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Bacterial Strategies to Maintain Zinc Metallostasis at the Host-Pathogen Interface*

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Among the biologically required first row, late *d*-block metals from Mn^{II} to Zn^{II}, the catalytic and structural reach of Zn^{II} ensures that this essential micronutrient touches nearly every major metabolic process or pathway in the cell. Zn is also toxic in excess, primarily because it is a highly competitive divalent metal and will displace more weakly bound transition metals in the active sites of metalloenzymes if left unregulated. The vertebrate innate immune system uses several strategies to exploit this "Achilles heel" of microbial physiology, but bacterial evolution has responded in kind. This review highlights recent insights into transcriptional, transport, and trafficking mechanisms that pathogens use to "win the fight" over zinc and thrive in an otherwise hostile environment.

Zinc is an essential micronutrient for all living organisms (1). Zinc is stable in the 2+ oxidation state (Zn^{II}) with a $3d^{10}$ outer electronic configuration and plays a wide variety of catalytic, regulatory, and structural roles in biology. Biological oxidation-reduction (redox) functions requiring reversible electron transfer are performed by other divalent metal ions of the late 3*d*-block series, from Mn^{II} ($3d^5$) to Cu^{II} ($3d^9$). As such, Zn^{II} (Zn) is nature's foremost Lewis acid, catalyzing a wide variety of hydrolytic reactions, and more generally, any reaction that requires activation of an otherwise poor nucleophile. Zn is incorporated into about 10% of all human proteins, and well over 300 enzymes are known to require Zn^{II} for catalytic or structural functions (1). The percentage of Zn-binding proteins in the bacterial proteome is lower (about 5–6%), largely due to the absence of canonical zinc finger transcription factors (1). Zn in bacteria is primarily used as a metalloenzyme cofactor with a total concentration in the 0.1–1.0 mM range (2–4). Zn^{II} acquisition, distribution, and efflux by pathogenic bacteria play central roles in the survival, pathogenesis, and virulence of these organisms in the vertebrate host.

Earth-abundant Zn^{II} is not readily accessible to the invading bacterium in the vertebrate host because during an infection, the host attempts to restrict the availability of essential micronutrients. This has long been known for iron (Fe) (5), but has been more recently established for Zn^{II} and Mn^{II} in a process generally termed "nutritional immunity" that is governed by sophisticated metal chelation strategies (6, 7). Some microbial pathogens, particularly those in an intracellular environment, *e.g.* following engulfment or persistence in macrophages, must, on the other hand, adapt to host-imposed zinc toxicity (8). Indeed, the relative importance of these mechanisms may be dependent on the site of bacterial colonization, *e.g.* Group A *Streptococcus* is suggested to face Zn toxicity during colonization of the nasopharynx, but Zn deprivation on the skin (8). Thus, the successful pathogen must have evolved ways to minimize the deleterious impact of metal ion excess (9) as well as metal deprivation during an infection.

This review describes the mechanistic principles that form the basis of our understanding of how bacterial pathogens counteract host-imposed zinc scarcity in acquiring the metal, while limiting the potential collateral damage of Zn toxicity (Fig. 1). Host-imposed zinc starvation is mediated by myriad innate immune system proteins that have evolved to coordinate transition metals with high thermodynamic and/or kinetic stabilities, thus effectively sequestering these essential metals from the bacterium. Many of the known chelators are derived from the S100 superfamily of Ca^{II}-binding proteins, and include the well studied neutrophil-derived S100 $A8_2A9_2$ heterotetramer, calprotectin (CP)[2] (6). CP is known to withhold Zn and/or Mn^{II} from the bacterial invaders, depending on the physiology of the organism and microenvironmental niche (10). CP has evolved transition metal-binding sites that feature unprecedented coordination chemistries and plasticities, which are activated by Ca^{II} binding to EF-hand type sites and are thus tuned to function as potent, host-derived extracellular chelators (11–13). CP also binds Fe^{II} with high affinity, hinting at a broader anti-bacterial function of this protein (14). S100 A7 (psoriasin) and A12 (calgranulin C) are other well studied members of this class of proteins with metal-sequestering antimicrobial activity (15, 16).

The response of the bacterial pathogen to host-imposed zinc starvation or poisoning can be grouped into three distinguishable strategies: 1) transcriptional regulation by metal-sensing metalloregulatory proteins; 2) Zn efflux and acquisition across cell membranes; and 3) Zn sparing and allocation of Zn to Zn-requiring enzymes, processes that are governed by Zn speciation in the cytoplasm. These will be discussed in turn.

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[2] The abbreviations used are: CP, calprotectin; Zur, zinc uptake repressor; AdcR, adhesin competence regulator; ABC, ATP-binding cassette; CDF, cation diffusion facilitator; SBP, solute-binding protein; BSH, bacillithiol; LMW, low molecular weight; OM, outer membrane; MT, metallothionein.

