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Metallochaperones and metalloregulation in bacteria

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Abstract

Bacterial transition metal homoeostasis or simply 'metallostasis' describes the process by which cells control the intracellular availability of functionally required metal cofactors, from manganese (Mn) to zinc (Zn), avoiding bothmetal deprivation and toxicity. Metallostasis is an emerging aspect of the vertebrate host–pathogen interface that is defined by a 'tug-of-war' for biologically essential metals and provides the motivation for much recent work in this area. The host employs a number of strategies to starve the microbial pathogen of essential metals, while for others attempts to limit bacterial infections by leveraging highly competitive metals. Bacteria must be capable of adapting to these efforts to remodel the transition metal landscape and employ highly specialized metal sensing transcriptional regulators, termed metalloregulatory proteins, and metallochaperones, that allocate metals to specific destinations, to mediate this adaptive response. In this essay, we discuss recent progress in our understanding of the structural mechanisms and metal specificity of this adaptive response, focusing on energy-requiring metallochaperones that play roles in the metallocofactor active site assembly in metalloenzymes and metallosensors, which govern the systems-level response to metal limitation and intoxication.

Introduction

The first row late *d*-block metal ions, including manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu) and zinc (Zn), function as essential and specific cofactors in metalloenzymes in the cell yet are toxic in excess [1–4]. The vertebrate innate immune system employs a number of strategies to exploit this metabolic requirement for essential metal ions in an effort to negatively impact microbial pathogen physiology [1], but the evolution of bacterial systems that control the homoeostasis of transition metals allows bacterial adaptation to these host-derived stresses. This response allows an organism to scavenge sufficient essential metal ions from the immediate microenvironment to ensure the proper metallation of metalloenzymes while adjusting cellular metabolism to match metal bioavailability and effluxing any excess metal from the cytoplasm of the cell. This process is quite selective for individual transition metal ions, and as a result, essentially parallel yet potentially intersecting pathways operate collectively in the cell to minimize 'cross-talk' of

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Competing interests

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metal homoeostasis and allocation systems. This ensures that the 'right metal gets to the right place at the right time'. Major players in the process are a panel of metalloregulatory proteins or 'metallosensors' that sense the bioavailability of a specific metal in the cell [5], and generally diffusible metallochaperones that protect the cell from aberrant metal chemistry, and effectively guide metal ions to specific protein targets or specific transporters for efflux [6].

One-third of all the proteins require metal cofactors for function, and as such, correct metallation of proteins is essential for all the living systems [7]. However, intrinsic metal binding preferences of many metalloproteins do not always match their catalytic requirements and as a result, nature exploits diverse mechanisms to ensure faithful maturation of metalloproteins. As a rule of thumb, chelate-binding affinities for Cu^{I} and Zn^{II} are generally higher than for earlier first row, late *d*-block divalent metals for a given ligand set, a trend known as the Irving–Williams series for divalent ions [8]. The bioavailability of Cu^{I} and Zn^{II} is therefore generally quite low and inversely proportional to their 'competitiveness' relative to other first-row metal ions (Figure 1A). This, in turn, dictates that the availability of Cu^{I} and Zn^{II} in cells be kept in check [9–12], which can be achieved by an overcapacity of the cellular cytoplasm to chelate these metals. Some fraction of these metals are found in low molecular weight (LMW), exchange-labile complexes with sulfur and nitrogen-based ligands [13–15]. Such a scenario precludes the existence of a 'pool' of hydrated 'free' metal ions that is readily available for the maturation of metalloproteins. This also illustrates the fact that *total* metal accumulated by cells is typically in significant excess relative to that 'free'metal that is made bioavailable in the cell (Figure 1A).

As a result, accessory proteins are often required to ensure that the maturation of metalloenzymes occurs with high specificity and kinetic efficiency, as a means to minimize mismetallation by non-cognate metals. This is particularly true under conditions of extreme metal starvation induced by the vertebrate host, where both the total and bioavailability of essential metals such as Zn^{II}, Mn^{II} and Fe^{II} to an invading pathogen is restricted by sophisticated metal-chelation strategies in a process termed as 'nutritional immunity' [16]. On the other hand, only ≤0.3% of a typical proteome requires Cu^{I} and as such, the host often exploits Cu cytotoxicity as a means to limit bacterial infections [17]. In mammalian cells and in fungal pathogens, the generally low intracellular bioavailability of Cu requires specialized metallochaperones for Cu, Zn superoxide dismutase and cytochrome oxidase that ensure metallation in these systems [18]. In any case, microbial pathogens must be capable of adapting to both host-mediated metal limitation as well as metal toxicity depending on the microenvironmental niche and the specific metal.

Proper metallation *in vivo* is favoured by a cytoplasm that defines a controlled microenvironment where the accessibility to transition metals is generally thought to be limited [19]. This control is achieved in part by 'metallosensors' that sense the bioavailability of a specific metal and induce expression of transporters that maintain the 'free' metal ion concentration in a range that is compatible with cellular needs [9]. The specific metal that is detected *in vivo* by each metallosensor is dictated by the degree to which they compete with other metal binding constituents, often small molecules and metabolites, of the cellular milieu [20]. Metal bioavailability controlled in this way

reinforces intrinsic metal selectivities of metallosensors governed by the tenets of inorganic chemistry, including the metal ion valence, ligand donor set, and coordination number and geometry. Formation of the first metal coordination shell, in turn, effects allosteric activation or inhibition of DNA operator binding, mediating an adaptive cellular response to too little or too much metal.

In this essay, we discuss our current understanding of the structure and function of two types of metallochaperones and 'one-component' metalloregulatory proteins that impact the inorganic chemistry of the cell, across the six major transition metals, from Mn to Zn (Figure 1B). Our interest in bacterial transition metal homoeostasis is strongly motivated by the impact of this process at the host–pathogen interface and how it is exploited by limiting bacterial infections. This will not, however, be discussed further here and we direct the reader instead to a number of recent reviews on this subject [17,21–23]. We provide sufficient mechanistic detail to pique the interest of the reader, while drawing general conclusions from recent findings in this rapidly expanding field [3,20,90,101].

Metallochaperones

Genetic analysis in microorganisms has revealed classes of proteins that are essential for post-translational metal cofactor insertion into specific metalloproteins [6,24]. These have been dubbed as metallochaperones by extension of the traditional concept of molecular chaperones that assist in protein folding. Metallochaperones differ from simple metal storage proteins, e.g. bacterioferritins and metallothioneins, in that their primary functional role is in the insertion of the metal or metal cofactor in to a specific apo-enzyme and therefore, actively facilitate their maturation. A subset of metallochaperones require nucleotide cofactor, e.g. GTP and use the energy of GTP hydrolysis to facilitate metal transfer or some other process in the metalloenzyme maturation cycle [25–27]. These energy-dependent metallochaperones are used to build enzyme active sites that employ Fe, Ni and/or Co (B_{12} or cobalamin). We place emphasis here on the G3E family of P-loop GTPases that couple metal (or metal cofactor) delivery to a target protein to GTP binding and hydrolysis. More recently, a subset of G3E GTPases have been proposed to be involved in maturation of selected Zn metalloenzymes under conditions of extreme Zn limitation [15].

G3E family GTPases, like most G-proteins, bind GDP and GTP using several short, conserved loops that often occur between an α-helix and a β-strand and often at a dimer interface, numbered G1–G5 [28] (Figure 2). G1, also known as the P-loop or Walker A motif binds to the phosphates in GDP. G2 and G3 are also known as switch I and switch II/Walker B motifs respectively, and interact with the phosphate and Mg^{II} in the active site, activate a water molecule for GTP hydrolysis and signal via a conformational change when hydrolysis has occurred. The G3E family gets its name from a Glu (E) in the G3 motif, ExxG instead of the canonical DxxG [29] (Figure 2). It is known that the glutamate directly participates in coordinating the Mg^{II} ion, whereas the more typical aspartate makes a water-mediated contact to the metal ion [29,30]; however, the functional impact of the D-to-E substitution remains unclear. Finally, the G4 and G5 motifs form contacts with the guanine base and are responsible for distinguishing GTP from ATP or other nucleotide triphosphates. G3E family members involved in metalloprotein maturation also contain a transition metal-

binding site, in between the G1 and G3 motifs, as in the case of HypB [31]. GTP hydrolysis induces a reorganization of the loops (G2, G3) in close proximity to the metal-binding residues [30,32] which may ultimately explain how GTP hydrolysis enforces metal selectivity; however, our understanding of metal selectivity determinants in these enzymes remains rather limited.

An historical extension of this definition of a metallochaperone as an 'insertase' involves a general 'shepherding' or chaperoning process, in which a specific metal present in excess is delivered to a transporter without dissociation into bulk solvent, followed by efflux from the cytoplasm. This latter definition describes most bacterial Cu^{I} chaperones quite well, as touched on briefly below. Both processes necessitate either stable or short-lived, transient *and* reversible protein–protein interactions to effect metal ligand exchange and metal transfer without dissociation into the bulk solution.

Copper metallochaperones

Copper chaperones are found in all kingdoms of life and function as high affinity Cu^{I}-binding proteins that deliver their cargo to specific molecular targets [33]. In bacterial pathogens, however, there is no known significant need for Cu^{I} in the cytoplasm and as a result, Cu^{I} is generally considered to be toxic as a result of possible Fenton chemistry in an oxidizing environment and mismetallation of other proteins and enzymes in cells [2]. In fact, Cu toxicity is used by the immune system to kill invading microorganisms, and adaptation to Cu^{I} stress generally involves channeling of Cu^{I} from donor to efflux transporter, for export to the periplasm (in Gram-negative bacteria) or the extracellular space [33].

Bacterial copper chaperones are found in both the cytoplasm and in the periplasm of Gram-negative bacteria and often adopt single domain ferredoxin-like [34] or cupredoxin-like [35] folds. Cytoplasmic Cu chaperones are typically soluble proteins, although *S. pneumonia* CupA ismembrane-anchored and all bindCu^{I} with high affinity ($K_{Cu} \approx 10^{18}\ M^{-1}$), and stabilize the Cu^{I} form in the cytoplasm of the cell [36]. Periplasmic Cu chaperones, on the other hand, bind Cu^{I} or both Cu^{I} and Cu^{II} in distinct sites, and include *Eschrichia coli* CusF, *Salmonella* spp. CueP, *E. coli* PcoC and *Pseudomonas* spp. CopC [33,35,36]. In both cytoplasmic and periplasmic Cu chaperones, Cu^{I}, although bound thermodynamically tightly, is kinetically labile in the presence of a target protein, and 'gives up its metal' to the target often down an affinity gradient, via metal-ligand exchange. In the case of delivery to an efflux transporter, this process becomes irreversible when coupled to ATP hydrolysis or for the periplasm-spanning efflux complex, the proton motive force, both of which drive Cu^{I} transport across a membrane against a concentration gradient [37]. Significant questions remain as to the structure and coordination chemistry of metal transfer intermediates and recent X-ray absorption spectroscopic approaches, coupled with high resolution structural analysis [38], promise to yield new molecular level insights into these Cu^{I}-transfer processes.

