Published in final edited form as:
Curr Opin Chem Biol. 2014 April ; 0: 59–66. doi:10.1016/j.cbpa.2013.12.021.

Recent Developments in Copper and Zinc Homeostasis in Bacterial Pathogens

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Abstract

Copper and zinc homeostasis systems in pathogenic bacteria are required to resist host efforts to manipulate the availability and toxicity of these metal ions. Central to this microbial adaptive response is the involvement of metal-trafficking and -sensing proteins that ultimately exercise control of metal speciation in the cell. Cu- and Zn-specific metalloregulatory proteins regulate the transcription of metal-responsive genes while metallochaperones and related proteins ensure that these metals are appropriately buffered by the intracellular milieu and delivered to correct intracellular targets. In this review, we summarize recent findings on how bacterial pathogens mount a metal-specific response to derail host efforts to win the "fight over metals."

Metal Ions at the Host-Pathogen Interface

Strict control of the homeostasis of transition metal ions is essential to all forms of life. The cellular balance of metal ions is orchestrated by proteins and small molecules, and when cellular physiology is disrupted by aberrant metal metabolism, human disease can occur [1]. This need for cellular control of metal homeostasis is exploited by the innate immune system during a bacterial infection. Here, the host attempts to restrict the availability of essential nutrients in a process generally termed nutritional immunity [2, 3•, 4] while inundating the bacterial cell with a wide range of toxic insults, including low pH, reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive chlorine species (RCS), and hydrolases [5,6]. A key aspect of this assault is an extensive perturbation of the availability of the four major transition metals required by the bacterium: iron (Fe), manganese (Mn), zinc (Zn), and copper (Cu). In contrast to host processes that attempt to limit a pathogen's access to Fe and Mn, recent work reveals that high Cu concentrations are

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Note added in proof

Although we focus on mechanisms of intracellular Cu resistance in this review, a recent paper (Chaturvedi *et al.*) shows that the extracellular siderophore, yersiniabactin (Ybt), protects intracellular *Escherichia coli* from copper-mediated killing in phagocytes as a result of a catalytic superoxide dismutase-like activity of Cu(II)-Ybt complexes.

Chaturvedi KS, Hung CS, Giblin DE, Urushidani S, Austin AM, Dinauer MC, Henderson JP: Cupric yersiniabactin is a virulence-associated superoxide dismutase mimic. *ACS Chem Biol* 2014, in press (doi: 10.1021/cb400658k).

used to kill microbial invaders, particularly intracellular pathogens (Figure 1) [7,8]. For Zn, the work taken collectively supports a role for both host-mediated toxicity [4,7] and sequestration [3•,9] as a means to restrict pathogen viability upon host infection. Although these microbial defense mechanisms disrupt transition metal homeostasis of most bacteria [3•,4,9], successful pathogens have evolved mechanisms of adaptation to these perturbations [4,10,11].

Molecular Basis of Cu(I) and Zn(II) Toxicity

Cu and Zn speciation is defined by the types of ligands encountered in the cell [12,13], while metal specificity is collectively dictated by metal coordination number and geometry, the rates of exchange in and out of metal complexes, and redox state (valence) [14]. On the basis of the binding affinities of selected targeting, trafficking, and metal-sensing proteins [12,15,16], Cu(I) is thought to be buffered by a typical cell in the attomolar range, while Zn(II) is buffered in the nanomolar [17•] to picomolar [16] range; however, these values may vary for different bacteria. As a rule of thumb, chelate binding affinities for Cu(II) and Zn(II) are generally higher than for earlier first-row divalent transition metals for a given ligand, a trend known as the Irving-Williams series for divalent ions (Figure 1) [18]. The bioavailability of Cu and Zn is therefore generally low and inversely proportional to competitiveness relative to other first-row metals, which dictates that their availability in cells be tightly regulated. Further, the major redox state of copper is monovalent Cu(I) in the bacterial cytoplasm due to the low reduction potential maintained by low-molecular-weight thiols relative to the Cu(II)/Cu(I) redox couple (−0.22V and +0.15V, respectively, relative to the normal hydrogen electrode) [13]. The sulfur-containing amino acids cysteine and methionine play important roles as soft bases that readily coordinate the soft acid Cu(I). These properties make unregulated Cu(I) highly toxic as evidenced by the ability of Cu(I) to mediate disassembly of iron-sulfur (Fe-S) clusters leading to dysfunctional cellular metabolism [19, 20]. The vulnerability of Fe-S clusters to Cu(I) remains to be validated as a general mechanism of Cu toxicity in other bacteria, particularly those that lack significant Fe-S cluster-containing proteins in their metallomes [21]. Our molecular-level understanding of intracellular zinc toxicity is far less clear, although a model invoking mismetallation of metalloenzymes through competition is a reasonable, albeit largely untested one [22].

