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Copper tolerance and virulence in bacteria

Erik Ladomersky[ab] and Michael J. Petris*[abc]

Copper (Cu) is an essential trace element for all aerobic organisms. It functions as a cofactor in enzymes that catalyze a wide variety of redox reactions due to its ability to cycle between two oxidation states, Cu(I) and Cu(II). This same redox property of copper has the potential to cause toxicity if copper homeostasis is not maintained. Studies suggest that the toxic properties of copper are harnessed by the innate immune system of the host to kill bacteria. To counter such defenses, bacteria rely on copper tolerance genes for virulence within the host. These discoveries suggest bacterial copper intoxication is a component of host nutritional immunity, thus expanding our knowledge of the roles of copper in biology. This review summarizes our current understanding of copper tolerance in bacteria, and the extent to which these pathways contribute to bacterial virulence within the host.

Introduction

Copper (Cu) has been used throughout much of human civilization as an antimicrobial agent. The earliest recorded medicinal use of copper can be traced to ancient Egyptian and Greek civilizations for the treatment of wounds and sterilization of water.[1] Today, the antimicrobial properties of copper are utilized in many different materials. Between 2008 and 2011, the Environmental Protection Agency (EPA) registered more than 300 copper alloys as antimicrobial, underscoring the increasing application of copper-based materials in the manufacture of surfaces where the presence of microbes could lead to nosocomial infections.[2] The realization that copper is used by the innate immune system is a relatively recent discovery. In contrast to other essential elements such as iron and manganese, which are withheld from the invading pathogen, host-derived copper appears to play a unique role in nutritional immunity by acting as a component of the antimicrobial arsenal produced by cells of the innate immune system. Several lines of evidence indicate that bacterial copper tolerance genes provide an important counter measure to this activity of the innate immune system. This review discusses our current understanding of bacterial copper tolerance pathways, and their contributions to pathogenesis within the host.

The essentiality of copper

The inclusion of copper within the repertoire of elements essential for life is thought to have first arisen within ancient photosynthetic cyanobacteria following the release of oxygen into the atmosphere. The ensuing decrease in the bioavailability of iron due to its oxidative precipitation as insoluble Fe(III) hydroxides allowed for the incorporation of copper into energy capturing systems such as cytochrome oxidase.[3] The essentiality of copper lies in its ability to undergo redox cycling between Cu(II), the oxidized cupric form, and Cu(I), the reduced cuprous form. As a soft Lewis acid, Cu(I) favors a tetrahedral coordination with soft bases such as hydrides, alkyl groups, phosphines, cysteinyl thiols and the thioether of methionine. Cu(II) is an intermediate Lewis acid that forms bonds with sulfates, nitrogen donors such as histidine, and oxygen donors such as glutamate and aspartate. The ability of copper to redox cycle between Cu(I) and Cu(II) endows copper-containing enzymes with redox potentials typically between +0.25 and +0.75 V,[3] permitting the removal of electrons from diverse substrates such as catechols, superoxide, ascorbate and iron. Consequently, copper-dependent enzymes across kingdoms function in diverse processes including oxidative phosphorylation (cytochrome oxidase),[4] iron homeostasis (ceruloplasmin, hephaestin),[5] pigmentation (tyrosinase; laccase),[6] superoxide dismutation (superoxide dismutases),[7] and connective tissue formation (lysyl oxidases).[8]

Mechanisms of bacterial copper toxicity

Despite the utility of copper as an enzymatic cofactor, its redox activity also creates a potential hazard to all life. Copper toxicity was probably an early and constant evolutionary pressure as suggested by the presence of homologous copper tolerance proteins within distantly related organisms such as bacteria and archaea.[9] Several mechanisms have been ascribed to the toxic properties of copper. Under aerobic conditions, copper is