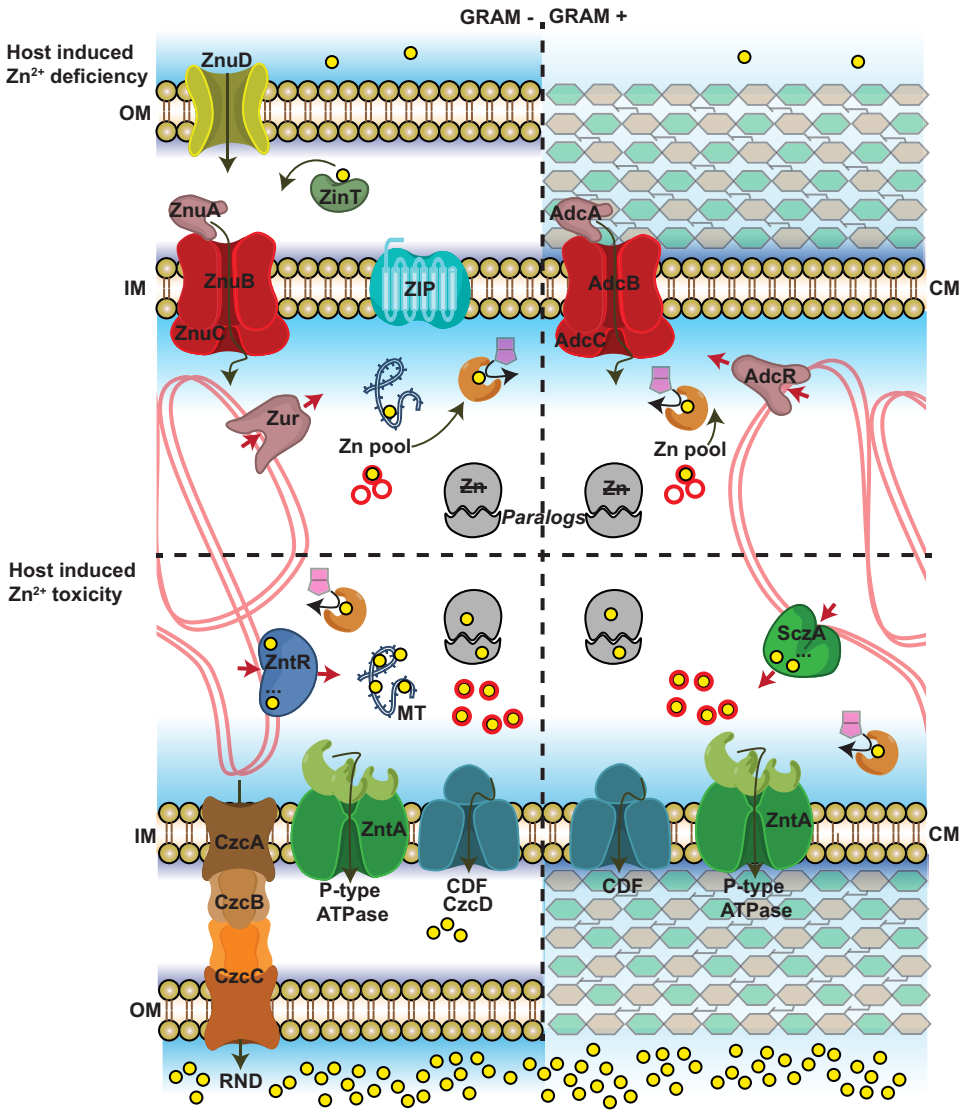


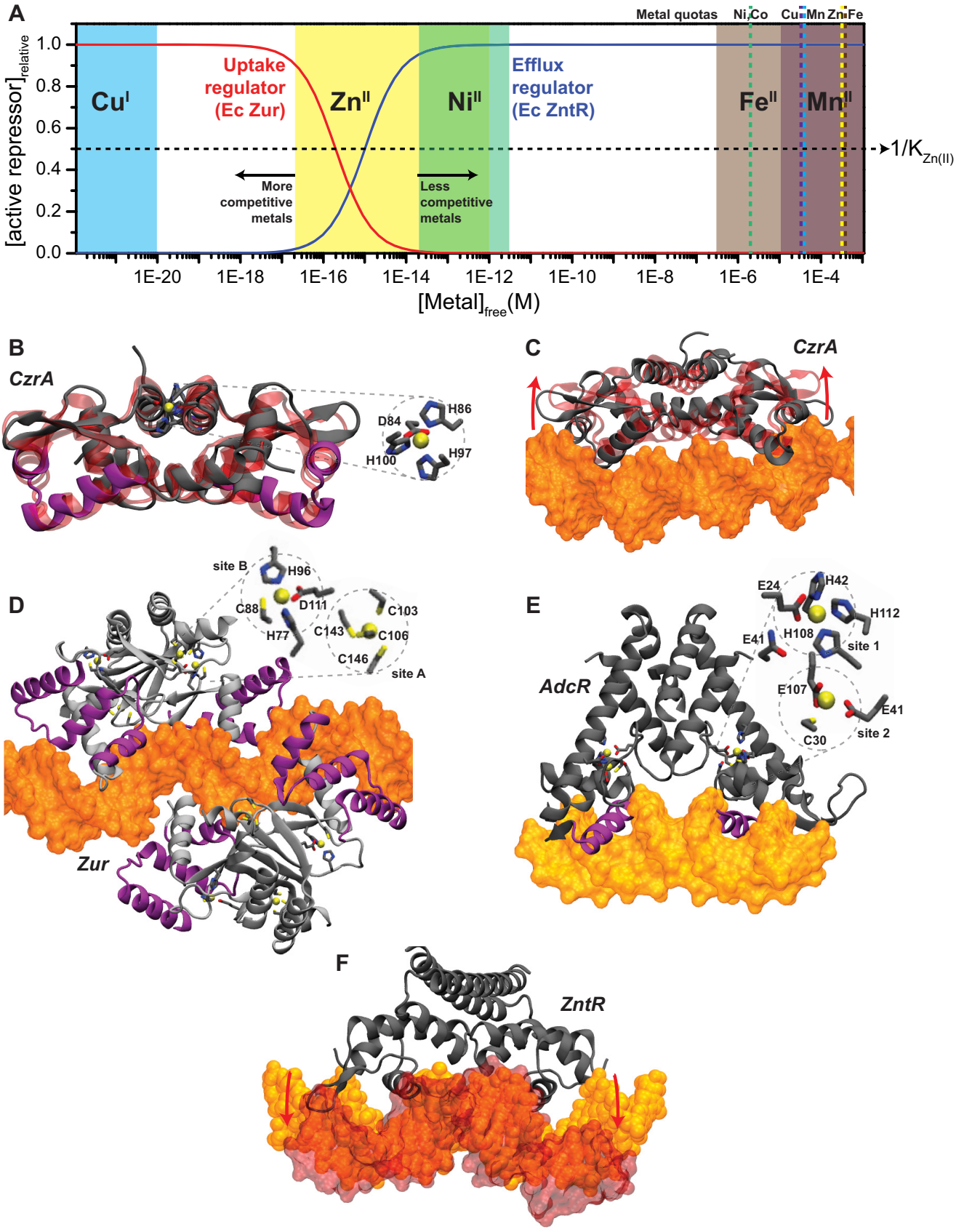
FIGURE 1. **Schematic rendering of the bacterial pathogen response to host-induced Zn^{II} deficiency (*top*) and toxicity (*bottom*) for representative Gram-negative (*GRAM −*) (*left*) and Gram-positive (*GRAM +*) (*right*) organisms.** Abbreviations used: *OM*, outer membrane; *IM*, inner (plasma) membrane; *CM*, cytoplasmic (plasma) membrane; *ZIP*, Zrt/Irt-like proteins found in bacteria (40, 41); *MT*, metallothionein; *CDF*, cation diffusion facilitator transporter; *RND*, resistance-nodulation-cell division transporter. Zn (*yellow circles*) homeostasis is governed by a pair of Zn-sensing regulators, an uptake regulator (exemplified by Zur or AdcR) and efflux regulator (by ZntR or pneumococcal SczA). The balance of Zn uptake and efflux activities establishes the total cytoplasmic Zn concentration (see text for details). The bioavailable or "buffered" pool of intracellular "free" Zn, defined here as that fraction of total Zn not tightly bound to protein and in rapid chemical exchange among small molecules (*red* circles), *e.g.* bacillithiol (94), metallothioneins in some cells (*blue ribbon*), and weakly bound proteome sites (not shown). These components collectively define Zn speciation in cells. Under Zn-limiting conditions, the shuttle of Zn to obligate Zn-requiring enzymes might require a specialized Zn chaperone (43) (*orange symbol*). In a Zn-sparing response under conditions of extreme Zn limitation, Zn-independent *paralogs* replace Zn-dependent ones, such as that which occurs with ribosomal proteins L31 and S14 (*gray* symbol) (78).