A second potential impact of copper toxicity is the chemistry of Cu(I) with host-mediated hydrogen peroxide (H_2O_2) or superoxide ($O_2^{-\bullet}$). Labile Fe(II) is accepted as a major source of intracellular oxidative damage in cells given its ability to heterolytically cleave H_2O_2 to form reactive hydroxyl radical OH• and oxidized Fe(III); this process becomes catalytic in the presence of cellular reductants [23,24]. Although uncomplexed Cu redox cycles faster than Fe *in vitro* [24–26], the degree to which Cu(I)-catalyzed Fenton chemistry is relevant *in vivo* remains uncertain due to the lack of a comprehensive understanding of Cu(I)-ligand speciation in the cell and how Cu(I)-complexes are modulated by myriad toxic insults at the host-pathogen interface.

Cu Sensing and Trafficking

Many pathogens accumulate micromolar levels of cell-associated Cu [12,22] despite possessing little or no clearly defined cytoplasmic need for the metal [27]. For Gram-negative bacteria, it is presumed that much of this Cu localizes to the periplasm and is bound to essential cuproproteins, although some pathogenic bacteria additionally harbor Cu(I) sequestration proteins in the cytoplasm [28,29]. Once inside the cell, bacterial copper chaperones generally represent a first line of defense against Cu(I) toxicity imparted by the host (Figure 2) [11]. The founding bacterial metallochaperone is *Bacillus subtilis* CopZ, which is structurally identical to Atx1 initially characterized in yeast (Figure 3a) [30]. CopZ adopts a ferredoxin-like fold where Cu(I) forms a *bis*-thiolato digonal coordination complex with a Cys-X-X-Cys loop sequence (where X is any amino acid) that is additionally capable of coordinating a small molecule from solvent. Metallochaperones buffer highly competitive Cu(I) to low levels in the cytoplasm [31] and shuttle Cu(I) to intracellular targets including cytoplasmic Cu(I) sensors and to Cu(I)-specific P-type ATPase effluxers with rapid exchange kinetics through an associative, ligand exchange mechanism that prevents release of Cu(I) into bulk solution (Figure 2) [10,32].

A new perspective on Cu(I) trafficking has been reported for the Gram-positive respiratory pathogen *Streptococcus pneumoniae* [33••]. This work reveals that the ancient cupredoxin fold [34] (Figure 3), known to play prominent roles in electron transfer and bacterial respiration, has been co-opted to function as a novel plasma membrane-anchored Cu(I) chaperone (CupA) that is capable of delivering Cu(I) to the N-terminal metal-binding domain (MBD) of the Cu(I)-effluxer CopA ($CopA_{MBD}$) [33••]. This Cu(I) transfer is thermodynamically favorable and flows from the low affinity S2 site on CupA to the high affinity S1 site on CopA, presumably via transient docking of electrostatically complementary domains that are otherwise isostructural and harbor identical binuclear Cu(I) binding sites (Figure 3b). Unlike soluble Cu(I) chaperones in other bacteria, CupA is essential for cellular copper resistance. This suggests either an obligatory transfer of Cu(I) from CupA to CopA for efflux (both Δ*copA* and Δ*cupA* strains accumulate excess copper), and/or CupA plays an as yet unknown function in Cu(I) delivery to other cellular targets, *e.g.*, the copper sensor CopY or other unknown targets [10]. In other bacteria, such as *Listeria monocytogenes*, loss of the copper chaperone has little impact on copper resistance or metallation of downstream targets when copper is replete. In these cases where Cu(I) chaperones are nonessential, the bioavailable pool of Cu(I) remains adequately regulated, potentially by other metal-binding proteins or by small molecules, *e.g.*, glutathione (GSH, see Figure 2) [31, 35•].