[a] *Department of Biochemistry, University of Missouri, Columbia, MO, 65211, USA. E-mail: petrism@missouri.edu; Fax: +1-573-884-2537; Tel: +1-573-882-9685*
[b] *The Christopher S. Bond Life Sciences Center, University of Missouri, Columbia, MO, 65211, USA*
[c] *Department of Nutrition and Exercise Physiology, University of Missouri, Columbia, MO, 65211, USA*

proposed to catalyze the production of hydroxyl radicals *via* the Fenton and Haber–Weiss reactions:[10]

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + OH^- + {}^{\bullet}OH$$

$$Cu(II) + {}^{\bullet}O_2{}^- \rightarrow Cu(I) + O_2$$

Because of the high standard reduction potential of the hydroxyl radical (>2 V),[11] copper-catalyzed production of $^{\bullet}OH$ is able to cause oxidative damage to most types of macromolecules.[11,12] Because of its very short half-life ($\sim 10^{-9}$ s), such damage would necessarily be diffusion-limited and thus restricted to macromolecules within the immediate vicinity of copper. Consistent with this notion, copper-mediated hydroxyl radical formation in *Escherichia coli* appears to be confined to the periplasmic regions where copper is most highly enriched.[13] However, studies suggest that in the absence of oxygen, various non-Fenton based mechanisms are more important processes of copper toxicity.[14] Such anoxic mechanisms involve the formation of adventitious Cu(I)–thiolate bonds, thus damaging enzymes that functionally depend on free cysteines or disulfide bonds.[15] In *E. coli*, targets of anoxic copper toxicity include families of iron sulfur cluster proteins that are required for intermediary metabolism.[14] In addition, excess copper is thought to lead to incorrect disulfide bond formation in the periplasm, as evidenced by studies showing that loss of the periplasmic disulfide bond isomerase, DsbC, which resolves incorrect disulfide bonds, renders *E. coli* more sensitive to copper toxicity.[15]

Mechanisms of microbial copper tolerance

To avoid copper toxicity, all organisms have evolved copper handling machinery to maintain a cytoplasmic milieu that is devoid of free copper. This concept was initially based on studies of the *E. coli* copper-responsive transcription factor CueR.[16] The finding that CueR induced the expression of copper tolerance genes at 10^{-21} M copper, which is many orders of magnitude lower than one free copper atom per cell, indicated that free unligated copper in the cytoplasm is not tolerated in bacteria. The principle mechanisms of copper tolerance in bacteria include: (1) transmembrane copper export, occurring from the cytoplasm into the periplasmic space or into the extracellular milieu; (2) copper sequestration by metallothioneins; and (3) oxidation of Cu(I) by multi-copper oxidases to generate the less toxic Cu(II) ion. Below is a general description of these mechanisms and their importance in bacterial virulence.

Copper export. While a number of different types of copper export proteins have been identified in bacteria, the most ubiquitous are the copper exporting P_{1B}-type ATPases. Examples of these proteins include CopA of *E. coli*, CtpV of *Mycobacterium tuberculosis*, CopA1 and CopA2 of *Pseudomonas aeruginosa* and CopA and GolT of *Salmonella typhimurium.* The principle function of these proteins is to prevent the cytoplasmic accumulation of copper by harnessing the energy derived from ATP hydrolysis to pump Cu(I) across the cell membrane.[9] In the case of Gram-positive bacteria, copper is exported across the plasma membrane,[9] whereas in Gram-negative bacteria, copper is exported across the inner membrane to the periplasmic space.[17] Signature elements of this family include cytoplasmic actuator-, nucleotide binding-, and phosphorylation domains, which are connected to six membrane-spanning regions.[18] Metal binding motifs, which typically include one or more Cys–X_2–Cys sequences, are located within the cytoplasmic amino terminal domain of these proteins. Other metal coordinating residues include His–Pro or Cys–Pro–Cys/His motif resides within the 6th membrane-spanning helix as well as YN and MXXS in other transmembrane segments.[17,19] The delivery of copper to these exporters is facilitated by copper chaperones, which are thought to interact electrostatically with the cytoplasmic amino terminal copper-binding domain of their cognate P_{1B}-type ATPases.[20] Although most copper chaperones are soluble proteins (*e.g.*, the CopZ protein of *Enterococcus hirae*), a recently discovered example of a membrane-bound variety is CupA, which has been found in *lactobacilli* and *streptococci* lacking a CopZ-like soluble metallochaperone.[21] CupA is also atypical in the use of a novel cupredoxin-like fold for copper binding, rather than the canonical ferredoxin-like fold present in the CopZ-like soluble metallochaperones.[21] In addition to their role in reducing copper concentrations, P_{1B}-type ATPases may also serve as a mechanism of copper delivery to periplasmic cuproproteins including cytochrome oxidase.[22] The CopA and GolT ATPases of *S. typhimurium*, are also necessary for copper delivery to the periplasmic superoxide dismutase, SodCII.[23] Copper delivery to SodCII is also dependent on the copper-binding protein CueP, suggesting a model in which copper is transferred from CopA/GolT to CueP for insertion into SodCII.[23]