Transcriptional Regulation by Metal Sensor Proteins

The Set-point Model

The set-point model is the simplest possible model that explains how bacteria maintain a characteristic total metal quota *and* metal bioavailability in the cell. This model is dictated by the sensitivity (K_{metal}) of the metalloregulatory or metal sensor protein, which transcriptionally regulates the expression of genes that encode metal transporters and other resistance proteins (17–19) (Fig. 2). In most cells, Zn homeostasis is maintained by pairs of Zn sensors that function collaboratively as uptake or efflux repressors. In this model, Zn^{II} bioavailability is maintained by these Zn sensors whose DNA operator binding or transcription activation functions are allosterically modulated by the direct binding of the metal ion. The affinities (K_{metal}) will therefore define a window of "free" or "bioavailable" transition metal concentration $[metal]_{free}$ in the cell, where $1/K_{metal} \sim [metal]_{free}$ (20, 21). Thus, the higher the regulator affinity for the cognate metal under the prevailing intracellular conditions, the lower the concentration of bioavailable metal in the cell (21). As the metal concentration rises above $1/K_{metal}$, changes in transcription result, with transporters enlisted to re-establish homeostasis by repressing uptake and activating efflux.

In most bacteria, the zinc uptake repressor (Zur) controls the expression of a small number of genes required to adapt to conditions of severe zinc depletion. When the intracellular zinc


A
Metal quotas
Ni Co
Cu Mn Zn Fe
[active repressor]relative
1.0
0.8
0.6
0.4
0.2
0.0
Cu^I
Uptake regulator (Ec Zur)
Zn^II
Ni^II
Efflux regulator (Ec ZntR)
Fe^II
Mn^II
1/K_Zn(II)
More competitive metals
Less competitive metals
1E-20
1E-18
1E-16
1E-14
1E-12
1E-10
1E-8
1E-6
1E-4
[Metal]free(M)
B
CzrA
H86
D84
H100
H97
C
CzrA
D
site B
H96
C88
D111
C103
H77
C143
C106
C146
site A
Zur
E
E24
H42
H112
E41
H108
site 1
AdcR
E107
E41
C30
site 2
F
ZntR

concentration is far below a critical threshold, $[metal]_{free} < 1/K_{metal}$, the zinc-free form of Zur has low affinity for the DNA operator, which overlaps the promoter, thus allowing unfettered access by RNA polymerase to transcribe the genes encoding a high affinity Zn uptake system(s) (Fig. 2). In addition, genes encoding the efflux system are transcriptionally repressed by the apo form of the Zn efflux repressor, ZntR in *Escherichia coli*, under these conditions. As levels of bioavailable Zn rise to $[metal]_{free} > 1/K_{metal}$ (zinc-replete conditions), the zinc-bound form of Zur binds tightly to the operator site, thus preventing transcription (22). Likewise, the efflux regulator, ZntR in *E. coli*, binds Zn and allosterically activates transcription of *zntA*, encoding a Zn-specific P-type ATPase efflux transporter, by converting a suboptimal promoter to an optimal one (23). In other bacteria, the Zn efflux regulator is a repressor whose DNA binding affinity is negatively regulated by Zn binding, thus driving transcriptional depression of downstream genes under conditions of excess Zn (24). In still others, *e.g.* SczA from *Streptococcus pneumoniae*, the efflux regulator is an allosteric activator of transcription (25), which may involve a metal-mediated physical interaction with RNA polymerase. In any case, the difference in K_{metal} between uptake and efflux regulators in the DNA-bound state thus defines the "window" of bioavailable Zn (Fig. 2*A*) (26).

Allostery

The zinc specificity of these "allosteric switches" (26) is defined largely by the metal coordination environment; however, metal sensors tend to bind divalent metals with a rank order of affinity that matches the Irving-Williams series for divalent metal-nitrogen/oxygen (N/O) chelates, regardless of which metal(s) is detected in the cell (17, 19, 21, 27). This raises questions about how a subset of sensors can detect weaker binding metals *in vivo* (21). One explanation is that metal binding kinetics rather than the thermodynamics dictate the sensor response, catalyzed by transient interactions with metallochaperones or other small molecules (see below). An alternative, earlier hypothesis implicates a metal-selective allosteric mechanism, *i.e.* formation of only the "correct" or "cognate" coordination geometry is capable of driving allosteric activation or inhibition of DNA operator binding (28). This by and large remains true, but the relative rank order of "set points" for individual transition metal sensors essentially enforces metal specificity in the cell (Fig. 2) (29, 30). Recent studies of the entire collection of *Salmonella enterica* metal sensors suggest that DNA binding occupancy of the apo (repressing) form of a particular efflux regulator can be tuned by taking into account its absolute concentration in the cell, coupled with knowledge of the allosteric coupling free energy (ΔG_c) (26) and relative affinities of the apo and metallated states (21). This *tour de force* reveals that experimental approaches that directly measure metal occupancy, DNA operator-promoter occupancy, and transcriptional regulation (repression, de-repression, or activation), as a sensitive function of $[metal]_{free}$ *in the cell*, will shed significantly more light on these processes.