In some bacterial pathogens, a Cu(I) chaperone has not yet been identified, a noteworthy example of which is *Mycobacterium tuberculosis*. However, this pathogen encodes two Cu(I) metallosensors and a cytoplasmic Cu metallothionein (MymT) that provides resistance to cytoplasmic Cu toxicity (Figure 2) [28,36,37]. CsoR is the founding member of a large family of repressors that adopt an all α-helical dimer of dimers architecture in solution and represses transcription in the apo state [38]. In the presence of elevated copper, CsoR binds four equivalents of Cu(I) per tetramer in a trigonal planar coordination geometry, which in turn leads to derepression of the *cso* operon (Figure 3c). The specificity of the Cu(I)

response appears to involve a second coordination shell of hydrogen bonding interactions and kinking of the long α2 helix, resulting in dissociation from the DNA via an as yet unknown structural mechanism [36,38,39]. A model of two CsoR tetramers bound to a single DNA operator has recently been reported for *Streptomyces lividans* CsoR and involves conserved residues that diagonally traverse an electropositive patch on one face of each tetramer (Figure 3c) [39,40]. *S. lividans* CsoR is most closely evolutionarily related to a second CsoR-like Cu(I) sensor in *M. tuberculosis*, regulated in copper repressor (RicR), which may play a more significant role in copper resistance than CsoR itself [37]. Similar sensor duality is also observed in the MerR family of transcriptional activators in *Salmonella* [41]. Why do two Cu(I) sensors of the same structure exist in the same cell? One hypothesis is that this arrangement, in tandem with Cu(I) chaperones, allows the evolution of multiple saturation “set points” of Cu(I) induction or finer combinatorial control of the copper stress response (Figure 2) [31,42•].

Copper Sensing without Cu(I) Binding?

It has recently been proposed that direct Cu(II) detection by the multiple antibiotic resistance regulator (MarR) in the *E. coli* cytoplasm underlies the microbial stress response to a range of antibiotics [43••]. In this work, Hao *et al.* propose that Cu(II) elicits transcriptional derepression of the MarR regulon via oxidative disulfide linkage of two MarR dimers into an inactive tetramer. Evidence was presented in this study that antibiotics lead to a change in Cu(I) speciation detectable by a fluorescent Cu(I) indicator, but intracellular Cu(II) was not directly detected. The mechanism of MarR oxidation by Cu(II) in the cell is not known, but could perhaps result from a thiol-disulfide redox imbalance where transient accumulation of copper-bound, oxidized glutathione (Cu(II)-GSSG) and/or Cu(II)-$MarR_2$ occurs as a result of redox cycling facilitated by molecular oxygen (O_2) [44]. Recent experiments suggest that antibiotic stress can occur in the absence of O_2 indicating that more than one mechanism of toxicity could be operative in bacteria [45,46]. In any case, this study connects perturbation of cellular Cu speciation to antibiotic stress, which may impact the metallation state of cuproproteins, *e.g.*, Cu,Zn-superoxide dismutase, in the extracytoplasmic compartment [47•].

Zn Homeostasis and the Host Response

Bacterial zinc homeostasis differs markedly from copper resistance in that bacteria have evolved both import and export mechanisms to strictly maintain accessible Zn(II) levels for zinc-requiring proteins and enzymes that function in diverse metabolic processes in the cell [48]. The need for sufficient bioavailable Zn(II) to supply a significant cellular demand while limiting Zn toxicity is maintained by the acquisition of zinc via ATP-binding cassette (ABC) transporters and cytoplasmic efflux through P-type ATPases or proton-coupled antiporters (Figure 4) [49,50••]. Therefore, host strategies to limit bacterial infection that exploit either zinc sequestration or zinc toxicity to override zinc homeostasis of microbial pathogens can be envisioned (Figure 1) [4]. The discovery of the involvement of neutrophil-derived calprotectin (CP) in metal homeostasis [9] and elucidation of its two metal-binding sites (S1 and S2) by structural and functional studies (Figure 3d) [51••,52••], as well as related calcium-activated S100 proteins [53], has brought into sharp focus the degree to

which the host and bacteria participate in a “tug-of war” over Zn(II) and Mn(II). The ability of CP to chelate Mn(II) and Zn(II) represents a powerful antimicrobial weapon [9, 52]; however, two important Gram-negative respiratory pathogens, *Acinetobacter baummanii* [54•] and *Neisseria meningitidus* [55••] have been shown to overcome the CP defense response. These pathogens express an outer membrane receptor under Zn(II) deplete conditions, which in the case of *Neisseria* is proposed to bind Zn(II) [or Mn(II)]-bound CP directly in order to strip the metal from CP, analogous to what happens in bacterial iron “piracy” from transferrin [56]. Interestingly, *Salmonella enterica* also has the capability to out-compete CP for zinc in the gut via the high-affinity ZnuABC transport system, providing another illustration of how bacteria adapt to extracellular host defense strategies [57].