Metallochaperone systems involving G3E family GTPases

A number of metal-specific, GTP-requiring metallochaperones involved in the maturation of Co, Ni and Fe-containing enzymes, have been isolated and characterized to date, and include

the metallochaperones UreE and HypB (see Figure 2) involved in the maturation of Ni-requiring enzymes urease and [NiFe]-hydrogenase respectively [26,27]. UreE and HypB contain either a C-terminal histidine-rich motif or an N-terminal cysteine-rich motif that binds the metal and aids in the delivery of Ni^{II} ions to these apo-enzymes. However, in contrast with the Cu^{I} chaperones, efficient metal transfer requires a suite of accessory proteins. Among these accessory proteins is at least one P-loop GTPase from the G3E superfamily (UreG and HypB; see Figure 2) and it has been proposed that GTP hydrolysis is coupled in some way to metal transfer and/or metal release. Other members of this family include MeaB from *Methylobacterium extorquens*, required for the activation of methylmalonyl-CoA mutase, one of two vitamin B_{12} or cobalamin-dependent enzymes [39], and members of the COG0523 protein family, a large and diverse subfamily of proteins with poorly defined functions [25]. A member of the COG0523 family [25] has been proposed to deliver Zn to obligatory Zn-dependent enzymes in response to host-induced zinc starvation in *Acinetobacter baumannii* [15]. The mechanisms by which these essential chaperones perform their functions, however, are not well established, due in part to the absence of structural information on complexes formed with their target proteins.

Ni chaperones for Ni-Fe cluster maturation in [NiFe] hydrogenase

The large subunit of [NiFe] hydrogenase contains a unique Ni-Fe active site that is assembled by an elaborate metal-insertion and protein-processing pathway [26]. Ni^{II} is inserted into the large subunit by a dedicated metallochaperone complex that is composed of HypA and HypB, in a process that consumes GTP [40]. After GTP hydrolysis, Ni transfer occurs rapidly and selectively from HypB to HypA facilitated by weakened nickel-binding affinity and stronger protein–protein interactions [40,41]. After Ni insertion into the large subunit, the HypC chaperone dissociates and the hydrogenase undergoes final processing before binding to the hydrogenase small subunit [26]. In *E. coli*, but not in all hydrogenase-containing bacteria, HypB also interacts with a peptidyl-prolyl isomerase (SlyD) which is thought to act as a reservoir of readily accessible, bioavailable Ni [42,43]. It has been proposed, based on protein–protein interactions and localization studies [44], that HypA obtains N^{II} directly from the NikABCDE importer, with the implication that this occurs via ligand exchange rather than indirectly through Ni release into the bulk cytoplasm. HypA exists in both monomeric and dimeric forms, and the dimer forms such that the ligands to each structural Zn^{II} or Ni^{II} site are donated by both protomers [45–47] (Figure 3). Zn^{II} is thought to play a structural role in stabilizing the dimer since the Zn^{II}-binding domain forms part of the interface with HypB in the HypA–HypB complex [40,48]. It has been suggested that the Zn^{II} domain might function to regulate Ni loading on to *H. pylori* HypA [46,47]. Bismuth-based drugs are known to target *H. pylori* HypB [49], which suggests that the hydrogenase maturation machinery might provide an avenue to the development of new antibiotics that negatively impact the energy requirements of the pathogen [50].

Ni chaperones for urease metallation

The maturation of the trimer of trimers urease, encoded by *ureABC,* involves the regulated insertion of two Ni^{II} ions per protomer to form the binuclear Ni^{II}_2 active site and requires at least four accessory proteins, UreE, UreF, UreG and UreH (or the UreH orthologue, UreD) [27]. The pathogenicity of the gut pathogen *H. pylori* has been shown to rely heavily on

urease, which converts urea into ammonia in an effort to neutralize the surrounding acidic milieu of stomach acids [51]. Although the interactions and mechanisms of action of these accessory proteins have been extensively studied and are known to be essential for urease maturation [30,52–63], the direct binding of a metallochaperone complex to the target apo-enzyme has yet not been demonstrated, perhaps due to the transient nature of the complex. However, there is a crystal structure of the *H. pylori* $(UreHFG)_2$ complex [30] and the complex with UreE has been modelled [56] (Figure 3).

UreG is a G3E family Ni-dependent GTPase that catalyses the hydrolysis of GTP necessary for the maturation of urease [63]. The *H. pylori* UreG show a largely disordered region in the central portion of the molecule that comprises the interaction site with UreE and the catalytic loops [57], and thus relies on structural plasticity to perform its function. Ni^{II} or Zn^{II} binding induce GTP-dependent dimerization of UreG [54,61] (Figure 2). The formation of $(UreE)_2$–$(UreG)_2$ complex is a prerequisite for recruitment of GTP to UreG and Ni^{II} transfer from UreE to UreG [53]. Although Zn^{II} binding has been well documented for UreE, UreG and the $(UreE)_2$–$(UreG)_2$ complex [54,61,64–66], the functional role of Zn^{II} binding remains enigmatic. Remarkably, these urease accessory proteins are also capable of receiving Ni^{II} from the proteins that function in the maturation of [NiFe]-hydrogenase. For example, UreE and HypA are capable of forming a ternary $UreE_2$–HypA complex [59] where HypA-derived Ni^{II} can be trafficked to UreE, which is subsequently donated to UreG [53]. How Ni^{II} moves from $UreG_2$ to the $(UreFD)_2$ complex in a GTP hydrolysis-dependent fashion and ultimately to the urease apo-enzyme is not yet known (see Figure 3).

Co-chaperones derived from the G3E family

In humans, the P-loop GTPase MeaB is required for the assembly of the adenosylcobalamin (AdoCbl)-dependent MCM [67]. A recent study reveals that candidate MeaB proteins from *Mycobacterium tuberculosis* and the related saprophyte *M. smegmatis* and *M. thermoresistibile* strains are *bona fide* MeaBs, uncovering a role for MeaB in a bacterial pathogen for the first time [68]. The crystal structures of all three bacterial MeaBs reveal the characteristic three domain, homodimeric MeaB architecture with GDP bound in the GTP-binding sites [68] (Figure 3). In order to deliver the AdoCbl cofactor to the MCM active site, target protein bound, and GDP-stabilized monomeric MeaB is proposed to undergo a GTP-dependent conformational change from an open to a closed conformation upon GTP hydrolysis, deduced from the crystal structures of the holo- and apo-forms of MeaB in a complex with its target protein isobutyryl-CoA-synthase (ICM) [39]. In these structures, MeaB is part of fused MeaB-ICM like homodimer, as a model for a maturation intermediate of MeaB bound to MCM [39] (see Figure 3). GTP hydrolysis appears to drive transfer of AdoCbl into the ICM active site. In this process, MeaB does not directly deliver the metal cofactor to the enzyme but instead facilitates MCM maturation by functioning as a chaperone rather than an insertase.

G3E GTPases involved in Fe^{III} active site assembly in nitrile hydratases

Nitrile hydratases (NHases) are heterotetrameric ($\alpha_2\beta_2$) enzymes that catalyse the broad spectrum hydration of nitriles to their corresponding amides in a process of significant industrial importance [69]. NHase α-subunits harbour an unusual non-haemFe^{III} or non-

corrin Co^{III} active sites that feature both cysteine sulfenate acid and cysteine sulfinate moieties as ligands to the active site metal. Each enzyme class, while structurally homologous, appears to employ very distinct activator or metallochaperone strategies that catalyse the assembly and oxidative maturation of the Fe^{III} compared with Co^{III} active sites. In the case of the Fe^{III} NHases, a G3E family P-loop GTPase, *Rhodococcus equi* TG328-2, found in the same genomic locus, has been recently characterized and shown to exhibit weak GTPase activity [70]. This GTPase binds Co^{II} to a CCIC (where C is cysteine) cysteine thiolate-rich motif and exhibits very high sequence similarity to *A. baumannii* ZigA, which we believe is representative of the COG0523 subfamily of P-loop GTPases that are up-regulated under conditions of extreme zinc limitation [15]. It remains to be seen if the metal specificity of TG328-2 is an intrinsic feature of the enzyme or is dictated by the intracellular milieu of *R. equi* relative to other low Zn-inducible COG0523 candidate metallochaperones [71,72].

Candidate G3E family Zn^{II} metallochaperones

In contrast with the metalloenzymes discussed above, the metabolic footprint of Zn^{II} on intermediary metabolism is considerable, and would seem to argue against the evolution of large numbers of enzyme-specific Zn^{II} metallochaperones. However, metallation of a small subset of Zn^{II} metalloenzymes might require such a metallochaperone, but only under conditions of extreme host-mediated Zn^{II} limitation [23], in the same way that the requirement for the Cu chaperone CCS for Cu, Zn-SOD can be largely bypassed by excess Cu [73]. No zinc chaperone has thus far been unambiguously functionally identified and characterized. It has been known for a number of years now that COG0523 proteins represent one of the four subfamilies of G3E GTPases and are predicted to function as magnesium- and zinc-binding metallochaperones and are typically up-regulated under the conditions of Zn deprivation [25]. An unbiased mutant screen of *A. baumannii* stressed with Zn^{II} limitation identified components of the zinc uptake regulator (Zur) regulon [71] (see below), among which was found a gene encoding a COG0523 protein, denoted ZigA [15]. ZigA is likely to be structurally homologous to Zn^{II}-bound *E. coli* YjiA whose nucleotide-free structure is known [74] (Figure 3), but is functionally uncharacterized. The ZigA assembly state, like other G3E family metallochaperones, is modulated by nucleotide binding, with GDP and GTP stabilizing the monomer in equilibrium with dimer, and thus may be functionally analogous to that of the AdoCbl-activating MeaB-MCM-like system (see Figure 3). ZigA binds two mol•equiv of Zn^{II} (and Co^{II}) with high affinity for a cysteine thiolate-rich site, which modestly stimulates GTPase activity but has no impact on assembly state.

The precise function of ZigA is not known but is connected in some way to histidine catabolism and mobilization of Zn^{II} from Zn^{II}–histidine complexes [71]. The enzyme that catalyses the rate-determining step in histidine catabolism, histidine ammonia lyase (HAL), is strongly stimulated by Zn^{II}, and the hypothesis is that ZigA may make Zn^{II} bioavailable for HAL and other obligate Zn-requiring enzymes under these challenging intracellular conditions of low Zn^{II}. The identification of all ZigA client proteins is of very high interest.