How allosteric communication propagates from the metal-binding site(s) to influence DNA binding affinity, and thus impact biological regulation, is also of considerable interest. There are a number of mechanisms of transcriptional regulation that have been described for a variety of Zn sensors. For example, the zinc efflux repressor *Staphylococcus aureus* CzrA is characterized by strong negative allosteric linkage between the binding of metal and the binding of operator DNA, with an allosteric coupling free energy, $\Delta G_c \sim 6.5$ kcal mol^{-1} (pH 7.0, 25 °C). The molecular origins of this negative coupling have proven enigmatic, partly due to the lack of a large structural change in the CzrA homodimer upon Zn binding (31). The apo and Zn-bound states adopt very similar "open" conformations, whereas DNA-bound CzrA adopts a more "closed" conformation, allowing the N terminus of the αR reading heads to reach into successive major grooves of the operator (32) (Fig. 2, *B* and *C*).

Zn^{II} binding to the regulatory site(s) in Zur induces an allosteric global conformational change that increases ($-\Delta G_c$) the binding affinity of Zur for the DNA operator (29, 33). Structural insights into this conformational change and operator recognition have been recently been revealed for a member of Zur family (22) (Fig. 2*D*). Molecular recognition of the DNA operator by Zn-Zur is also based, as it is for CzrA, on protein conformational plasticity (22). In addition to allostery, the roles that multiple metal-binding sites play in multi-domain repressors have been suggested to impact differential set points, thus expanding the dynamic range of sensing Zn^{II} in the cell in both *Streptomyces coelicolor* Zur (33) and *Bacillus subtilis* Zur (29).

The MarR (multiple antibiotic resistance) family zinc uptake repressor AdcR (competence regulator) from *S. pneumoniae* is another example where Zn binding to two distinct metal sites activates DNA binding, but to varying degrees (34). AdcR shares the winged-helix DNA-binding fold of CzrA (Fig. 2*E*), but possesses an additional C-terminal α6 helix in the dimer interface, giving rise to an extended, triangularly shaped homodimer. In Zn-replete conditions, AdcR represses the expression of the Zn uptake system and contributes to streptococcal virulence (35). For all other members of the MarR superfamily, ligand binding attenuates DNA binding; for AdcR, the

FIGURE 2. *A*, graphical representation of the "set-point" model for metal homeostasis. In this model, the metal affinity for individual or pairs of metal sensor proteins (*red* and *blue solid lines*) defines the ability of the cytoplasm to buffer biologically required transitions metal ions. The K_{Zn} of the pair of regulators set the boundary of free Zn concentration in the cytoplasm, where $1/K_{Zn} \sim [Zn^{II}]_{free}$. The free metal concentration follows the Irving-Williams series (*shaded areas*). The total concentrations are represented in *dashed lines* (color coded in the graph). The metal affinity constants and concentrations were taken from Ref. 19. *B*, *S. aureus* CzrA in the Zn-bound form (Protein Data Bank (PDB): 2m30) with the Zn-binding site in the zoomed region. The apo form (PDB: 1r1u active repressor) is superimposed in *red*, showing minimal structural difference (31). *C*, the *S. aureus* CzrA in DNA-bound form (PDB: 2kjb) docked on a DNA operator (32). The apo form (PDB: 1r1u active repressor) is superimposed in *red*, representing the induced fit that the protein undergoes upon DNA binding. *D*, *E. coli* Zn_4-Zur_2-33-mer DNA complex (PDB: 4mte) (22). *E*, *S. pneumoniae* AdcR in the Zn state (PDB: 3tgn) docked on the DNA operator (34). *F*, the ZntR (PDB: 1q90) docked on the DNA operator based on the *E. coli* CueR structure (PDB: 4wls). The bent DNA conformation (PDB: 4wlw) is represented in *red* to show the conformational change at the level of DNA responsible of changes in gene expression upon Zn binding (23). In all the structures, the putative DNA-binding region is colored in *purple* and DNA operators are shown in *orange*. *Red* arrows indicate movement upon metal/DNA binding.

ligand (Zn) is used as an allosteric activator of DNA binding consistent with its biological function as uptake repressor (36).

Zinc Efflux and Acquisition

The cellular demand for bioavailable Zn^{II} while limiting Zn toxicity by this highly competitive metal is governed by the relative rates of Zn acquisition by ATP-binding cassette (ABC) transporters and cytoplasmic efflux via P-type ATPases or proton-coupled antiporters (37–39). More recently, other transporters have been shown to function in Zn uptake in *S. enterica* and *Vibrio cholera* (40, 41). As discussed above, host strategies to limit bacterial infection exploit both zinc toxicity, perhaps best understood for intracellular niches (3, 42), and severe zinc limitation (starvation) (43).

Zinc Efflux

In the model system *E. coli*, the efflux regulator ZntR (Fig. 2*F*) regulates transcription of genes encoding three types of exporters: a P-type ATPase ZntA (Fig. 1), cation diffusion facilitator (CDF) family transporters ZitB, which augments zinc tolerance mediated by ZntA (44), and YiiP (discussed further below), and the periplasm-spanning "efflux guns" CzcD and CzcBCA (for a recent review, see Ref. 45). The CDFs constitute a large family of divalent metal, proton-coupled antiporters that play important roles in global intracellular metal homeostasis in all three kingdoms of life (46). The most extensively structurally characterized bacterial CDF to date is *E. coli* YiiP, an established Zn/Cd transporter (38), which is also known to efflux Fe^{II} (44, 47) (Fig. 3). An alternating access model of transport where the exchange between Zn^{II} and H^+ leads to a conformational change enables Zn to move through the membrane (48). Despite an extensive characterization of YiiP, how metal selectivity is achieved for the CDFs in general remains unclear. The functional and structural diversity of CDFs may well be significant (49), thus motivating efforts to solve the structures of other CDFs to obtain a clearer understanding of how metal selectivity is achieved. A recent *in vivo* study that compares the metal selectivity of *E. coli* YiiP and *S. pneumoniae*-specific Zn and Mn CDFs (CzcD and MntE, respectively) shows that the first coordination shell of A (membrane)-site coordination ligands, *e.g.* an Asn in Mn-specific CDFs, is a primary specificity determinant that enhances the transport of cognate *versus* noncognate or competing metal ions (50) (Fig. 3). Similar findings characterize the Mn-specific human CDF, ZnT10 (SLC30A10) (51), mutations in which cause parkinsonism and related neurological and liver dysfunction (52).