Zn Sensing and Trafficking

The extraordinary control of intracellular Zn(II) bioavailability is maintained by pairs of zinc sensors whose DNA operator-binding or activation functions are allosterically modulated by the direct binding of Zn(II) [12,16]. *Uptake repressors* facilitate repression of genes encoding uptake transporters upon zinc binding while *efflux regulators* control the expression of genes encoding zinc efflux systems upon zinc binding, either via transcriptional depression or activation [58] (Figure 4). The zinc specificity of these “allosteric switches” [58] is defined by the metal coordination environment and metal-induced structural changes that drive the appropriate allosteric response; in some single domain repressors a second-sphere hydrogen-bonding network appears to physically connect the metal- and DNA-binding sites [48,59••], analogous to CsoR discussed above (Figure 3c). For example, disruption or destabilization of this network in the zinc efflux repressor *Staphylococcus aureus* CzrA produces variable degrees of uncoupling of the allosteric response without effecting the binding free energies of either ligand (Zn(II) or DNA) [59••]. Another example involves the MarR-family zinc uptake repressor AdcR (adhesin competence regulator) from *S. pneumoniae* that features a second-coordination shell in the Zn(II)-bound state that is postulated to play an important role in allosteric coupling (Figure 3e) [60,61]. In addition to allostery, the roles of multiple metal-binding sites in multidomain repressors has been suggested to impart differential set points for expanding the dynamic range of sensing Zn(II) based on new crystallographic data of the zinc uptake repressor (Zur) that is proposed to be controlled by two functional sites (S1 and S2) (Figure 3f) [42•, 62].

Although *bona fide* intracellular Zn(II)-specific chaperones have yet to be positively identified in bacteria, an unbiased mutant screen of *A. baumannii* stressed with CP-induced Zn(II) limitation identified components of the *zur* regulon, including a gene encoding a COG0523 family member [54•]. GOG0523 proteins are putative P-loop GTPases, one of which is a known Ni(II)-chaperone for urease in the pathogen *Helicobactor pylori*, [63] suggesting a role for this family of proteins in Zn(II) homeostasis [64,65•]. In addition to potential *bona fide* Zn(II) chaperones, copper chaperones may moonlight as Zn(II) binding proteins *in vivo* [66]. For example, the Zn(II)-mediated heterocomplex formed between Atx1 and the MBD of the PacS Cu(I)-transporting ATPase is significantly more stable than the corresponding Cu(I) complex [67]. Exchange of Cu(I) and Zn(II) on metalloregulators has

also been proposed to occur under both unstressed and zinc-stressed growth conditions providing further support for the idea that cells must carefully control inappropriate access to these highly competitive metals [35•,66].

Conclusions and Perspectives

Transition metal ion homeostasis in pathogenic bacteria is characterized by the strict control of intracellular Cu(I) and Zn(II) speciation by metallosensors, metallochaperones, and metal transporters and is central to the adaptive response to host-mediated metal toxicity or restriction. The discovery and characterization of new bacterial copper- and zinc-trafficking proteins and host proteins that function in “metal-centric” nutritional immunity promises to provide further insights into this emerging aspect of the host-pathogen interface. The development of new methods to probe metal ion speciation in intact cells [68], particularly for spectroscopically challenged d^{10} metal ions Zn(II) and Cu(I) discussed here, remains a significant challenge, with low-throughput biochemical fractionation methods still state-of-the-art [69•]. The development of new synthetic and genetically encoded metal sensors, coupled with new biophysical/analytical methodologies that allow both temporal and spatial measurement of total metal and protein identification is certain to help unravel the complexities of transition metal homeostasis in liquid culture and in infected tissues [70•]. Obtaining such a sophisticated understanding of the dynamic *in vivo* metallome will further direct *in vitro* structural and functional studies, which in turn, may allow the rational design of new antibiotics that could provide a significant advantage to the host cell in the “fight over metals”.

Acknowledgments

Studies carried out in the Giedroc laboratory on bacterial metal homeostasis are supported by funding from the US National Institutes of Health (R01 GM042569 to D.P.G).

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Highlights

- The “fight over copper and zinc” at the host-pathogen interface is discussed.
- Proteins involved in copper and zinc trafficking and sensing are discussed.
- Cellular speciation of metals remains a significant challenge to address.
- Characterizations of new host and bacterial players promises new biological insights.