Metalloregulatory proteins

In bacterial cells, a panel of DNA binding transcriptional regulators, termed metalloregulatory protein or metallosensors, collectively 'manage' the bioavailability of transition metals in the cell cytoplasm [75]. They do this by forming coordination complexes with a specific metal, or in some cases, a number of closely related metal ions; metal coordination, in turn, allosterically activates or inhibits binding to a specific DNA sequence termed a DNA operator that often overlaps with the promoter. Metalloregulatory protein binding here regulates either physical access or the activity of RNA polymerase, thus controlling the expression of downstream genes. Downstream genes typically encode metal transporters, metallochaperones (*vide supra*) or metal-storage proteins thus connecting metallosensor metal binding to transcriptional control of the adaptive response to too little or toxic concentrations of a specific metal. Cognate metal ion binding can drive transcriptional derepression (mediated by allosteric *inhibition* of DNA operator binding by metal), transcriptional activation (mediated by metal-dependent conformational changes on the bound DNA), or transcriptional co-repression (mediated by allosteric *activation* of DNA binding) of downstream genes [75,76].

Metallosensors are therefore functionally subdivided into two broad groups of regulatory proteins. One group mediates the up-regulation of metal efflux systems upon metal binding in response to metal toxicity generally via transcriptional derepression or activation mechanisms (Figure 4). The second group consists of metalloregulators that down-regulate metal uptake systems via transcriptional co-repression in response to cognate metal binding (Figure 5). The affinities for different metals generally follows the Irving–Williams series. Metal responsiveness is dictated by ligand set and metal coordination number and geometry, rather than intrinsic metal-binding affinity since formation of the 'right' coordination geometry upon binding cognate metal is a key feature of metal-mediated allosteric inhibition or activation of DNA operator binding. As discussed above, metal speciation in the cell reinforces these general trends, with the bioavailability of highly competitive metals (see Figure 1A) minimized. 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 [77]. This model, for which there is now strong evidence [20], is dictated by the sensitivity (K_{Me}) of individual metalloregulatory proteins for their cognate metals, where $K_{CuI} > K_{Zn} > K_{Ni} > K_{Co} > K_{Fe} > K_{Mn}$, thus establishing that cytoplasmic bioavailable (free) metal follows this trend: $[Cu^{I}]_{free} << [Zn]_{free} < [Ni]_{free} < [Co]_{free} < [Fe]_{free} \approx [Mn]_{free}$ (Figure 1A).

Among the more than 20 structural classes of so-called 'one-component' transcriptional regulatory systems in prokaryotes (where direct 'ligand' binding regulates DNA binding), no fewer than 11 of these structural families contain members that are metallosensors. In addition, most of these 11 structural classes also harbour representatives that control the adaptive response to cellular ROS, RNS, RES and the recently described RSS. These are also discussed here, given potential insights into the evolution of new functional specificities within a single protein family, and the interconnectedness of metal and highly reactive small molecule stressors that generally employ cysteine thiol oxidative chemistry [78]. Seven of these 11 structural families constitute major families of metallosensors, and include metal

efflux regulators derived from the arsenic repressor (ArsR), mercuric ion repressor (MerR), CsoR and CopY families (Figure 4), and metal uptake repressors from the Fur, diphtheria toxin repressor (DtxR) and nickel-responsive regulator (NikR) protein families (Figure 5). Four additional structural families of transcriptional regulators are such that the majority is not strongly associated with metallostasis and include members of the multiple antibiotic resistance repressor (MarR), tetracycline repressor (TetR), lysine repressor (LysR) and gluconate repressor (GntR) families (Figure 6). We discuss each of these protein families in turn as organized in Figures 4–6, with a description of metal efflux regulators (Figure 4) followed by primary uptake regulators (Figure 5), and finally the four families of repressors within which metallosensors are significantly under-represented (Figure 6).

Metal efflux regulators

ArsR family

Individual members of the ubiquitous ArsR family of ≥3000 transcription repressors have evolved to sense a remarkably diverse array of metal, metalloid and non-metal inducers (Figure 4) employing distinct sites on a relatively simple, homodimeric winged-helical scaffold [75,79,80] (see Figure 7, below). Metal binding and inducer recognition exclusively drive transcriptional derepression of genes associated with the efflux, sequestration and/or detoxification of excess metals or other stressors.

The metal-binding sites and the positions of chemically reactive thiols are named according to the secondary structure elements from which inducer sites are derived [79] and are now characterized by five structurally distinct sensing sites (see Figure 7, below). The α3 and α4 sites are composed of cysteine residues that are used to coordinate highly polarizable (soft) metals such as Cd^{II}, Pb^{II}, Bi^{III} and As^{III}, employing an S_3 (As^{III}) or S_4 ligand set (Cd^{II}, Pb^{II}, Bi^{III}) [81–84]. An alternate α3 site is also known to coordinate Zn^{II} in some cases with an additional ligand from the N-terminal region [85–87], often a histidine to form an S_3N donor set. The C-terminal α5 site in ArsR repressors, represented by *S. aureus* CzrA [88], is composed of an all N/O-donor ligand set used to coordinate harder metals such as Zn^{II}; in some cases, the α5 site is further elaborated to bind Ni^{II} and Co^{II} with octahedral coordination geometries, achieved by the recruitment of N/O donors from the extreme N-terminal tail [89]. It is becoming increasingly clear that ArsRs are not restricted to metal sensing [90–98], with individual members now known to have evolved to sense ROS [93,94,98,99] and RSS [90,92] via a conserved cysteine pair in the α2 and α5 helices, while RES appear to be sensed by a cysteine in α1 helix [91]. It is important to point out here that redox-sensing ArsR family members are often misannotated as MarR family members due to known or anticipated functional similarities (*vide infra*). MarRs as a distinct protein family are readily distinguished from ArsRs by the presence of an extended four-helix bundle regulatory domain involving the C-terminal α5 and α6 helices of both protomers within the homodimer (see Figure 7, below).

Structural changes in ArsRs upon metal binding or oxidative modification are generally small, with the exception of some reactive oxygen, sulfur and electrophile species which appear to induce somewhat larger conformational changes in the homodimer that allosterically inhibit DNA binding [91–93]. It therefore remains challenging to understand

how sensing is physically connected to regulation of DNA binding. It has been proposed that that metal coordination induces changes in the dynamical properties of the repressor that impact the ability of the active repressor form to explore an excited state conformation that is compatible with DNA binding [100]. Recent methyl-relaxation based NMR experiments carried out with the Zn^{II} sensor CzrA suggests that this picture may be an oversimplification [101]. In that work, it was shown that Zn^{II} coordination induces a redistribution of conformational entropy that is a key feature of the inhibition of DNA binding. Allosterically impaired mutants simply perturb this entropy redistribution in a way that fails to trigger dissociation of the repressor from the DNA [101].

MerR family

MerR is a Hg^{II} sensor and founding member of a class of MerR family regulators that function nearly as exclusively as transcriptional activators via a metal-induced allosteric model that converts a suboptimal promoter into an active promoter via a DNA underwinding mechanism [102] (Figure 4). MerR metalloregulators alleviate metal toxicity induced by a wide range of biologically required metals, metal ion pollutants and oxidative stressors, by triggering the expression of specific efflux or detoxification system upon metal detection [102–104]. All MerR proteins possess an N-terminal winged-helical domain composed of a canonical winged helix-β hairpin structure, followed by a long dimerization helix that forms an antiparallel coiled coil in the homodimer (Figures 4 and 7). MerRs represent a remarkably functionally diverse family of regulators that exploit a common DNA-based allosteric switching mechanism, for which structural details are now in hand for CueR [105] and the Pb^{II} sensor PbrR [103]. In single molecule tracking experiments in living *E. coli* cells, the apo- and metal-bound holo forms of the Cu^{I} sensor CueR and the Zn^{II} sensor ZntR show distinct concentration-dependent dissociation kinetics from their DNA operators, findings that suggest a novel mechanism of facilitating transcriptional turn-off compared with activation in cells [106].

The functional specificity of a single sensing site is achieved by precise remodelling of coordination number, ligand type and geometry in MerRs [103,107,108]. MerRs bind inducing metals ions via coordination by a minimum of two conserved cysteine residues present in a C-terminal metal-binding loop, and Cu^{I}, Ag^{I} and Au^{I} all adopt this *bis*-thiolato coordination structure, e.g. in CueR and GolS. Divalent metal ion MerR family sensors, including the prototypical Tn501 plasmid-encoded MerR and ZntR, add a third ligand to the coordination sphere, often found at the N-terminal region of the dimerization helix; in CueR and GolS, this cysteine is a serine [107,108]. More recent studies reveal that MerRs have evolved to sense a wide range of stimuli, and include the photosensory transcriptional regulator LitR [109], SoxR, which sense oxidative stress via reversible oxidation–reduction in a [2Fe-2S] cluster assembled in the same region, and *B. subtilis* BmrR and *P. aeruginosa* BrlR, which bind bulky organic molecules to a far larger regulatory domain [110–112]. NmlR (*Neisseria* MerR-like regulator) senses oxidation of a conserved cysteine located in a pocket in the hinge region between the N-terminal DNA-binding domain and the central dimerization helix by neutralizing cytotoxic compounds produced by oxidative stress [113]; the mechanism of sensing however is as yet unknown. AdhR (alcohol dehydrogenase regulator) from Gram-positive organisms has also been proposed to utilize cysteine thiol

chemistry to respond to reactive electrophiles (RES) such as formaldehyde [113] (Figure 3). Similarly, *Haemophilus influenzae* NmlR is a thiol-dependent transcription factor that detects formaldehyde and functions by DNA unwinding, in the absence of the C-terminal sensing-site region [114], the mechanism of which remains undefined.

CsoR family

CsoR [115] and RcnR (resistance to cobalt and nickel repressor) [116] are founding members of the CsoR transcriptional repressor family [117]. CsoR has been shown to be essential for resistance to copper toxicity in *M. tuberculosis* [118] and *S. aureus* [119]. These proteins adopt a disc-shaped, all α-helical dimer of dimers, tetrameric architecture, with a four-helix bundle as a key structural subfeature [115,120,121] (Figures 4 and 7). Individual CsoR-family paralogues have been shown to coordinate Cu^{I} or Ni^{II}/Co^{II} [20] or differentially exploit cysteine sulfur oxidative chemistry to specifically sense reactive electrophiles (RES) including formaldehyde [120,121] or RSS [122]. Much like the MerR family [117], the biological specificity towards different stressors derives from subtle variation on a single peripheral sensing site, in this case a 'WXYZ' amino acid fingerprint with the X-residue an absolutely invariant cysteine residue [123–125]. Metal binding or cysteine oxidative chemistry appears to subtly alter the shape of the tetramer to a more hydrodynamically compact structure [126] while altering the surface electrostatic potential distribution of the tetramer [123,127], leading to disassembly of the operator DNA–CsoR complex [123,126,128]. A recent study on the Ni^{II} sensor InrS shows that the substitution of metal-binding residues from the 'WXYZ' amino acid fingerprint decreases the affinity for the metal ion with no effect on regulation, thus defining a different 'set-point' that controls Ni^{II} availability in cells [20]. The structure/reactivity features that distinguish the formaldehyde (RES) sensor FrmR, from the persulfide (RSS) sensor CstR, froma Cu^{I} or Ni^{II} sensor are only incompletely understood, although there has been significant recent progress on this question [119,123]. It also seems likely that as yet uncharacterized CsoR family sensors will appear that increase the functional diversity of this still-growing class of metal- and inducer-sensing repressors [128].