The P-type ATPases, like the CDFs, are a large family of integral membrane proteins that include those from the P_{1B} clade that mediate Zn^{II} efflux from the cytoplasm in bacteria and plants (45). The physiological functions of these pumps are not restricted to Zn^{II} efflux and include Cu^{I} (53), Co^{II}/Ni^{II} (54), Fe^{II} (55), and Mn^{II} (56) effluxers, while also mediating resistance to toxic metals Pb^{II} and Cd^{II} (57) and Ag^{I} (58). Metal selectivity has been shown to be dictated by the metal coordination geometry, which impacts overall rates of translocation. Some experimental details concerning the mechanism of Zn transport by P_{1B}-type ATPases have recently been unraveled. The crystallographic structure of ZntA from *Shigella sonnei* in its Zn-free E2 conformation reveals a negatively charged funnel and a candidate extracellular metal release pathway (37) (Fig. 3). It is thought that a conformational change in the transmembrane helices as part of a canonical Post-Albers cycle, where the ATP hydrolysis is coupled to interconversion between E1 and E2 states, prevents the reverse flow of the transported ion(s); however, a detailed mechanism of metal transport remains to be elucidated (37). The mode of Zn coordination is of course not defined by this structure, but a tetrahedral $S_2(O/N)_2$ model involving the Cys-Pro-Cys (CPC) motif in transmembrane helix 4 (TM4) emerges from the biochemical and x-ray absorption studies of *E. coli* ZntA (59, 60). These studies of ZntA complement parallel studies of the bacterial Cu^{I}-specific P_{1B}-ATPase CopA, for which an enhanced structural and mechanistic understanding of the Cu^{I} capture, transport, and efflux is emerging (61–63). Additional work is clearly required to fully understand metal specificity and transport by this ubiquitous family of transporters.

Zinc Uptake

Nearly all bacteria employ tripartite, high affinity ABC Zn transporters consisting of a periplasmic or extracellular solute-binding (lipo)protein (subunit A; solute-binding protein; SBP), a transmembrane-spanning permease (subunit B), and a cytoplasmic ATPase (subunit C) (Fig. 3). ABC transporters are twofold pseudosymmetric and typically adopt an AB_2C_2 stoichiometry; in some cases, the two B-subunits and two C-subunits are encoded by different genes as a result of gene duplication. The prototypical bacterial Zn-specific ABC transporter is encoded by *znuABC*. A significant body of structural work reveals that the SBP subunit harbors all significant features required for metal (ligand) specificity of Zn-dependent ABC transporters (64, 65). In contrast, there is comparatively little known about how ATP hydrolysis is coupled to Zn^{II} transport by an intact transporter, with most models based on high resolution structures of the *E. coli* vitamin B_{12} (cobalamin) and related transition metal transporters (66, 67). These models generally invoke an alternating access mechanism driven by ATP binding, hydrolysis, and product release (Fig. 3). Targeting the SBP (ZnuA) may represent an excellent strategy to identify new antibiotics against Gram-negative bacteria (68).

In Gram-negative bacteria, the ZnuABC system is essential for zinc uptake, but its expression does not necessarily promote competitive advantages over the host microbiome (41). In these cases, bacteria use alternative or additional Zn capture systems that function alongside ZnuABC, whereas others express a "supercharged" ZnuA that harbors additional Zn-coordinating residues, *e.g.* poly(His)-rich sequences and/or a second soluble domain, *e.g.* ZinT, appended onto ZnuA. Alternatively, the expression of other zinc-binding proteins is up-regulated under extreme zinc deficiency, *e.g.* ZinT/ZitB (Fig. 1) (69) or polyhistidine triad (Pht) proteins (70) that likely aid in Zn capture in both Gram-positive and Gram-negative organisms (68, 71).

There is now strong evidence that metal-specific outer membrane (OM) receptors, reminiscent of OM porins that transport Fe^{III}-siderophore complexes or cobalamin in a TonB-dependent fashion, also function in Zn uptake in Gram-negative bacteria (65, 72). The nature of the specific Zn species that is trans-