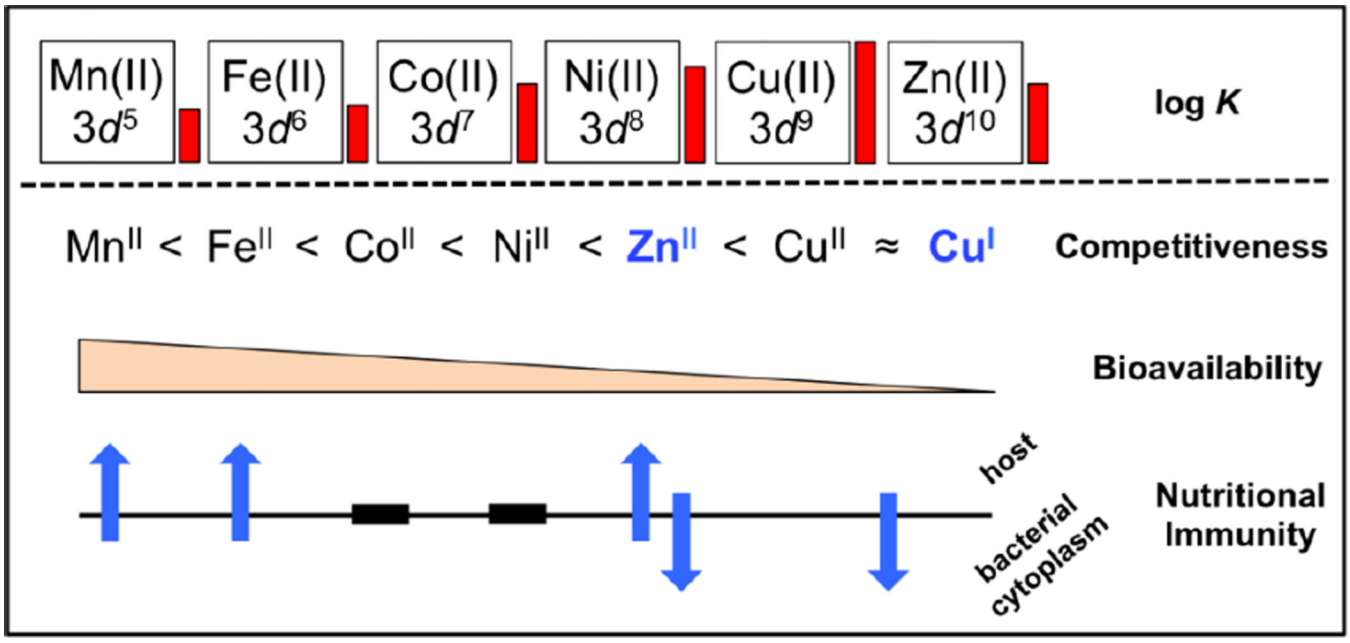

Figure 1.

Bioinorganic chemistry at the host-pathogen interface. Cu(II) has the highest affinity for a given ligand compared to other first row transition metals as exemplified by the height of the *red* bars which depict the NIST approved log *K* values relative to the Cu(II)-aspartic acid complex [Cu(II)-aspartic acid, log *K* = 8.9]. This empirical relationship is described as the Irving-Williams series and relates to the relative competitiveness of first-row transition metals in a cellular environment [18]. Cu(I) predominates in the cytoplasm and like Zn(II), forms high-affinity complexes with softer acids (histidine, cysteine, methionine), and is therefore also considered a highly competitive metal. Bioavailability is inversely proportional to competitiveness and has been roughly approximated on the basis of the relative binding affinities of metal-dependent transcriptional regulators [16]. Metal-centric nutritional immunity is defined as the host's attempt to both sequester metal ions from cells (*upward-facing blue arrows*) and/or bombard the bacterial cytoplasm with metal-ion stress (*downward-facing blue arrows*). Roles of Co and Ni in nutritional immunity are not yet known (*black bars*).

