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Review

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Biochemical and Biodiversity Insights into Heavy Metal Ion-Responsive Transcription Regulators for Synthetic Biological Heavy Metal Sensors

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To adapt to environmental changes and to maintain cellular homeostasis, microorganisms adjust the intracellular concentrations of biochemical compounds, including metal ions; these are essential for the catalytic function of many enzymes in cells, but excessive amounts of essential metals and heavy metals cause cellular damage. Metal-responsive transcriptional regulators play pivotal roles in metal uptake, pumping out, sequestration, and oxidation or reduction to a less toxic status via regulating the expression of the detoxification-related genes. The sensory and regulatory functions of the metalloregulators have made them as attractive biological parts for synthetic biology, and the exceptional sensitivity and selectivity of metalloregulators toward metal ions have been used in heavy metal biosensors to cope with prevalent heavy metal contamination. Due to their importance, substantial efforts have been made to characterize heavy metal-responsive transcriptional regulators and to develop heavy metal-sensing biosensors. In this review, we summarize the biochemical data for the two major metalloregulator families, SmtB/ArsR and MerR, to describe their metal-binding sites, specific chelating chemistry, and conformational changes. Based on our understanding of the regulatory mechanisms, previously developed metal biosensors are examined to point out their limitations, such as high background noise and a lack of well-characterized biological parts. We discuss several strategies to improve the functionality of the metal biosensors, such as reducing the background noise and amplifying the output signal. From the perspective of making heavy metal biosensors, we suggest that the characterization of novel metalloregulators and the fabrication of exquisitely designed genetic circuits will be required.

Keywords: Heavy metal, transcriptional regulator, biosensor, synthetic biology, genetic circuit

Introduction

Metal ions participate in many indispensable biological processes, including as cofactors for hydrolytic enzymes and oxidoreductases, in electron transfer, and in structural centers for stabilizing the folding of proteins [1]. In fact, nearly half of the proteins structurally characterized so far require metals [2]. Due to their importance, biologically essential metals such as Cu, Zn, Fe, Mn, Ni, Al, and Co are referred to as micronutrients or trace metals, and low concentrations are often sufficient to enable them to fulfill their functions. On the other hand, excessive concentrations of essential metal ions can cause toxicity to cells; for example, the Fenton reaction with Fe and Cu can generate reactive oxygen species that cause cellular damage [3]. In addition, some heavy metals such as Hg, As, Cd, Cr, Pb, and Sn do not have a biological role and cause extreme toxicity. Therefore, all life, including microorganisms, need to maintain the intracellular concentration of essential metal ions at the desired level and to exclude toxic heavy metals.

Prokaryotes have evolved a metal homeostasis system composed of metal uptake, efflux, metallochaperones, detoxification by oxidation or reduction, and sequestration [4–8], which are usually regulated at the transcriptional level. Metal-ion-responsive transcriptional regulators, or

metal sensory parts for short, play pivotal roles by orchestrating the expression of homeotic and/or detoxifying genes in response to metal ions. However, this is not a simple job for a transcriptional regulator because several complexities pose hurdles that need to be overcome. The first obstacle comes from the fact that there are many metal species inside the cytoplasm and only the correct one should be recognized and regulated. Metalloregulators must discriminate between metals of similar physical and/or electrochemical characteristics. The second difficulty is that in the case of non-functional toxic heavy metals, metal-responsive regulators are required to bind very sensitively to prevent cellular damage. Cellular concentrations of Zn and Fe are in the range 10^{-4} to 10^{-3} M, while Mn and Cu are 10-fold lower and Ni and Co are another 10-fold lower [9, 10]. Besides, non-functional toxic heavy metals such as Hg and As should be detoxified at a much lower concentration. Last, cellular requirements for metal ions do not always follow the natural order of stability of metal complexes, the so-called Irving-Williams series [11]. It describes that metal complexes are stable in the order of Cu, Zn>Ni, Co>Fe, Mn>Ca, Mg. However, bioinformatic analysis has shown that the order of the abundantly used metal species as cofactors is Mg>Zn>Fe>Mn [2]. Besides, cellular needs can be changed conditionally because the use of metal ions is biased by enzymes; for example, most oxidoreductases (E.C. number 1) need Fe and Cu while most transferases (E.C. number 2) use Mg and Mn. Another layer of complexity is added when two regulators compete for the same metal ion; *e.g.* Zn-responsive transcriptional regulators, Zur and ZntR, controlling the expression of Zn uptake and pumping out, respectively. Despite these hindrances, prokaryotic cells proliferate as they perform metal homeostasis successfully at any given moment. Therefore, it is of great interest and essential to address the question of how metalloregulatory proteins sense each metal ions specifically and regulate homeotic and/or detoxifying genes elaborately.