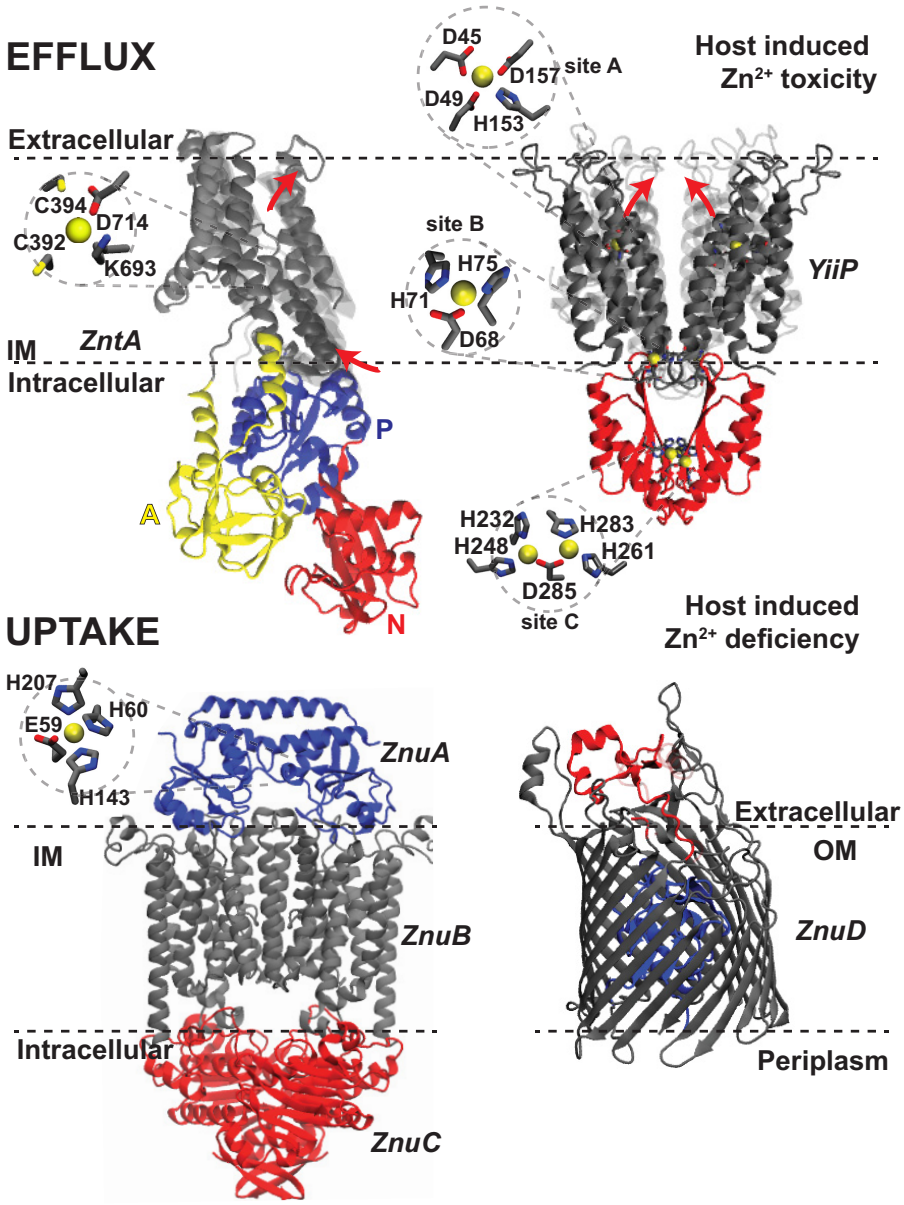


FIGURE 3. **Membrane transporters for Zn^{II} efflux (*top*) and uptake (*bottom*).** Abbreviations used: *OM*, outer membrane; *IM*, inner (plasma) membrane. *Top, left:* the P_{1B}-type ATPase is *S. sonnei* ZntA in the Zn-free "E2" ground state (PDB: 4umv) (37). Actuator (*A*, *yellow*), phosphorylation (*P*, *blue*), and nucleotide-binding (*N*, *red*) domains are shown. The transmembrane domain is shown in *gray* superimposed with the phosphorylation intermediate (PDB 4umw, *transparent*), representing the closure of an extracellular release pathway. A model of the intramembranous high-affinity Zn^{II}-binding site based on spectroscopic and functional studies (60) is shown in the *inset*. The *red arrows* indicate the putative metal pathway accompanied by protein rearrangements. *Top, right:* the CDF protein is *E. coli* YiiP (PDB: 3h90). The functional dimer is shown. The Zn-bound form of the cytoplasmic dimerization domain is shaded *red*. The Zn^{II} chelates shown represent the three distinct metal-binding sites (A, B, and C1/C2). Note that functional studies of *S. pneumoniae* CzcD are consistent with a single C-site metal site, not a binuclear cluster as indicated (50). The inward facing state (PDB 3j1z, *transparent*) is shown to represent the mechanism of metal release for this transporter. *Bottom, left:* the model for ZnuABC is based on the structure of BtuCDF (PDB: 4fi3) for cobalamin uptake. The detailed metal coordination displayed in the *inset* is taken from the crystal structure of ZnuA (PDB: 2osv) with the residues numbered according to the UniProt sequence. The solute-binding protein (ZnuA, *red*), permease (ZnuB dimer, *gray*), and ATPase (ZnuC dimer, *blue*) are shown. The model of ZnuD is from *Neisseria meningitidis* (PDB: 4rdr) (72). The "plug" domain (shaded *blue*), the β-barrel domain (shaded *gray*), and the metal-sensing extracellular loop 3 (shaded *red*), with the Cd^{II}-bound form transparent; PDB: 4rdt) are shown.

ported by these systems is generally not known, although for *Neisseria* ZnuD, it has been argued on the basis of structural and computational studies that free, hydrated Zn^{II} is the transport substrate (72) (Fig. 3). In an escalation in the Zn acquisition "arms race" between microbe and host, an OM porin designated CbpA, a candidate bacterial receptor for CP-Zn complexes (73), is thought to capture this CP-bound Zn, consistent with a direct role in zinc piracy analogous to iron piracy practiced by many bacterial pathogens (74).

More recent work reveals that the general strategy that bacteria use to acquire Fe, as Fe^{III}-siderophore (or more generally, chelator) complexes, may not be limited to Fe, but is used to capture other transition metal ions, including Cu, Zn, Co, and Ni, as zincophores or more generally, metallophores (75). In addition, previously characterized Fe siderophores likely have moonlighting functions, a notable example of which is yersiniabactin, which binds Cu^{II} and protects uropathogenic *E. coli* from extracellular reactive oxygen species (76). *S. aureus* is now