CopY family

The copper-specific metallosensor CopY is proposed to be a member of MecI/BlaI family due to the high sequence similarity in the N-terminal DNA-binding domain and identical cognate DNA operator sequences [129]. The DNA-binding domain adopts a canonical winged-helical domain like that found in ArsR, MerR and MarRs, as determined by the solution structure of the N-terminal domain of *L. lactis* CopR [130]. The C-terminal domain functions as the regulatory and dimerization domain [131] and is of unknown structure and responsible for the coordination of Cu^{I} (Figure 4). CopYs regulate copper efflux and the oxidative stress response in a small number of bacterial pathogens from closely related Firmicutes, derived from the *Lactococcus, Streptococcus* and *Enterococcus* spp. [132–135]. CopYs have a conserved a single C-terminal **CXC** or duplicated **CXC**XXXX**CXC** motif that is known to coordinate Cu^{I} or Zn^{II} in distinct coordination geometries and stoichiometries [134]. Zn^{II} is reported to function as an allosteric activator of DNA binding required for full repression of the *cop* operon in the absence of Cu^{I} stress; as Cu^{I} levels in the cell rise, two Cu^{I} displace Zn^{II} to form an S_4-Cu_2 cluster that impairs DNA binding. A

fascinating question for future work is *how* differential occupancy of the C-terminal metal site by Cu^{I} compared with Zn^{II} differentially allosterically impacts DNA binding and *cop* operon expression.

Metal uptake regulators

Fur family

In most bacteria, members of the ubiquitous Fur superfamily are involved in the regulation of Fe and Zn metabolism [136] via transcriptional control by Fur [137] or Zur repressors. A third Fur family member, the peroxide stress response repressor PerR is present in many Gram-positive organisms, establishing a tight functional interconnectedness with the global regulator Fur, often present in the same cytoplasm[75]. Other Fur family repressors have been functionally characterized and shown to function in metal homoeostasis [138], including the manganese uptake regulator (Mur) involved in Mn^{II} and Fe^{II} sensing [139], the nickel responsive regulator (Nur) [140], and the haem iron responsive regulator (Irr). Like other metalloregulatory protein families, Fur proteins harbour an N-terminal winged-helix DNA-binding domain, with the regulatory and structural metal-binding sites found in the carboxy domain of the protein; this C-terminal domain also enables dimerization [136] (Figure 5).

Fur family structures generally reveal up to three metal-binding sites per protomer [138]. The primary metal sensing site region derives from a conserved histidine- and cysteine-rich motif, **HHH**X**H**X_2**C**X_2**C**, located in the N-terminal region of the C-terminal dimerization domain. In PerR, Fe^{II} binds to this same region but binds H_2O_2 to an open coordination site of the Fe^{II} which performs Fenton chemistry on the Fe chelate, leading to histidine ligand oxidation, dissociation of the Fe and PerR dissociation from the DNA operator, inducing transcriptional derepression [141]. In contrast with the regulator families described above, all Fur family repressors bind metal and none are known to performthiol oxidative chemistry in response to ROS, RNS or RSS (Figure 5). The Fur family provides an excellent illustration of how transition metal selectivity can be tuned by evolving a single primary metal site to adopt distinct coordination numbers and geometries optimized to mediate a metal-selective allosteric response, much like the MerR regulators. A second CXXC motif near the C-terminus forms a $Zn^{II}Cys_4$ structural site that stabilizes the dimer (see Figure 7, below). A third (secondary) metal site is found in some Fur-family regulators, and employs ligands derived from the C-terminal regulatory domain only and is ‘‘interdigitated’’ with metal ligands from the histidine patch that coordinate the primary metal ion.

It is generally believed that metal coordination to the primary site stabilizes a ‘closed caliper’ form of the dimer that stabilizes its interaction with the DNA operator (Figure 5). A specialized feature of Fur-family repressors is the ability to control the expression of diverse operons as a global regulator. This has been rationalized for *B. subtilis* Fur in a study that explores the conformational plasticity of Fur bound to different ligands, metals ions or small molecules and the DNA operator [142]. The Fur and Zur homodimers are capable of forming a number of differentially metallated complexes. Apo-Zur, for example, generally refers to a form with Zn^{II} bound to the structural Cys_4 site with the primary and secondary (if present) metal sites empty, while Zn_2 and Zn_4 forms refer to distinct holoforms with Zn^{II}

bound to the primary compared with primary and secondary (third) sites respectively. The precise metallation status of the repressor appears to differentially impact the binding of Fur or Zur to different DNA operators, enabling a graded response to increasing degrees of cellular metal limitation [143,144].

DtxR family

The DtxR and *M. tuberculosis* IdeR [145] are the prototypical members of a family of Fe^{II}- and Mn^{II}-regulated repressors that control the transcription of virulence genes in pathogenic bacteria [146]. In general, metal binding represses the expression of metal uptake systems. Crystallographic studies of DtxRs reveal that the active form of the repressor is a homodimer consisting of an N-terminal winged-helical DNA-binding domain and C-terminal domain harbouring often two structurally distinct, yet conserved metal-binding sites per protomer [147–152] (Figure 5). One of these sites (site B) is often structural, yet metal binding here maximizes allosteric activation of the primary metal sensing site A (Figure 5). The difference in metal-binding stoichiometries in the primary sensing site (binuclear compared with mononuclear) originally suggested a mechanism for selectivity in the Mn^{II}-specific sensor, MntR, from *B. subtilis* [152], since non-cognate metal ions, Fe^{II}, Zn^{II} and Co^{II}, could only form mononuclear metal complexes. However, both one- and two A-site metal-containing DtxR family Mn^{II}-specific sensors have been structurally and functionally characterized [153,154]. Several mechanisms of allosteric control of DNA operator binding have been proposed and it is generally accepted that metal binding induces major structural rearrangements dependent on recruitment of carboxylate ligand donor from the extreme N-terminus of each protomer, e.g. Asp^{7} in the Mn^{II} sensor *S. pneumoniae* PsaR (Figure 5). This leads to a reorientation of the DNA-binding helices and allosteric activation of DNA binding [147,155].

NikR family

NikR is a Ni^{II}-activated homotetrameric, dimer of dimers [156–158] and only known metal-sensing member of the ribbon-helix-helix superfamily (Figure 5). *E. coli* NikR represses the expression of a Ni^{II}-specific ABC-type importer, while *H. pylori* NikR also acts as a transcriptional activator of *ure* operon expression (*vide supra*) [159]. A number of crystal structures of NikRs in various allosteric states reveal four Ni^{II} ions bind with picomolar affinities, as measured by chelator competition assays [160,161]. The C-terminal regulatory domain hosts the four symmetry related regulatory metal-binding sites, each consisting of three conserved histidines and one cysteine located at the tetramerization interface.

Three distinct conformations of NikR have been observed in crystal structures depending on the conformation of a flexible linker between the DNA binding and regulatory domains: open, *trans* and *cis*. Residues that form non-specific electrostatic contacts with the DNA have been shown to be critical for Ni^{II}-activated DNA binding [162]. This has led to the proposal that activation of non-specific DNA binding initiates a 1Dsearch for the operator-promoter sequence, a model supported by structural and computational studies [163]. The work on *H. pylori* NikR posits an allosteric model, in which formation of the square planar, tetragonally distorted Ni^{II} coordination complex specifically increases the interdomain dynamical fluctuations in order to promote DNA operator binding, i.e. via an ‘unlocking’

mechanism [164,165]. Cu^{II}, which adopts a coordination geometry that is very similar to that of Ni^{II}, is also a potent allosteric activator; the bioavailability of Cu^{II} in the reducing cell cytoplasm, however, is vanishingly small, thus making NikR a Ni^{ii}-specific switch (*vide supra*). Non-cognate metal ions Zn^{II} and Co^{II} bind with different affinities, stoichiometries and form non-native coordination geometries and ligand sets that do not support a metal-mediated allosteric response [160,166]. Ni^{II} also binds to lower binding affinity Ni^{II} sites leading to an enhancement of DNA binding affinity [166,167], while different conformations have been shown to bind different promoters [168–170]. NikR thus provides an exceptional illustration of the importance of metal coordination geometry as a key feature of cognate metal sensing by a metalloregulatory protein, that is reinforced by the prevailing intracellular metalmilieu. In addition, the presence of lower affinity Ni^{II} suggests the possibility of a graded response to Ni^{II} stress, as has been documented for Zur (*vide supra*) [143].

Other metalloregulatory protein families

TetR family

The TetR family of all α-helical repressors (Figures 6 and 7), like the MarR family described below, are widely associated with antibiotic resistance and the regulation of genes encoding small molecule exporters [171]. TetRs also play a far broader metabolic regulatory role, controlling the expression of genes involved in central carbon metabolism, antibiotic production, quorum sensing and many other aspects of prokaryotic physiology. There are more than 200000 proteins identified as TetRs to date and more than 200 high-resolution structures have been deposited in the Protein Database (PDB). Canonical TetRs function as ligand mediated, homodimeric repressors, and each subunit harbours an N-terminal three-helix DNA-binding domain (α1–α3), followed by a much larger C-terminal regulatory domain (α4–α9) that incorporates a number of independently evolved ligand-binding cavities that are readily accessible from the side, front or top of the dimer (Figure 7).

Three TetR family proteins have thus far been characterized as responsive to changes in cellular metal concentration and speciation. The clearest example of a TetR-family metallosensor is the transcriptional activator of the *S. pneumoniae* Zn^{II} efflux system, SczA [172]. The metal binding affinity, stoichiometry and coordination chemistry have recently been reported for *S. pneumoniae* SczA, and suggest an allosteric activation model that requires the filling of two pairs of metal sites per dimer [3]. SczA activates the expression of the gene encoding the Zn^{II} effluxer, CzcD in response to Zn^{II} binding. This contrasts sharply with the transcriptional regulator HrtR, the haem-regulated transporter regulator, which binds haem and induces DNA dissociation and transcriptional derepression of a haem-efflux system in *L. lactis* [173,174]. Finally a putative Cu-responsive TetR has been identified as ComR in *E. coli* [175,176]. Although it is clear that ComR is involved in the Cu stress response in some way, direct Cu binding by ComR has not yet been reported.

