SH32*), encoding a Cu(I)-translocating P-type ATPase (Yu et al. 2014). This strain was also shown to contain a putative cadmium resistance determinant *cadXD*, encoding for a Cd(II) transporter as well as a transcription regulator of the ArsR family (Yu et al. 2014). A recent study has reported that plasmid SAP078A in methicillin-resistant *S. aureus* CC22 SCC*mec*IV (EMRSA-15) contains *cadCA*, *mco*, and *copB* in addition to an *ars* operon conferring resistance to cadmium/zinc, copper, and arsenic, respectively (Loeffler et al. 2013). It was also shown that plasmid SAP078A is widespread among both human and animal

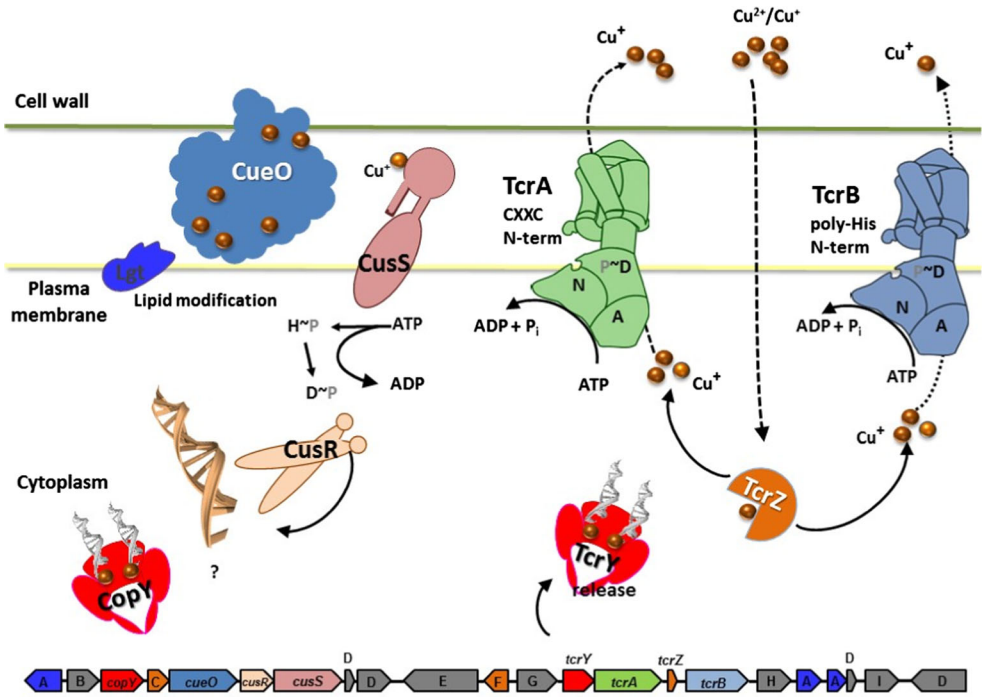

Fig. 3 Copper fitness island in *Enterococcus faecalis*. Proposed genes and protein products forming the molecular mechanisms of copper detoxification in *E. faecalis*. The *bottom line* indicates the genes and their transcriptional and translational directions. TcrY regulates expression of *tcrYAZB*, encoding for the repressor, a cytoplasmic chaperone (TcrZ), and two P-type ATPases (TcrA and TcrB) responsible for Cu^{+} export. In close proximity to *tcr* genes encoding a two-component regulatory system (CueRS), a multicopper oxidase (CueO), a predicted metal chaperone (no homology to TcrZ and labeled "C"), and transcriptional repressor (CopY) have been identified. CueO is predicted to oxidize Cu^{+} to Cu^{2+}. It is not clear to what extent the predicted chaperon (C) might be involved in copper detoxification. It has not yet been established if transcription of these genes is controlled by the two-component regulatory system (CusRS) responding to external copper concentration, CopY, as a response to internal copper concentrations, or both. Adjacent to and separating the two copper resistance determinants, genes encoding prolipoprotein diacylglyceryl transferase (*A*), integral membrane protein (*B*), hypothetical proteins (*D*), transposase (*E*), disrupted P-type ATPase (*F*), integrase (*G*), adenylate kinase (*I*), and resolvase (*I*) have been identified. The extent to which some of these proteins might be involved in copper detoxification has not been analyzed