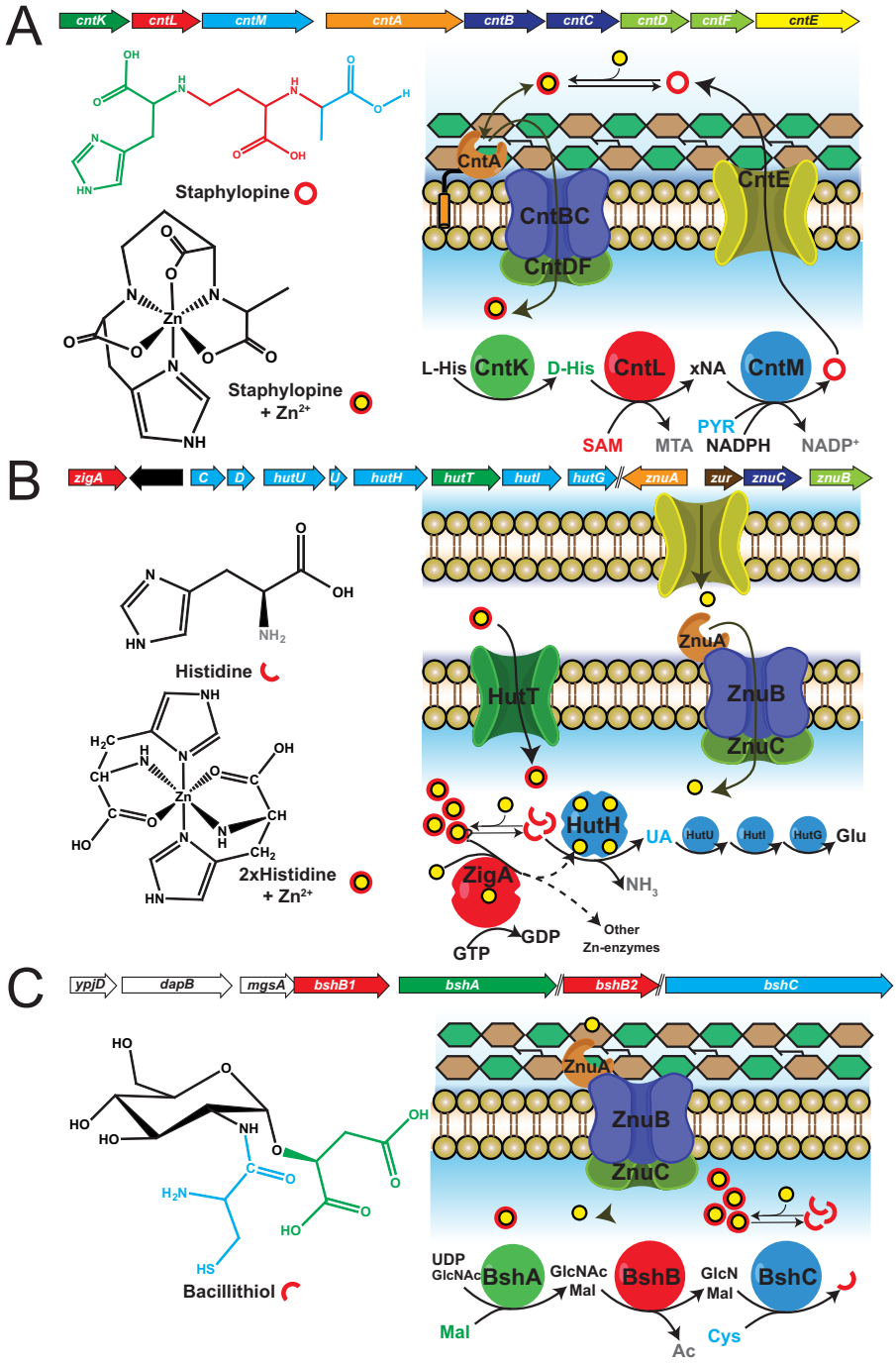


FIGURE 4. **Small molecules involved in Zn homeostasis and Zn speciation in cells.** *A*, model of staphylopine biosynthesis and staphylopine-mediated transition metal acquisition by this broad-spectrum metallophore (77). Abbreviations used: *SAM*, *S*-adenosyl-L-methionine; *MTA*, 5′-methylthioadenosine; *xNA*, nicotianamine intermediate; *PYR*, pyruvate. Staphylopine is biosynthesized from L-His by the combined activities of CntK, a histidine racemase, CntL, a nicotianamine synthase-like (NAS-like) enzyme, and CntM, a staphylopine dehydrogenase. The metal-free metallophore is proposed to be exported through CntE, a major facilitator superfamily (MFS) transporter, and then imported as the metallated complex via a specific ABC transporter, CntABCDF. *B*, model of how histidine uptake and catabolism impact the labile histidine-Zn pool in *A. baumannii* under conditions of host-mediated Zn starvation (43). His is proposed to be taken up as His_2-Zn complex through HutT. ZigA is a Zn^{II}-activated GTPase that may directly activate the Zn-stimulated metalloenzyme HutH (histidine ammonia lyase), which converts L-His to *trans*-urocanic acid (*UA*) and ultimately glutamate (*Glu*) via the sequential activities of HutUIG, all encoded by the *hut* (histidine utilization) operon. Urocanic acid (43) and glutamate have lower affinities for Zn than histidine, thus potentially mobilizing Zn from the labile or rapidly exchanging pool for use in Zn-requiring processes. *C*, schematic model of bacillithiol (BSH) biosynthesis (99) and known role of bacillithiol in buffering cytoplasmic Zn (94). *BshA*, a glycosyltransferase that condenses *N*-acetyl glucosamine (GlcNAc) with L-malic acid (*Mal*); *BshB* (BshB1 and BshB2 are partially redundant), a deacetylase; *BshC*, a cysteine ligase.

known to harbor a gene cluster, *cntKLM*, that encodes the biosynthetic pathway for a broad-spectrum metallophore, staphylopine from L-histidine, the core structure of which is chemically similar to the plant chelator nicotianamine (77) (Fig. 4*A*). Although absolute metal affinities and the impact on cellular metal quota have not yet been measured for *cnt* mutants, staphylopine appears to be a *bona fide* metallophore that is capable of coordinating a wide range of divalent transition met-

als. This suggests an important function in the competition between the infected host and microbe for nutrient metals.

Zinc Sparing and Zn Allocation under Conditions of Extreme Zinc Limitation

Zinc is so integral to much of metabolism that a number of specialized Zn-specific adaptations have evolved to ensure that critical processes run smoothly under conditions of extreme host-mediated Zn scarcity, beyond that which can be overcome via increased transcription of the Zn uptake machinery (Fig. 1). This is particularly true for bacterial pathogens where Zn availability is tightly restricted by the innate immune system (see above).

One mechanism is termed Zn sparing, commonly used in bacteria to increase the expression of non-Zn-requiring proteins to replace essential Zn-dependent enzymes and proteins, which was first described for ribosomal proteins (78, 79). This strategy ensures that the metabolic functions of key Zn-dependent enzymes are maintained under conditions of Zn scarcity. A well established example is the replacement of Zn-containing ribosomal subunits L31 and S14 with non-Zn-containing subunits (80). Zn sparing involves the use of often structurally distantly related enzymes or proteins that either show a relaxed metal specificity or dispense with the metal cofactor altogether. Enzymes involved in this process include threonyl-tRNA synthetase, GTP cyclohydrolase I (FolE), porphobilinogen synthase (PBGS), and DksA (81–84). In the case of the GTP cyclohydrolases, the Zn-requiring (Ia) enzyme is a better catalyst with a very high affinity for Zn; the 1b enzyme, on the other hand, has modest affinity for many divalent metal ions, each of which support variable levels of activity (82). In this case, Zn sparing is clearly a "workaround" to access a crucial metabolic process, *i.e.* folate biosynthesis, under extreme cellular zinc limitation. It seems possible that there are other examples of Zn sparing yet to be discovered in bacteria.