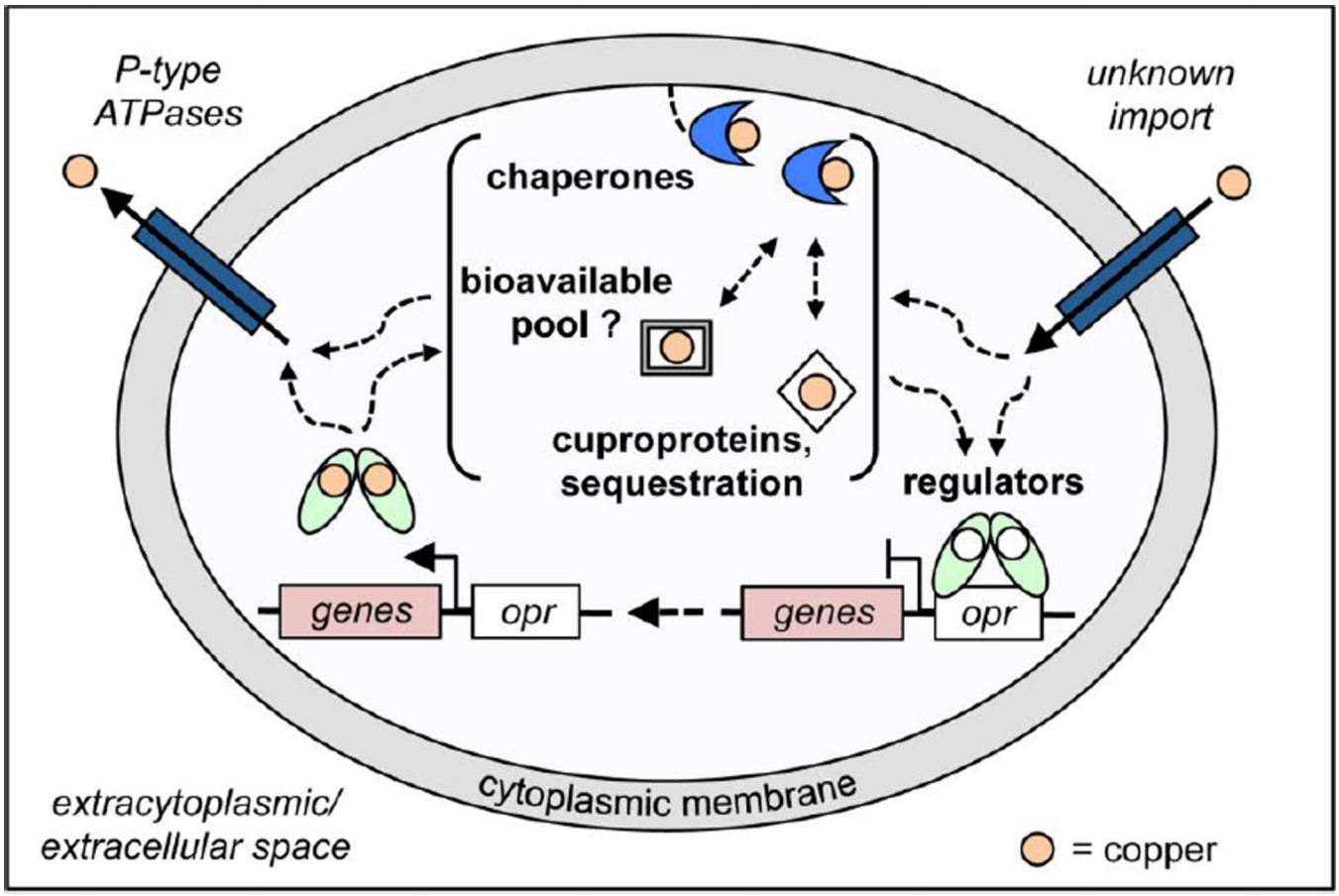

Figure 2.

Overview of copper sensing and trafficking within the bacterial cytoplasm. Copper enters the cytoplasm through largely unknown mechanisms. Copper speciation within the cell depends on the relative concentrations of Cu(I) bound to the bioavailable pool, *e.g.*, copper bound to low-molecular-weight thiols, cytoplasmic binding proteins, *e.g.*, MymT [28] and CutC [29]), chaperones, and Cu(I) sensors. The thermodynamics and kinetics of Cu(I) speciation remain incompletely understood and may be dictated by the concentrations at which copper homeostasis proteins become saturated. Importantly, Cu(I) overload must ultimately be sensed by Cu(I)-dependent metalloregulators (*light green* calipers) causing transcriptional derepression as a result of dissociation from the DNA operator-promoter region (*white* rectangle, opr) (or transcriptional activation) and expression of Cu(I) resistance genes (*pink* rectangle, labeled genes). It is these upregulated copper resistance proteins that ultimately function in Cu(I) resistance, either via sequestration or export through P-type ATPases.