isolates of *S. aureus* (Loeffler et al. 2013). Moreover, given the role of transition metals in the mammalian immune response, the presence of *cadCA* and *copB*/*mco* provides corresponding strains with an advantage (Hood and Skaar 2012). Interestingly, the epidemic ST22-IV has been replacing other MRSA clones from hospitals possibly due to enhanced virulence. Detailed studies revealed that ST22-IV has a significantly higher capacity to invade the A549 cells and a higher virulence in a murine model of acute lung infection causing severe inflammation and determining death in all the mice within 60 h (Baldan et al. 2012, 2015). We suggest that severe pathogenicity of ST22-IV might be partially attributed to increased transition metal resistance of this strain.

Cadmium and silver resistance—mutation plus selection equals evolution to new resistance?

Due to much lower environmental distribution of cadmium and silver, they are not essential micronutrients for bacteria and no data has been published on their involvement in bacteria/host interactions. Therefore, it is unlikely for bacteria to develop specific resistance mechanisms to these metals to a large extent. At the same time, most of the metal resistance determinants described to date are involved in detoxification of multiple substrates, e.g., conferring resistance to both copper and silver as well as to both zinc and cadmium (Rensing et al. 1999; Rosenzweig and Arguello 2012). Moreover, both *E. coli* and *Salmonella* contain detoxification systems for zinc and copper in addition to the possible plasmid-encoded resistance determinants on their chromosomes. In other words, it is quite unlikely that the evolutionary pressure comes exclusively from metal-contaminated environments. Rather, resistance to silver is a by-product of copper resistance and cadmium is a by-product of zinc resistance. In fact, resistance studies with *S. aureus* were unable to produce silver-resistant strains even after 42 days of continuous passage in the presence of $AgNO_3$ (Randall et al. 2013) Similar results were observed for some Gram-negative organisms, whereas in *E. coli* strains, silver resistance arises as a result of mutations in both *ompR* and *cusS* or mediated through the *sil* system (Randall et al. 2015).

Metal homeostasis in bacteria is a delicate balance, especially since metals like zinc and iron have been found to be essential for the pathogenicity of these organisms (Cerasi et al. 2014; Gradassi et al. 2013; Pesciaroli et al. 2011). The fact that protozoan grazing might be a strong force on keeping or gaining resistances against copper or zinc in nature together with the data suggesting that the concentrations necessary to maintain resistance plasmids within a population are well below the minimal inhibitory concentration (MIC) of the non-plasmid containing susceptible stain (Gullberg et al. 2014) might explain the prevalence of resistance mechanisms. Increasing metal contamination caused by anthropogenic metal use in agriculture and other sectors might also result in increased ability of protozoans to utilize copper and zinc to kill phagocytized bacteria. This, in turn, can cause a higher level of resistance in bacteria—a mechanism that has been primarily selected to avoid protozoan killing. In addition, copper is known to induce viable but non-culturable (VBNC) state in some bacteria, causing an increase in their survival rates (Ordax et al. 2006). It has been reported that cells of the VBNC state might have been responsible for the recent *E. coli* O104:H4 outbreak (Aurass et al. 2011).

It is worth noting here that copper-induced resistance combined with high toxicity and non-selectivity of redox processes induced by copper presence in cells present major challenges for developing copper-based antimicrobial therapy. Recently, Festa et al. have published a novel approach that allows accumulation of copper in pathogen cells without activating its Cu-resistant mechanisms and significantly increases selectivity of the treatment (Festa et al. 2014). In other strategies, Cu(II) ions have been either utilized as carriers for known antibiotics, allowing them to bypass existing efflux-mediated resistance to drugs (Manning et al. 2014; Lopes et al. 2013; Shams et al. 2014), or as chelators that upon binding to a ligand, change its conformation to the "active" mode (Haeili et al. 2014). Several potent copper chelators with activity against MRSA and *Mycobacterium tuberculosis* strains have been identified through drug screening assays specifically designed for identification of copper-dependent antimicrobial compounds (Speer et al. 2013) with, potentially, more discoveries on the way.

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Conflict of interest The authors declare that they have no competing interests.

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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