Another strategy to retain critical metabolic functions under extreme Zn limitation is to ensure that those enzymes that are obligatorily Zn-dependent can be metallated under these conditions. One might anticipate that this role might be played by a zinc chaperone(s), reminiscent of Cu chaperones that metallate Cu-requiring enzymes under conditions of extreme Cu limitation (85); however, this area of zinc metabolism remains poorly understood. A number of Zn chaperones have been proposed, with most recent efforts focused on candidate G3E family GTPases from the COG0523 subfamily, whose expression is under the transcriptional control of Zur (86, 87). An unbiased mutant screen of *Acinetobacter baumannii* stressed with CP-induced Zn^{II} limitation identified components of the Zur regulon (10), which was found to include a gene encoding a COG0523 protein, denoted ZigA (43) (Fig. 4*B*). Several other G3E family GTPases play roles in metallocenter assembly consistent with the hypothesis that the Zur-regulated COG0523 GTPases may function as Zn chaperones (88–90). *A. baumannii* ZigA and *E. coli* YeiR have been biochemically characterized, and both possess intrinsically low GTPase activity that is only modestly stimulated by Zn binding (43, 91). The conjecture is that this activity will be strongly stimulated upon physical, even transient, association with an apo enzyme target or client protein, thus providing a driving force for intermolecular Zn transfer. Although there is as yet little direct support for this hypothesis, recent work in *A. baumannii* reveals clearly that ZigA impacts the labile Zn pool by stimulating histidine degradation under conditions of CP-induced Zn limitation (Fig. 4*B*) (see below) (43).

Zinc Speciation in the Bacterial Cytoplasm

As discussed above, although the total cell-associated Zn concentration in bacterial cells is in the millimolar range (Fig. 2*A*), the bioavailable or "buffered" Zn concentration in the bacterial cytoplasm is predicted to be in the pico- to nanomolar range, largely as estimated by the metal sensitivities ($1/K_{metal}$) of the uptake and efflux regulators (92) (Fig. 2). This 10^6-fold difference in concentration establishes that the cell has an overcapacity to chelate Zn and that access to the metal might be restricted by the specific nature of these poorly defined "buffering" components. Unfortunately, detailed chemical insights into Zn speciation and how this might change under different environmental conditions are largely lacking, and this remains a significant analytical challenge given the lability of small molecule-metal complexes.

Glutathione (GSH) and bacillithiol (BSH) (Fig. 4*C*) are low molecular weight (LMW) thiols established as important players in redox homeostasis. Recently, a new role for the cell-abundant reduced forms of these LMW thiols in buffering transition metals has been uncovered (93, 94). Although the deletion of glutathione biosynthetic genes has no effect on Zn tolerance in *E. coli*, GSH was shown to become important when Zn efflux systems are not expressed (93). Similarly, BSH, as the most abundant LMW thiol in *B. subtilis* (95), serves as a major component of the labile Zn pool in that organism (94). In addition to these thiols, histidine has recently been shown to contribute to the labile Zn pool in *A. baumannii* (Fig. 4*B*) (43). Histidine is a formidable Zn chelator, forming complexes with ~10 μM affinity (43) with high intracellular histidine concentrations potentially tying up the metal. The histidine utilization operon (*hut*) is slightly up-regulated in *A. baumannii* strains lacking Zur (10) with the resultant expression of the major histidine transporter HutT contributing not only to the cytoplasmic histidine pool, but also to the cellular Zn status by transporting His_2-Zn complexes (Fig. 4*B*) (43). The fact that histidine protects *A. baumannii* against Zn toxicity, as found previously in eukaryotic organisms (96), *and* Zn scarcity in a way that is linked to ZigA-stimulated histidine catabolism, reflects the demand for a highly dynamic pool of labile Zn that can be rapidly altered to meet cellular needs (43). Clearly, other small molecules such as other LMW thiols, amino acids, nucleotides, inorganic phosphate, and citrate are all candidates as contributors to Zn speciation of a labile Zn pool, which remains undefined for most bacteria.

Finally, in some bacteria, including the cyanobacteria and *Mycobacterium tuberculosis*, Zn/Cd- or Cu-binding LMW cysteine-rich polypeptides termed metallothioneins (MTs) are found, where they generally play roles in resistance to Zn^{II} or Cu^{I} toxicity (97). Extensive studies of the vertebrate and plant MTs reveal that the redox chemistry of Cys and a high degree of conformational plasticity enable MTs to dynamically (dis)asso-

ciate multiple Zn ions; as a result, MTs are believed to play roles in Zn storage and delivery to metalloproteins, as well as buffering cytosolic Zn in these systems (98). The full extent to which *bona fide* Zn-specific MTs contribute to Zn speciation and homeostasis in bacteria remains unclear, although they appear to have physical properties consistent with such a function (97).

Conclusions

In this Minireview, we summarize recent efforts to understand the degree to which important human bacterial pathogens adapt to host efforts to remodel the transition metal landscape and thus limit the impact of a bacterial infection. Zinc acquisition and efflux play central roles in this "fight over metals" due to the tremendous footprint of Zn on cellular metabolism, coupled with its toxicity that derives from its position near the top of the Irving-Williams series (27). A more sophisticated understanding of the structure and dynamics of cellular small molecule and proteome Zn speciation is the next frontier, and will depend on significant analytical advances in mass spectrometry (21, 43, 77) and related techniques. These advances, coupled with detailed mechanistic and physicochemical understanding of Zn sensing, uptake, efflux, and allocation, promise exciting discoveries to come in zinc metallostasis at the host-pathogen interface. This knowledge, in turn, will be leveraged to identify new molecular targets for the development of antimicrobial agents as alternatives to traditional antibiotics.

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