While all bacteria appear to possess at least one copper exporting P_{1B}-type ATPase, there is considerable diversity when it comes to alternative copper exporters among different bacteria. The *E. coli CusABC* complex is a large tripartite copper exporter found in the majority of gamma proteobacteria.[24] Studies in *E. coli* demonstrate that the CusABC complex and its metallochaperone, CusF, mediates copper export across the inner and outer membranes *via* proton motive force,[25,26] and is required for tolerance to moderately high copper concentrations, especially under anaerobic conditions.[25] Interestingly, recent studies show that CusF acquires copper directly from the CopA suggesting it can function as a periplasmic target of this Cu(I)–ATPase.[27] The Cus complex is comprised of an inner membrane proton–substrate carrier (CusA) and an outer membrane pore (CusC), which are connected by a linker protein, CusB in the periplasm.[28–30] Recent studies suggest that copper-bound CusB facilitates cuprous ion delivery from the CusF metallochaperone to the CusABC complex.[31,32] In the case of mycobacteria, copper export is dependent on the P_{1B}-type ATPase, CtpV, located within the inner membrane,[33] as well as MctB a pore-forming protein originally identified in the outer or inner membrane,[34] although its precise role and function remain unknown. Mutation in either CtpV or MctB results in reduced copper tolerance due to hyperaccumulation of the metal.

There have been additional plasmid-encoded copper tolerance proteins identified in certain bacteria isolated from environments with extremely high copper concentrations. The *pcoABCDRSE* system of *E. coli* was initially discovered in a plasmid pRJ1004 within bacterial isolates from pigs fed a copper-supplemented diet.[35] A homologous system, *copABCDRS*, was later found in plasmid pPT23D isolated from *Pseudomonas syringae* growing on tomato plants treated with copper-based fungicides.[36,37] The *pco* and *cop* systems share four structural genes, *pco*/*copABCD*. PcoA and CopA are soluble periplasmic proteins with homology to multi-copper oxidases,[38] and may function in a similar manner to the chromosomally encoded CueO multi-copper oxidase (see below). PcoB/CopB are located in the outer membrane with putative roles in copper translocation. CopC/PcoC are copper binding proteins located in the periplasm and may deliver copper to PcoD/CopD proteins located in the inner membrane.[39,40] There is genetic evidence that PcoD may function in copper transport into the cytoplasm,[41] however, this would appear to be at odds with its role in copper tolerance and remains to be demonstrated biochemically.