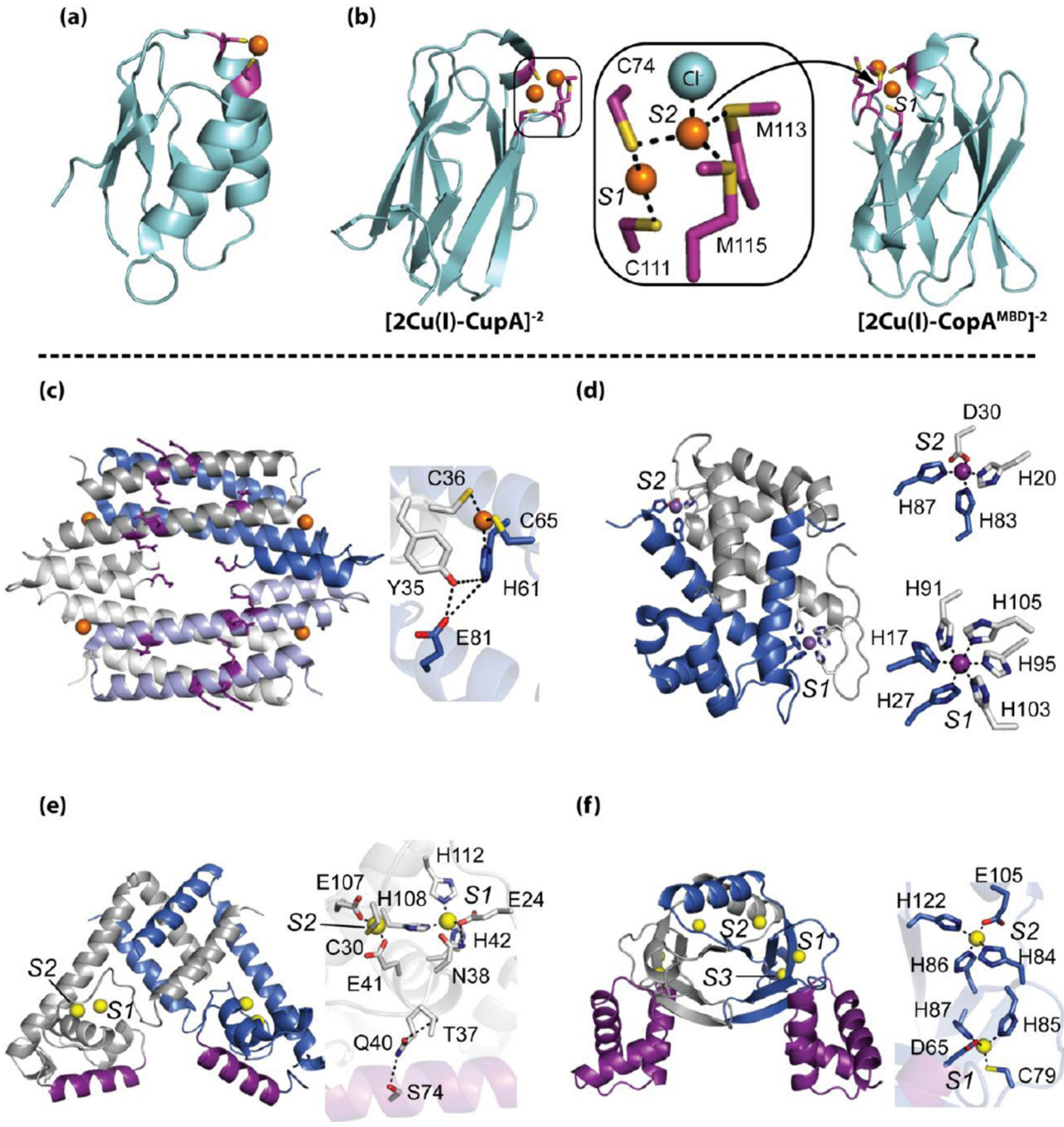

Figure 3.

Structural insights into the bioinorganic chemistry of proteins operating at the host-pathogen interface. *Top panel*, Cu(I)-trafficking proteins: **(a)** Cu(I)-bound CopZ [PDB 1K0V]; **(b)** binuclear Cu(I)-bound soluble CupA (sCupA) [PDB 4F2E] transfers Cu(I) from *S1* Cu site to *S2* Cu site of $CopA_{MBD}$ [PDB 4F2F]. *Inset*, Cu(I) binding sites of sCupA [33••]. *Bottom panel*, crystal structures of (**c**) Cu(I)-bound *M. tuberculosis* CsoR (PDB 2HH7) [36,39], (**d**) Mn(II)-bound calprotectin S100 A8/A9 heterodimer [51••] (Ca ions not shown for clarity) (PDB 4GGF), **(e)** Zn(II)-bound AdcR homodimer [PDB 3TGN] [60], and **(f)** Zn(II)-bound *Streptomyces* Zur homodimer [PDB 3MWM] [42•]. In each case, protomers are shaded *blue* and *grey* with

known or probable DNA binding domains/residues shaded *purple*. Labels for metal-binding sites are in italics. *Insets*, metal-binding sites with dashed lines indicating either first-coordination sphere or second-sphere hydrogen-bonding interactions.