Investigation of the structures of a number of TetR family repressors in different allosteric states suggests a model where ligand binding induces a pendulum-like motion in the dimer that significantly increases the distance between the two N-terminal DNA-binding domains on each protomer, relative to a DNA-binding competent conformation [171,177,178] (Figure 7). Ligand binding has also been shown to increase the folding cooperativity between

different domains [179]; in HrtR, haem binding induces a coil-to-helix transition in a long α4 interdomain connecting helix, resulting in a rigid body motion of the DNA-binding domain into an orientation that is incompatible with DNA binding [174]. A fascinating question for future work is the degree to which these conformational transitions characterize other metal-sensing TetRs, and how metal specificity is built into this as yet small number of metal-sensing TetRs.

MarR family

The MarR family of transcriptional regulators is named for *E. coli* MarR [180,181]. This large 12000 member protein family shares the same winged-helical DNA-binding fold of ArsRs, but harbours an additional C-terminal (α6) helix in the dimer interface that alters the quaternary structure of the dimer relative to ArsR family repressors (Figures 6 and 7). MarRs that repress the expression of drug efflux pumps may bind antibiotics or other substrates leading to the transcriptional depression of the genes associated with an adaptive response to antibiotic stress, e.g. efflux systems [180]. Other MarRs may contribute to virulence by regulating the activity of genes involved in a number of diverse stress responses, or the degradation of toxic molecules [182–187]. While some MarRs activate transcription, the vast majority repress transcription in their apo- or ligand-free states [180]. For many MarRs, the binding of organic molecules occurs in a cleft between the DNA binding and dimerization domains [180,188]; however, the physiological significance of this binding mode in *E. coli* MarR has recently been called into question [182]. In other MarRs, cysteine oxidation is known to occur in distinct sites in the molecular scaffold, dependent on the nature of the stressor [182–185] (Figure 6). In *E. coli* MarR, this reactive cysteine is positioned in the DNA-binding helices giving rise to what is reported to be a Cu^{II} dependent disulfide-crosslinked tetramer that physically blocks DNA binding by the homodimer [182]. Although it has been argued that this chemical modification can be considered (local) Cu^{II} sensing (Figure 6), the ability of other oxidants to induce oxidation of these cysteines has not yet been thoroughly investigated.

In contrast with the extraordinary functional diversity of stress-sensing MarRs, MarR family metallosensors are restricted to a single known regulator of Zn^{II} uptake [177,178,189], exemplified by AdcR from *S. pneumoniae* and closely related *Streptococcus* and *Lactococcus* spp. [177,187,190] (Figures 6 and 7). AdcR possesses two closely spaced pseudotetrahedral Zn^{II}-binding sites that bind Zn^{II} with different affinities (Figure 7). Zn^{II} is an allosteric *activator* of DNA operator binding, in strong contrast with all other members of the MarR superfamily, consistent with its biological function as uptake repressor at high intracellular Zn^{II}. A number of distinct allosteric mechanisms have been proposed for this large family [180,181,183, 191–194], from a domino-like response [183], to ligand binding-mediated effects on asymmetric motions [192]. The structural versatility required to accommodate binding of a wide range of ligands to different sites, coupled with the wide distribution of oxidation sensing cysteine residues and low overall sequence similarity, collectively suggest a model where ligand binding induces a conformational change in the dimer that cannot be explained by a single molecular pathway, and is thus reminiscent of ArsR family repressors (*vide supra*) [101] (Figure 7).

LysR family

The LysR-type transcriptional regulators (LTTR) are named for the LysR, which regulates lysine biosynthesis and comprise the largest family of transcriptional regulators in prokaryotes. Members of this family possess the same domain-based molecular architecture as found in other regulators discussed here, i.e. an N-terminal DNA-binding domain and C-terminal globular ligand-binding domain. LTTRs are involved in the regulation of basic metabolic pathways or virulence gene expression. *E. coli* OxyR is the prototypical redox-responsive LTTR that mediates transcriptional activation of an oxidative stress regulon in response to nanomolar hydrogen peroxide. OxyR exploits cysteine sulfenylation chemistry to affect transcriptional activation of the expression of target genes. OxyR can also respond to superoxide and nitrosative stresses, although these functions are presumed to be ancillary to its primary role as H_2O_2 sensor [195,196]. Metallosensors identified for this family are limited thus far to the sensing of the oxyanion molybdate (Mo^{VI}) by ModE, which represses the transcription of Mo^{VI} uptake system in *E. coli* and other bacteria [197,198]. Crystal structures of both apo- and molybdate-bound forms of ModE reveal [199] a homodimeric architecture where molybdate bound in the C-terminal specificity domain alters the relative orientation of the N-terminal DNA-binding domains from each protomer into a DNA-binding competent state (Figure 6). No LTTR is known to bind transition metals directly. A recently proposed ‘sliding dimer’ model of transcriptional activation for a homotetrameric LTTR that activates transcription (DntR) suggests inducer binding alters the quaternary structure of the tetramer and changes the supramolecular features of the DNA-bound complex, converting the LTTR from a repressor into a transcriptional activator. This model of transcriptional activation is somewhat reminiscent of MerR-family regulators [200].

GntR family

The GntR family is named for the gluconate operon repressor from *B. subtilis* [201] and currently comprises some 50000 sequences. GntRs also possess a domain-based architecture, like LTTRs (Figure 6). The inducer typically impacts the assembly state of the regulator [201] and it has been shown that changes in dynamics are associated with ligand sensing [202]. Several repressors that belong to the FadR subfamily of GntRs, named for the acyl-CoA regulator, have been shown to bind metals with nanomolar affinities [203], particularly Zn^{II} and Ni^{II} in a conserved histidine-rich binding pocket [203–206]. However, the relatively buried location of the bound metal suggests that metal binding may play a structural role, such as facilitating the binding of a specific metabolite. A recent structure of a regulator of citrate metabolism, CitO, from *Enterococcus faecalis*, shows that both citrate and a metal ion (Ni^{II} and Zn^{II} to a lesser extent) binding are necessary for formation of the DNA complex [204]. These results suggest that the location of the metal-binding site in GntR family may be related to its role as ‘dual’ sensor. In addition to CitO, a global regulator in *M. smegmatis* has been shown to respond, directly or indirectly, to Cu^{II} stress [118]. However, direct Cu^{II} binding to this regulator has not been reported, and could be sensing collateral oxidative stress induced by this pro-oxidant, as in the case of *E. coli* MarR (*vide supra*).

Perspectives

A tug-of-war between the mammalian host and bacterial pathogen for nutrients, including first-row transition metals, occurs during infection. In order to propagate and cause infection, microbial pathogens must both acquire these metal nutrients from the host [1] and prevent toxic effects of metals that are used as bactericidal weapons, e.g. Cu and in some niches, Zn [2,3]. Therefore, the pathogen adaptive response to host-induced stress conditions has to be highly metal-specific in a way that minimizes the potential cross-talk between homoeostasis systems devoted to a particular metal. To do this, biology optimizes foundational tenets of small molecule inorganic chemistry, e.g. the nature and number of coordination bonds, specific geometries and metal reactivities, to fine tune metal-responsive sites in proteins, while leveraging an intracellular milieu that features exchange-labile small molecule- and metabolite-metal complexes to 'buffer' transition metals to an extent dictated by their relative competitiveness (see Figure 1) and cellular needs [15,20]. An exciting future direction is to obtain a comprehensive understanding of transition metal speciation in the cell, how this differs from bacterial cell to cell, and how this ultimately impacts metallostasis.

Metallochaperones and metallosensors discussed here collaborate to mediate the adaptive response to too much or too little metal in the cell, employing an impressive array of molecular scaffolds (Figures 3–7). A subset of these metallosensor metal-sensing sites have been further elaborated to detect small molecule stressors, including reactive oxygen, nitrogen, electrophile and sulfur species. In contrast with our relatively mature understanding of metal selectivity of metallosensors summarized here, how nature evolves 'chemical selectivity' in small molecule stress sensors is not well established. This represents an exciting future research direction where we hope to achieve a level of understanding on par with what, for example, distinguishes a metallosensor from a metal-based redox sensor, e.g. a Fur from a PerR [136,141] (see Figure 5)or a CueR from a SoxR [105,207] (see Figure 4).

Note added in proof (5 May 2017)

We direct the reader to a paper that appeared during the preparation of this manuscript that highlights structural work on a specific member of the Rrf2 superfamily not discussed here [211]. Rrf2 family repressors regulate iron-sulfur cluster biogenesis (IscR), cysteine metabolism (CymR), iron homeostasis (RirA) and the sensing of reactive nitrogen species via a [4Fe-4S) cluster (NsrR).

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Abbreviations

AdcR	adhesin competence regulator
AdoCbl	adenosylcobalamin
ArsR	arsenic repressor
CopY	copper-responsive repressor
CsoR	copper-sensitive operon repressor
CzrA	chromosomal zinc-regulated repressor
DtxR	diphtheria toxin repressor
Fur	ferric uptake regulator
GntR	gluconate repressor
HAL	histidine ammonia lyase
HypB	[NiFe]-hydrogenase accesory GTPase
ICM	isobutyryl-CoA-synthase
LTTR	lysine repressor-type transcriptional regulator
LysR	lysine repressor
MarR	multiple antibiotic resistance repressor
MCM	methylmalonyl-CoA mutase
MeaB	MCM accesory GTPase
NHase	nitrile hydratase
NikR	Nickel responsive regulator of the nik operon
NmlR	*Neisseria* MerR-like regulator
Nur	Fur family nickel uptake regulator
RES	reactive electrophile species
RNS	reactive nitrogen species
ROS	reactive oxygen species
RSS	reactive sulfur species
TetR	tetracycline repressor
UreG	urease accessory GTPase
Zur	Fur family zinc uptake regulator.

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Summary

- Transition metal homoeostasis, metallostasis, is an emerging aspect of the vertebrate host–bacterial pathogen interface, during which the host attempts to ‘remodel’ the landscape of transition metal availability and toxicity as a means to limit infections.
- Metallochaperones function in metal allocation and delivery to select metalloenzymes so as to enhance the metal specificity of these processes while minimizing deleterious side reactions.
- Bacteria deploy specialized metalloregulatory proteins or ‘metallosensors’, that detect changes in intracellular metal bioavailability, leading to a transcriptional response to too much or too little cellular metal.
- Metallosensor protein families include representative members that drive allosteric inhibition or activation of DNA binding in response to reactive oxygen, reactive nitrogen, reactive electrophile and RSS using cysteine thiol chemistry.
- How metallosensors evolve new metal or inducer specificities while enforcing metal selectivity of what are essentially massively parallel metal homoeostasis systems in cells is an important aspect of future work in this field.