Copper sequestration. Yet another mechanism of copper tolerance in bacteria involves sequestration by cysteine-rich metallothioneins. Although commonplace in eukaryotes, metallothioneins are relatively rare in bacteria. The best characterized of these proteins is MymT of the *Mycobacteriaceae* family that has been shown to confer copper tolerance in *Mycobacterium tuberculosis* and protection against reactive oxygen species.[42] The periplasmic CusF protein of *E. coli* may similarly function as a copper buffer in addition to its role in copper delivery to the CusBC complex for export across the outer membrane.[26]

Recently, a novel mechanism of copper tolerance was identified in studies of yersiniabactin, a siderophore produced in *Yersinia* species and found in *E. coli* isolates from patients with urinary tract infection.[43] Siderophores are small high-affinity iron chelating compounds secreted by microorganisms to scavenge iron within the host. Although required for iron acquisition, yersiniabactin is also capable of binding Cu(II); an interaction that confers copper tolerance by preventing the formation of the toxic Cu(I).[43] In addition, recent studies using yersiniabactin-expressing *E. coli* demonstrated that the copper-bound siderophore possesses superoxide dismutase activity, which protects against the respiratory burst of macrophages.[44] Thus, it would appear that yersiniabactin is a highly versatile siderophore capable of counteracting multiple host defenses including iron limitation, copper toxicity and the oxidative burst.

Multi-copper oxidases. The periplasm contains the most numerous and diverse copper-dependent enzymes in bacteria. Accordingly, this compartment is most at risk of copper-induced damage, which is exacerbated under anoxic conditions.[13] To reduce the potential for damage by copper, bacteria have evolved multi-copper oxidases that protect against copper toxicity. Members of the multi-copper oxidase family typically contain four copper atoms, each of which participates in the single electron oxidation. Oxygen is reduced to water to complete the reaction cycle. Despite their common structure, multi-copper oxidases exhibit a considerable diversity of substrates including iron, phenols, diamines, catecholates and ascorbate. Bacterial multi-copper oxidases that are known to confer copper tolerance include CueO in *E. coli* and *S. typhimurium*, and MmcO in *M. tuberculosis*.[45–47] However, the mechanism by which these proteins confer copper tolerance is not fully understood. CueO of *E. coli* exhibits cuprous oxidase activity, which is thought to protect against the toxic effects of Cu(I) by increasing its conversion to Cu(II).[45,46] In addition, CueO has been shown to oxidize the catecholate groups of 2,3 dihydrobenzoic acid, a precursor to the iron scavenging siderophore, enterobactin.[48] Because enterobactin is known to sensitize *E. coli* to copper through its ability to act as a copper reductant,[48] CueO-dependent oxidation of enterobactin may be an additional mechanism of preventing the generation of toxic cuprous ions, albeit at the expense of the iron scavenging activity of the siderophore. As discussed below, this function of the CueO protein may be specifically required during host infection to defend against copper toxicity used by the innate immune response.

Regulation of copper tolerance gene expression. The expression of bacterial copper tolerance genes is typically increased under excess copper concentrations *via* the action of copper-sensing transcription factors. The CueR transcription factor in *E. coli* and *S. typhimurium* mediates the copper-induced expression of *cueO* and *copA* genes as well as *cueP* (in *S. typhimurium*).[49–51] *S. typhimurium* also possesses a second transcription factor GolS, which is responsive to copper (and gold) and induces the expression of GolT, a second P_{1B}-type ATPase involved in copper export into the periplasm.[52,53] The *E. coli cusCFBA* operon is regulated by a two-component signal transduction system involving the periplasmic CusS copper sensor and the CusR transcriptional regulator.[54] In certain bacteria, inhibition of a transcriptional repressor by high copper concentrations enables the increased expression of copper tolerance genes. This derepression mechanism underlies the activity of CsoR found multiple bacterial specifies including *M. tuberculosis*, *Bacillus subtilis*, *Corynebacterium glutamicum* and *Staphylococcus aureus.*[55–58] *M. tuberculosis* also contains a second transcriptional repressor, RicR, that is derepressed by elevated copper concentrations to increase expression of copper tolerance proteins MymT (a metallothionein), and Mmco (a multi-copper oxidase).[59,60]