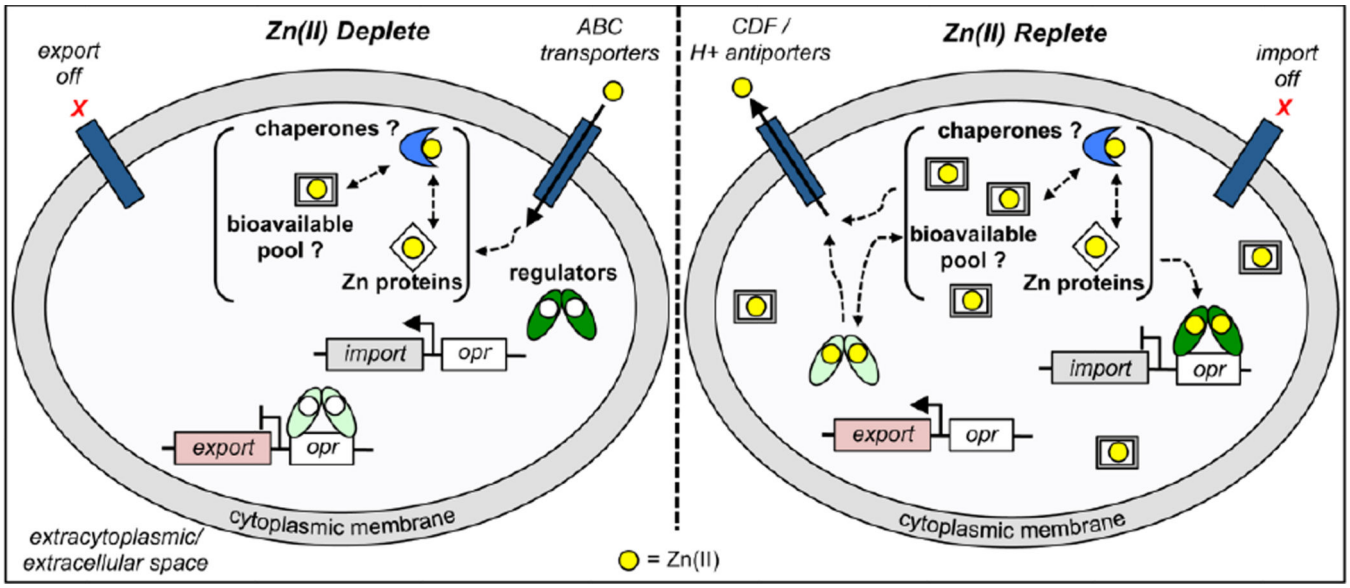

Figure 4.

Cellular response to either limited (*left*) or toxic (*right*) Zn(II) concentrations is mediated by the coordinate action of zinc uptake (*dark green calipers*) and efflux (*light green calipers)* transcriptional regulators that control the expression of import (*grey* boxes) and efflux (*pink* boxes) genes, respectively, as a result of a Zn(II)-regulated binding (activation or inhibition, respectively) to their DNA operators (*white* boxes, opr). *Left panel*, Zn(II) uptake regulators have low affinity for their DNA operator sequence in the apo state under zinc limiting conditions which allows for the expression of import genes. Zn(II) efflux regulators have high affinity for their DNA operator in the absence of Zn(II) and repress efflux. This response allows the cell to maintain a bioavailable concentration of Zn(II) that is sufficient for cellular needs. Trafficking of Zn(II) to selected proteins may involve the action of zinc chaperones, for which there is no definitive evidence. *Right panel*, under conditions of high extracellular zinc, the uptake regulators are metallated and bind with high affinity for their DNA operator, thereby repressing import. In the presence of Zn(II), efflux regulators dissociate from their operator sequence (shown), or become transcriptional activators, in the Zn(II)-bound state, resulting in the transcription of export genes. Zinc speciation in the cytoplasm is projected to involve small molecules, Zn-requiring metalloproteins, and possibly zinc chaperones, as indicated. There is some evidence that zinc-uptake and -efflux regulation occurs at distinct zinc concentrations added to cells, with repression of uptake genes occurring at lower total zinc relative to derepression/activation of export genes [22].