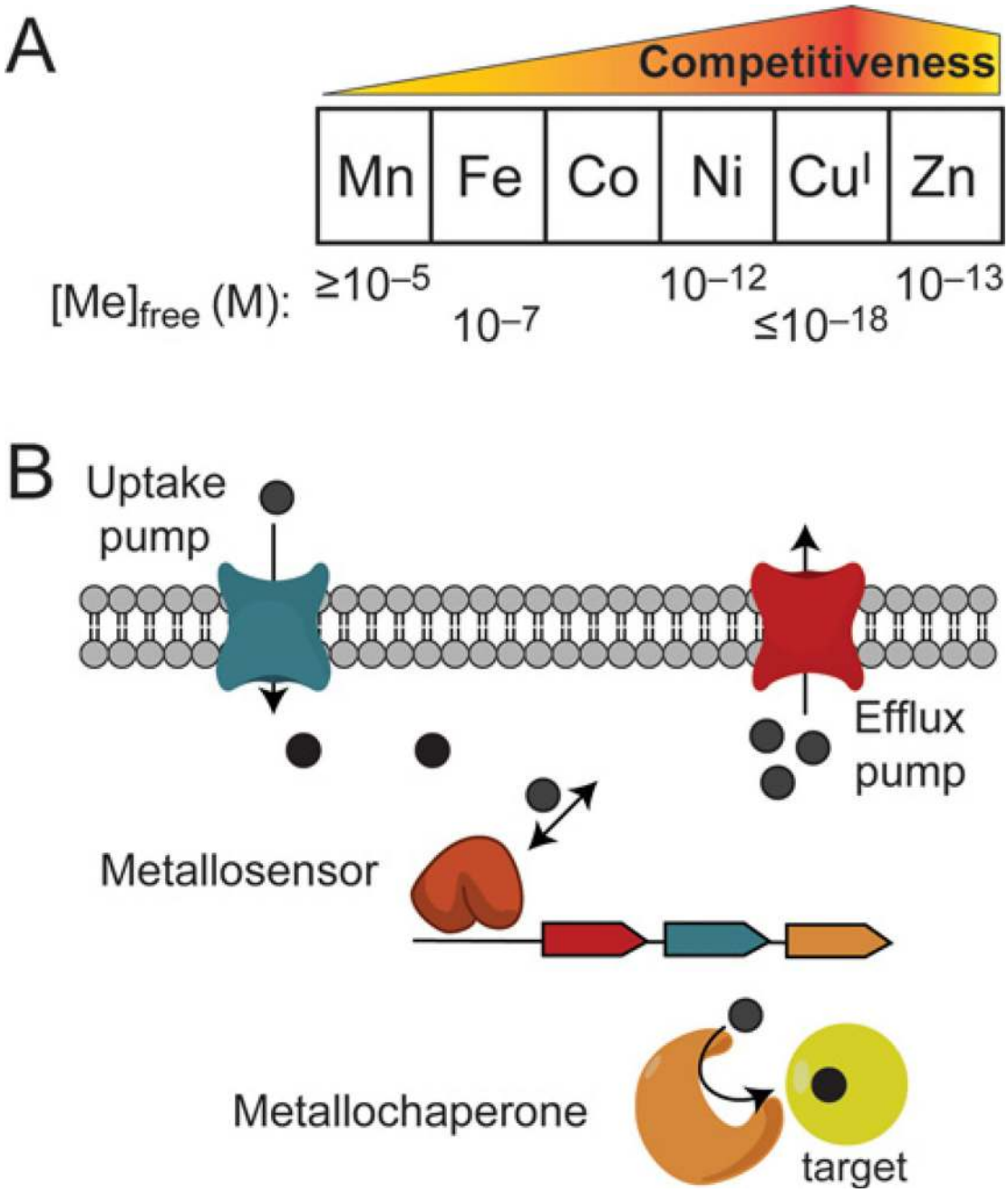

Figure 1. Overview of the central concepts of transition metal homoeostasis

(**A**) Biologically important late *d*-block first-row transition metals extracted from the periodic table from Mn to Zn. The competitiveness in a cellular environment is inversely related to 'bioavailability' of each metal in the cell. Bioavailability is roughly based on reported metal sensor affinities for their cognate metal and is not a direct measure of rapidly exchangeable metal in cells [23]. (**B**) Schematic representation of bacterial response to metal-induced stress, highlighting the roles of sensor proteins and metallochaperones discussed here. Note that a metallochaperone may not necessarily function via a direct

interaction with the target or client protein; in fact, much of the work on the G3E GTPases discussed here argues against a direct insertion mechanism (see text for details).

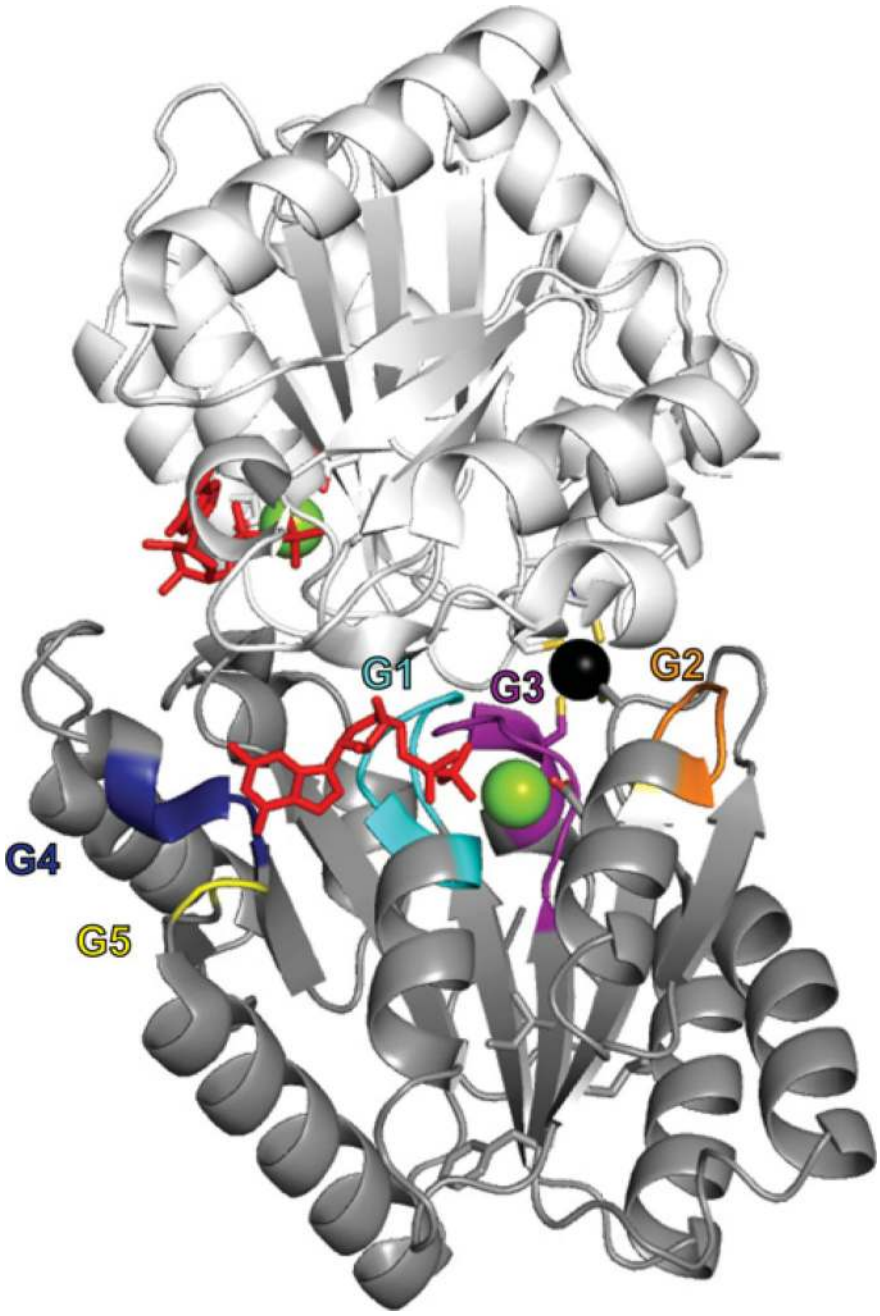

Figure 2. Ribbon representation of the structure of homodimeric *Helicobacter pylori* HypB (4LPS) with one subunit shaded *grey*, the other shaded *white* as representative of a G3E P-loop GTPase

Signature motifs G1–G5 are indicated on the structure shaded *cyan, orange, magenta, blue* and *yellow* for G1–G5 respectively. G1, P-loop/Walker A motif, GxxxxGKS/T; G2, switch I, with only a threonine nearly universally conserved; G3, switch II/Walker B motif, with the minimal sequence DxxG, but ExxG in G3E family GTPases; G4, base recognition motif/ base specificity loop, (S/N/T)KxD; G5, guanine base specificity determinant, with the sequence motif SAK, but only weakly conserved in G3E GTPases. Mg^{II} ions, *green*; GTP, *red* stick; tetrathiolate (S_4) subunit bridging transition metal-binding site bound to Ni^{II}

(shaded *black*); Zn^{II} in a related bacterial HypB is also known to bind here with a different coordination structure and stoichiometry [208].