Copper tolerance as a determinant of virulence. Several lines of evidence from both host and pathogen support a general model in which the host innate immune system uses copper to kill invading pathogens (Fig. 1A). Copper-regulated genes such as *ctpV* in *M. tuberculosis* and *copA* in *S. typhimurium* are induced upon phagocytosis by macrophages, suggesting that bacteria are exposed to elevated copper levels within the host phagosome.[61–63] Other studies suggest that, in general, bacterial virulence is attenuated by mutations that cause copper sensitivity, particularly mutations that affect the copper transporters. For example, copper hypersensitivity caused by loss of the CtpV copper exporter renders *M. tuberculosis* less virulent in both mice and guinea pig models of infection.[64] Similarly, mutation of the *mctB* gene of *M. tuberculosis* results in a marked decrease in copper tolerance as well as attenuated virulence in both mice and guinea pig models of lung infection.[33,34] However, when it comes to mutations in other copper handling genes of *M. tuberculosis* that

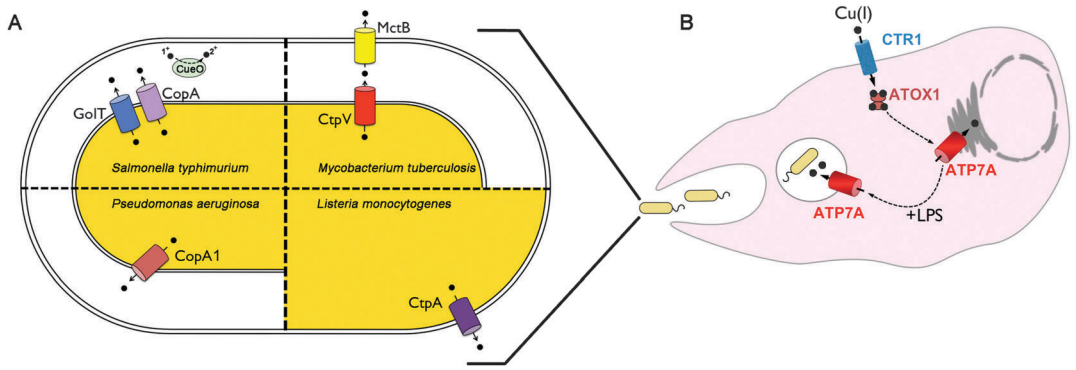

Fig. 1 Model of copper homeostasis at the host–pathogen interface. (A) Copper handling proteins are shown in various species of gram negative and gram positive bacteria that have been shown to be required for both copper tolerance and survival within either cultured macrophage cells or animal hosts. (B) Model of copper-mediated bacterial killing in macrophages. Inflammatory agents such as lipopolysaccharide released from invading bacteria induce the expression of the CTR1 copper importer, which mediates copper uptake across the plasma membrane. The copper chaperone ATOX delivers copper to the ATP7A copper pump in the trans-Golgi network. ATP7A undergoes partial relocalization from the Golgi to phagolysosomes, loading bactericidal copper into this compartment.

also cause copper hypersensitivity, not all correlate with reduced virulence *in vivo*. For example, loss of the MymT metallothionein, or the MmcO multi-copper oxidase both cause hypersensitivity to copper, however, neither mutation reduces virulence of *M. tuberculosis* in mice.[42,47] The underlying basis for differences in virulence between different copper sensitive mutant strains is currently unclear, however, it is possible that within the host, alternative pathways of copper tolerance may compensate for the loss of specific genes. Consistent with this concept, recent studies have demonstrated mutation of the entire copper responsive *ricR* regulon was required to attenuate virulence of *M. tuberculosis*, whereas mutation of individual *ricR* target gene targets alone was without effect.[60]