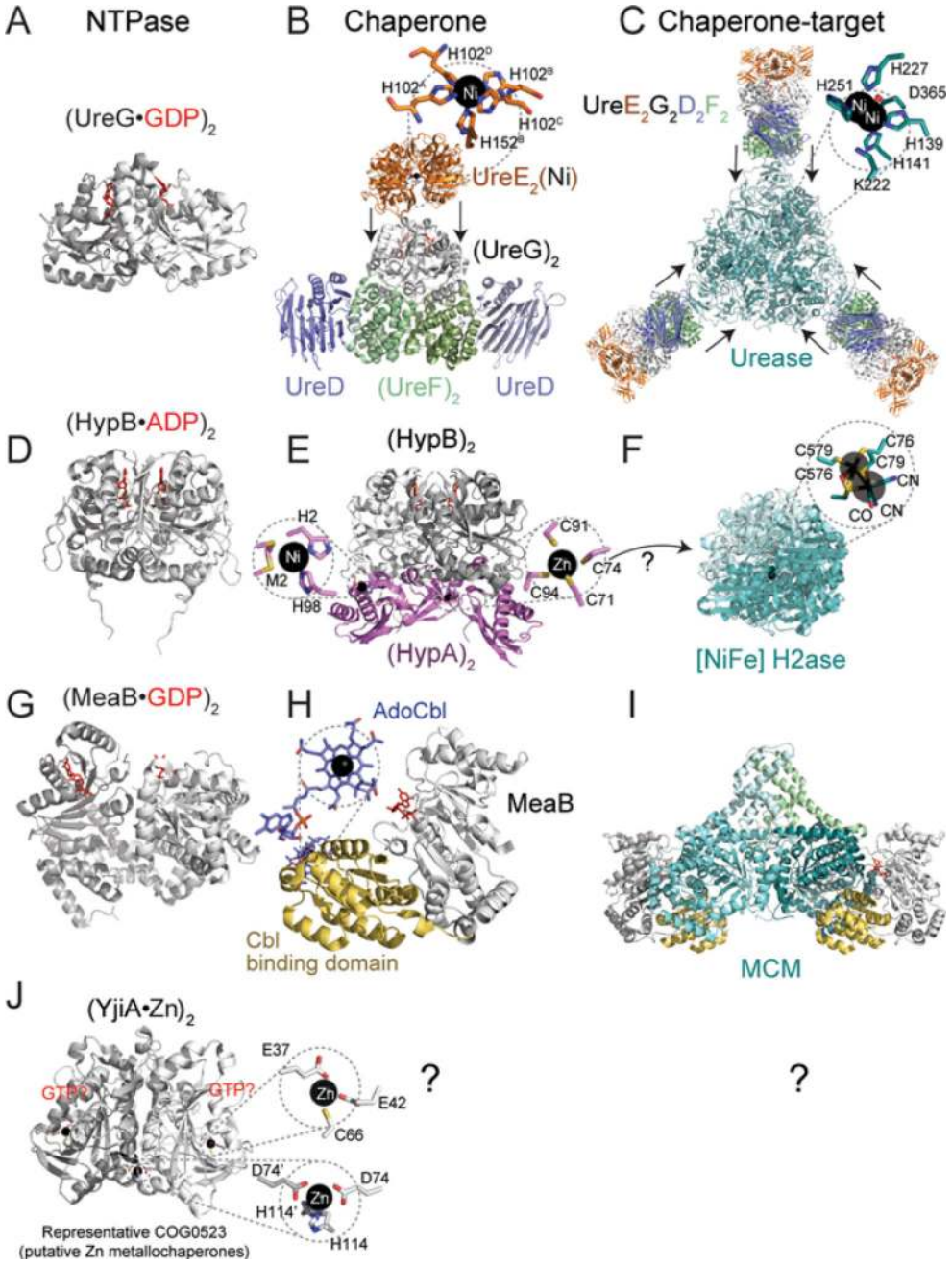

Figure 3. G3E family P-loop GTPases involved in the maturation of metalloenzymes

(**A**) *H. pylori* urease accessory protein UreG (protomers shaded *grey* and *white*) bound to GDP (pdb code 4hi0). (**B**) *H. pylori* urease metallochaperone complex of UreD (*light blue*), UreF (*green*) and UreG (*grey*) (4hi0), including dimeric UreE (*orange*; 3yn0) in an orientation described in a previously reported docking model [56]. (**C**) The trimer-of-trimers urease apoprotein (UreA, UreB, UreC; *cyan*, 4z42) from *Yersinia enterocolitica* either sequentially binds UreD, UreF and UreG or binds a preformed $UreD_2F_2G_2$ complex. Formation of the active enzyme requires GTP binding and hydrolysis by UreG and Ni^{II} delivery by UreE [27]. (**D**) *Thermococcus kodakarensis* homodimeric HypB (*grey* and

white) bound to ADP (5aun) [48]. (**E**) *T. kodakarensis* chaperone complex $HypA_2B_2$ (HypB dimer shaded *pink*) (5aun) [48]. (**F**) *E. coli* mature [NiFe] hydrogenase (5a4f) highlighting the active site. (**G**) Human MeaB GDP bound homodimer (2www). (**H**) *Cupriavidus metallidurans* chaperone complex formed by a MeaB monomer in a complex with the holo-Cbl-binding domain (*yellow*) (4xc6). (**I**) *C. metallidurans* chaperone complex with the methylmalonyl-CoA mutase (MCM, *cyan*; 4xc6). (**J**) *E. coli* Zn^{II}-bound dimer of YjiA, a COG0523 subfamily member of unknown function (4ixm) [74]. Nucleotides are shown in *red* stick and metals are shown as *black* spheres. Zn^{II} ligand C66 is derived from the C64-x-C66-C67 (CxCC) motif conserved in COG0523 proteins, which is close to the G2 loop (switch I) containing the Zn^{II} ligand E37; ?, chaperone-accessory protein and chaperone-target protein complexes for YjiA and other COG0523 GTPases are as yet unknown. The coordination structures of the bound metals are expanded in each panel.

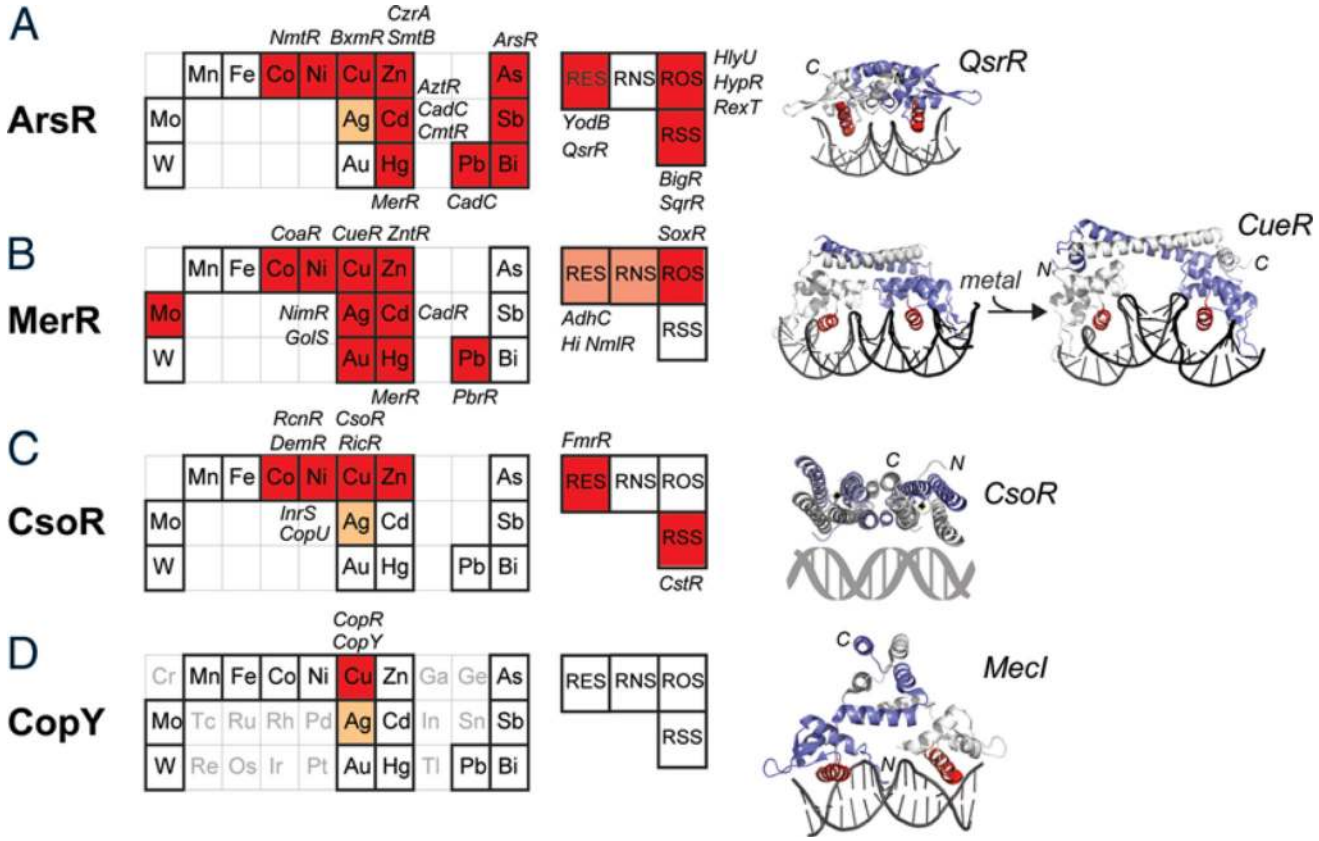

Figure 4. Structural families of metal efflux regulators

For each family, boxes for metals that are known to be sensed are shaded *red* on the abbreviated periodic table, while boxes on the right denote family members that are known to react with small molecule reactive species, also following the order of the periodic table as C, reactive electrophile species (RES); N, reactive nitrogen species (RNS); O, reactive oxygen species (ROS); and S, reactive sulfur species (RSS). Boxes identifying putative metal and non-metal sensors that are likely not physiologically relevant are shaded *pink* or *yellow* respectively. The four-letter designations for individual proteins that perform the function listed in the nearby box are shown (see text for details). Ribbon representations of representative sensors in the DNA-bound state are shown on the *right* with individual protomers shaded *white* and *blue* in each case, with the DNA-binding motif shaded *red* on both protomers. Metal ions are shaded in *black*. Structures are from *top* to *bottom* are (**A**) *Staphylococcus aureus* QsrR in the apo form (4hqe) [209] (**B**) *E. coli* Cu^{I}-sensor CueR with the apo (*right*, 4wls) and Cu^{I}-bound forms shown, with Cu^{I} ions in *black* (*left*, 4wlw) [105] (**C**) *Geobacillus thermodenitrificans* Cu^{I}-sensor copper-sensitive operon repressor (CsoR) with regulatory Cu^{I} ions shown The DNA cartoon is shown to represent the proposed DNA-binding mode (4m1p) [128] (**D**) *S. aureus* MecI as a model for *Enterococcus* copper sensor (CopY) (2d45) [210].

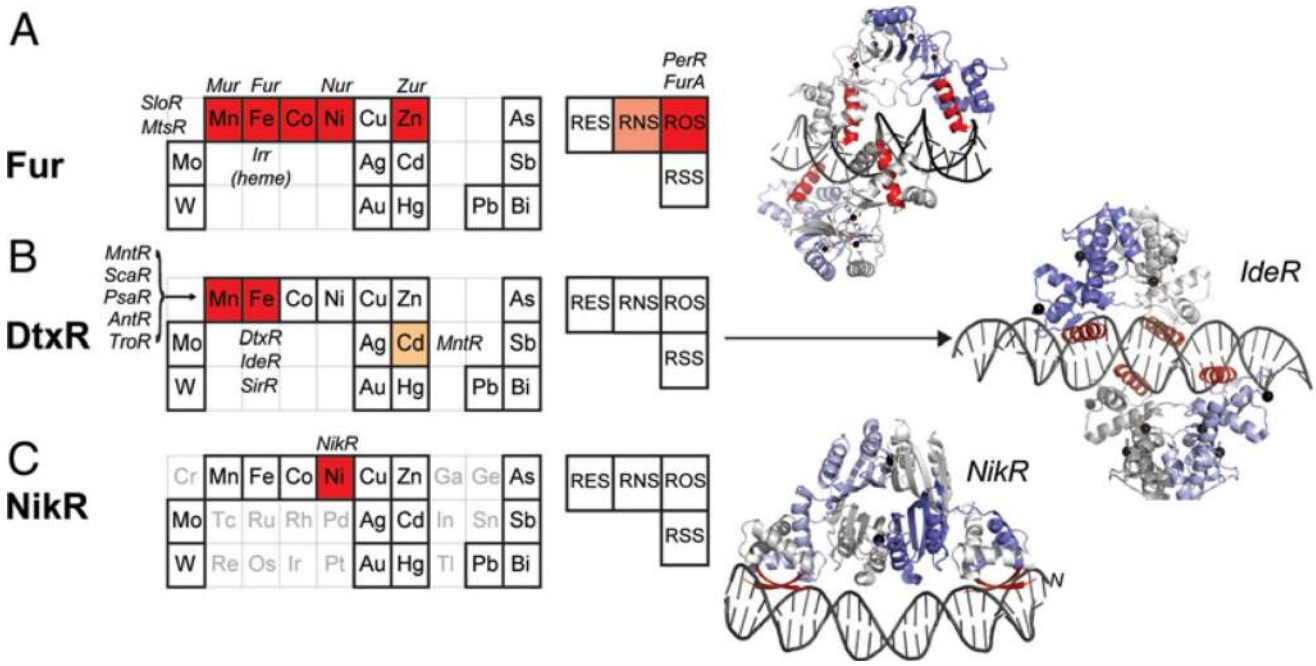

Figure 5. Structural families of metal uptake regulators

Boxes, colours and labels follow the same convention as in Figure 4. Structures are from *top* to *bottom*: (**A**) *Magnetospirillum gryphiswaldense* MSR-1 ferric uptake regulator (Fur)-Mn^{2+} (4rb1) (**B**) *M. tuberculosis* IdeR (2isz) (**C**) *E. coli* NikR (2hzv).