While less information is available for other bacterial species, in general there is a positive correlation between copper sensitivity and virulence (Fig. 1A). Mutations in the *P. aeruginosa copA1* gene (formerly *cueA*) renders this pathogen sensitive to high copper concentrations when grown *in vitro*, and significantly attenuates virulence as determined by bacterial colonization of the spleen in mice, and the number of bacteria required to kill mice.[65] Similarly, mutation of the CtpA copper exporting P-type ATPase in *Listeria monocytogenes* significantly reduces liver colonization of in mice,[66] and loss of the CopA copper exporting P-type ATPase of *Streptococcus pneumonia* reduces colonization of the lung and nasopharynx of infected mice compared to wild type strains.[67] In *S. typhimurium*, loss of both the CopA and GolT copper exporting P-type ATPases causes copper hypersensitivity and reduces bacterial survival within cultured macrophages.[63] However, such mutations did not affect tissue colonization using a systemic infection model in mice.[63] In contrast, loss of the CueO multi-copper oxidase in *S. typhimurium* was found to reduce colonization of the lung and spleen following orogastric infection of mice, however, there was no effect on bacterial survival in cultured RAW264.7 macrophage cells.[68] Taken as a whole, these studies indicate a strong, albeit imperfect, correlation between copper tolerance and virulence in the host.

Host-derived copper as a mechanism to control infections

Copper is important for both adaptive and innate immune function. Human infants with the copper deficiency disorder, Menkes disease, exhibit higher incidences of lung and bladder infection.[69–72] Copper deficiency in animals has been shown to impair the production of antibody-producing cells,[73] suppress the respiratory burst of neutrophils and macrophages,[74,75] and limit the ability of the host to combat infection.[76–81] It has been known for decades that infection or inflammation induces marked changes in copper homeostasis in the host. For example, copper concentrations in the serum are significantly elevated in response to inflammation.[82–93] This is due, in part, to the increased production and secretion of ceruloplasmin, an acute phase response protein which contains approximately 85% of total serum copper.[94] Copper accumulates at sites of inflammation and injury,[95] including within granulomatous lung tissues of guinea pigs infected with *M. tuberculosis*.[34] While the underlying mechanisms by which copper deficiency limits the ability to fight infection are not fully understood, a supply of copper to the phagocytic cells of the innate immune system appears to be of particular importance. Copper concentrations are known to accumulate within the phagolysosomal compartments of interferon-gamma activated peritoneal macrophages challenged with different mycobacteria species.[96] Insight into the underlying molecular basis of these changes came from studies of RAW 267.4 macrophage cells in which activation with interferon-gamma or bacterial lipopolysaccharide (LPS) was found to stimulate copper uptake by increasing the expression of the copper importer, CTR1[97,98] (Fig. 1B). Moreover, these same inflammatory conditions triggered the increased expression and trafficking of the ATP7A copper pump from the Golgi complex to cytoplasmic vesicles and the phagolysosome, thus providing a possible mechanism for concentrating bactericidal copper within this compartment during infection[97] (Fig. 1B). Consistent with this model, studies have demonstrated the ability of RAW264.7 cultured macrophages to kill *E. coli* was found to be dependent on the expression of ATP7A.[97] It will be important to test the extent to which ATP7A is required for killing other bacterial pathogens and whether it plays a significant role in whole animal studies of infection.

Concluding remarks

Whereas nutritional immunity is a term that has been historically applied to metals such as iron and manganese that are withheld from invading pathogens, the unique roles of copper in host immune defense now expand this concept to encompass nutrient intoxication. There are several outstanding questions to be addressed in the coming years: What is the mechanism by which host-derived copper kills bacteria? Why do certain mutations cause copper sensitivity in bacterial pathogens without affecting virulence? What is the role of copper-containing ceruloplasmin in host immune function and does this protein provide a means of mobilizing systemic copper to sites of infection? Can drugs that are designed to inhibit bacterial copper tolerance proteins, or enhance copper delivery to the pathogen,[99,100] give rise to new classes of antibiotics? To what extent is host-derived copper effective against non-bacterial pathogens? Does the widespread use of copper as a dietary supplement in livestock contribute to the virulence of enteric pathogens that may enter the human food supply?[101] The answers to such questions will require multidisciplinary approaches to unravel the genetic and physiological basis of copper handling pathways within both host and pathogen.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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