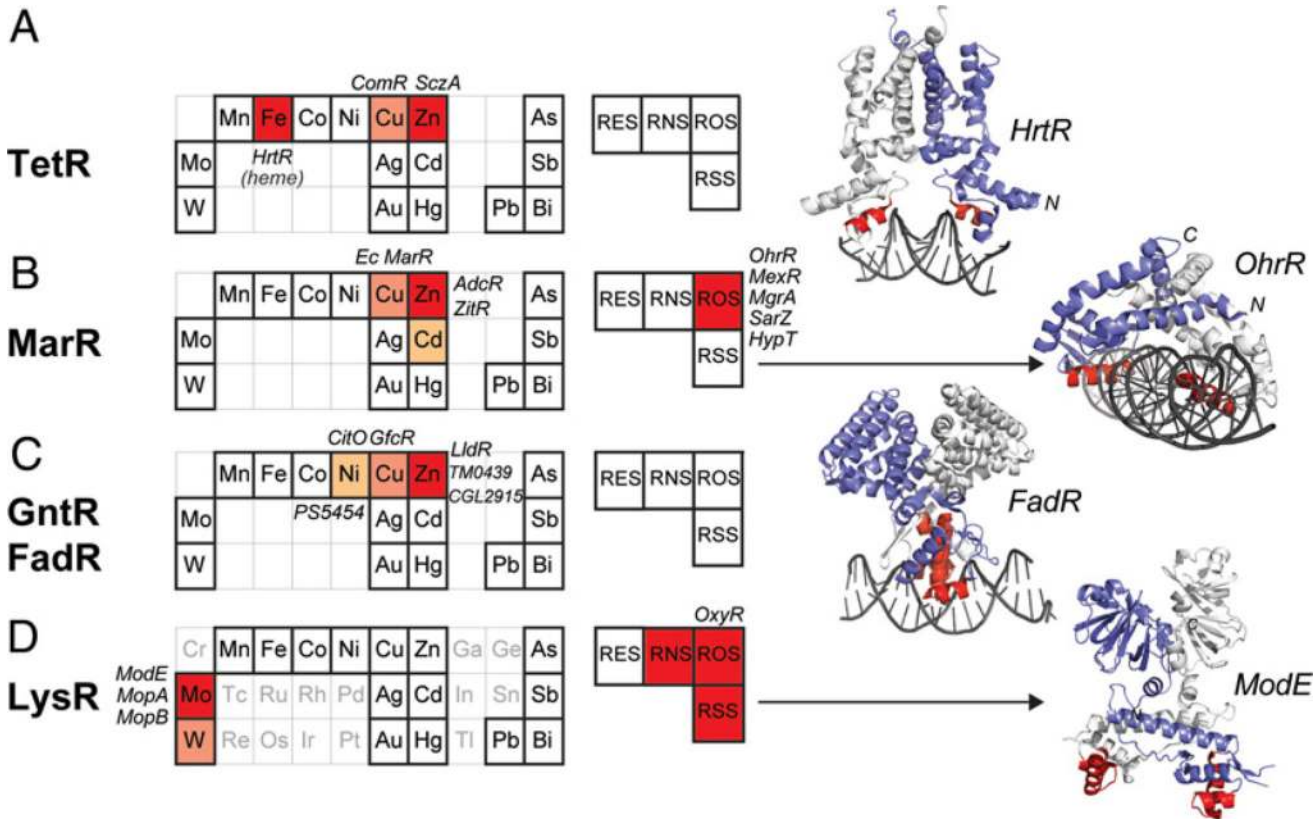

Figure 6. Other structural families of transcriptional regulators where metallosensors have been identified

Boxes, colours and labels follow the same convention as in Figure 4. Structures are from *top* to *bottom*: (**A**) *Lactococcus lactis* HrtR (3vok) (**B**) *Bacillus subtilis* OhrR (1z9c) (**C**) *Vibrio cholerae* FadR as a model of the DNA bound form of metal-binding proteins from the FadR family. (4p9u) (**D**) *E. coli* ModE in molybdate-bound form without DNA (1b9m).

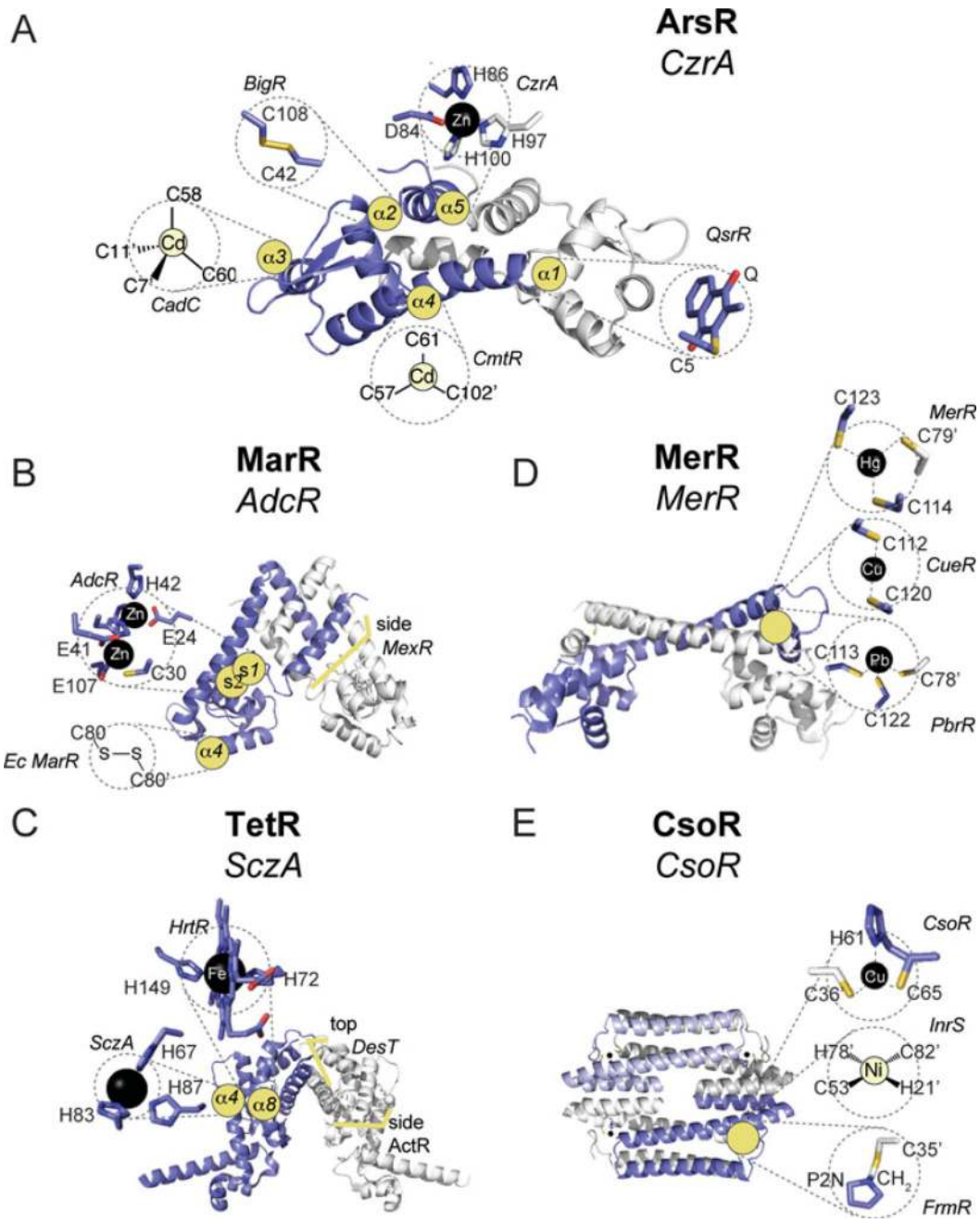

Figure 7. Ribbon representations of five representative metalloregulatory protein families

The approximate location of metal sites schematized on each structure, and the chelate or disulfide of representative members of each family shown in atomic detail on the *insets*. Subunits are shaded blue and grey, with the regulatory family indicated on *top*, and the specific protein designation indicated directly below. Ligand sets are as defined for the specific protein indicated. For MarR and TetR proteins (panels B and C) the pockets for organic ligands are displayed with a yellow line. Structures are from *top* to *bottom*: (**A**) *S. aureus* CzrA in the Zn form (2m30), *Xylella fastidiosa* BigR in the oxidized form (*inset*, 3pqk), *S. aureus* QsrR-menadione complex (*inset*, 4hqm) (**B**) *S. pneumoniae* adhesin

competence regulator (AdcR) in the Zn form (*inset*, 3tgn) (**C**) *Streptococcus agalactiae* SczA in the metal-bound form (3kkc) [3], *L. lactis* HrtR in the haem-bound form (*inset*, 3vp5) (**D**) *Pseudomonas aeruginosa* MerR in the Hg bound form (5crl), *E. coli* CueR in the Cu bound form (*inset*, 1q05), *Ralstonia metallidurans* PbrR in the Pb bound form (*inset*, 5gpe) (**E**) *Geobacillus thermodenitrificans* CsoR in the Cu bound form (4m1p), formaldehyde-treated *E. coli* FmrR containing a methylene bridge (CH_2) that links C35′ and the N-terminal P2 residue of the adjacent protomer (*inset*, 5lbm).