Humans cannot be excluded from the necessity for metal homeostasis and managing the toxicity of heavy metal ions. It is estimated that humans are exposed to 35 metals in everyday life and 23 of them are heavy metals, including As, Pb, Hg, Cd, Cr, Co, Ni, Zn, U, Cu, Mn, V, Ag, Sb, Bi, Ce, Ga, Au, Fe, Pt, Te, Tl, and Sn [12]. Historically, humans have suffered from heavy metal toxicity and have tried to reduce and prevent heavy metal pollution through international cooperation such as the Minamata convention. Many agencies such as the Environmental Protection Agency, the UN Environment Programme, the Agency for Toxic Substance and Disease Registry, and the US Department of Labor have placed heavy metal pollution as a primary concern. Despite enormous effort, heavy metal pollution has been reported in drinking water, food, and irrigation [13–15]. To prevent environmental pollution and toxicity from heavy metals, monitoring their concentrations from various sources is an important task, and indeed, many analytical methods based on spectrometry, electrochemical voltammetry, and chemical sensors have been developed and used [16]. However, they often require an expensive instrument, a highly skilled workforce, and intensive chemical treatment of the samples, and moreover, they might not be suitable for the selective detection of the target metal ions in the presence of other metal ions. Therefore, alternative methods other than chemical- and instrument-based methods are required.

Biosensors have several advantages over chemical methods in terms of selectivity, simplicity, low manufacturing and maintenance cost, ease of use, and portability. A recent report has demonstrated that the use of biosensors for heavy metals is compatible with analytical devices as the former have demonstrated limits of detection in the nanomolar range, which is much lower than that necessitated by environmental regulation [17]. The construction of a biosensor often requires the combination of a transcriptional regulator, a DNA-binding operator sequence, and a reporter gene from various sources. Hence, the optimization of the biosensor should consider the kinetics of cellular processes such as transcription, and translation, and binding affinity with metal ions or DNA-binding sequences of different host strains. Even though a lot of heavy metal biosensors have been made over the past decades, there is still room for improvement in performance by tuning such steps for sensing heavy metals and generating output signals. The resources for the biosensor development have been provided from the accumulated biochemical data of the diverse heavy metal transcriptional regulators and the novel concepts for genetic circuit design. Therefore, we may need to progressively apply the principles of synthetic biology on the basis of solid understanding of heavy metal-sensing transcriptional regulators.

In this review, we summarize the accumulated knowledge on heavy metal ion-responsive transcriptional regulators. Even though metal-specific regulators can be categorized into at least 10 families based on their structural similarity [18, 19], we focus on the two major

families, SmtB/ArsR and MerR, because their abundance and diversity are overwhelmingly outpacing the other regulators, and the two families regulate the most toxic heavy metal ions such as As, Pb, Hg, and Cd and essential metals including Zn, Cu, and Co as well. The application of metal-responsive regulators to biosensors, from simple genetic circuits to their sophisticated design, is also reviewed and strategies to improve the performance of heavy metal ion biosensors are discussed.

The SmtB/ArsR Family

The SmtB/ArsR family is a major metalloregulatory protein family in which SmtB/ArsR-type regulators generally function as transcription repressors. In the absence of toxic levels of cognate heavy metal ions, the apo-form proteins can bind to DNA operator sequences to prevent the expression of the regulated genes. When the concentration of heavy metal ions increases, they bind to specific amino acid residues in the protein, thereby causing conformational changes, and the regulator protein dissociates from the DNA operator region to allow the expression of heavy metal homeostasis/resistance proteins such as efflux pumps, metallothionein, and metal reductase [20]. The targeted heavy metals and the target genes of the SmtB/ArsR family proteins are summarized in Table 1.

The SmtB/ArsR family proteins regulate genes in response to diverse heavy metal ions including As(III), Sb(III), and Bi(III) by the ArsR of *Escherichia coli*; Cd(II), Pb(II), and Zn(II) by CadC of *Staphylococcus aureus*; Ni(II) and Co(II) by NmtR of *Mycobacterium tuberbulosis*, and Cu(III) and Ag(III) by BmxR of *Oscillatoria brevis* [21–24] (Table 1). Typically, one regulator regulates the resistance genes in response to 2 to 3 heavy metal ions at different concentrations. For example, the allosteric regulator of *S. aureus* CzrA responds in the order Zn(II)>Co(II)>Ni(II) whereas *M. tuberculosis* NmtR has the opposite affinity for the same metals in the order Ni(II)>Co(II)>Zn(II) [25].

The most intriguing questions concerning the SmtB/ArsR metalloregulatory protein family are i) how do they differentiate between metal ions having different ion radii and charges and ii) how do they couple metal binding and negative allosteric regulation. To address these questions, we summarize the structural and biochemical data of the metal-binding sites in the regulators along with the conformational changes in the regulators upon binding of metal ions to dissociate from the operator sequence. Additionally, the evolution of the SmtB/ArsR family is discussed based on the location and functionality of the metal-binding sites.

Metal-Binding Sites

The first crystal structure of the SmtB/ArsR family investigated from *Synechococcus elongatus* PCC 7942 SmtB contains five α-helices plus two β sheets (α_1-α_2-α_3-α_4-β_1-β_2-α_5), and a winged helix-turn-helix (HTH) motif for DNA binding [26]. Since this discovery, the metal-binding motifs of other SmtB/ArsR homologs have been termed as the location of the metal-binding sites on the secondary structure; currently, 13 have been identified, namely α3, α3N, α5, α3N-α5, α5c, α53, α4c, α4c2, α3N-2, α5-4, α55, α2-α52, and α3-4, along with a non-metal-binding motif, α33 [20].

All SmtB/ArsR proteins have one or two pairs of metal-binding sites and are considered to be homologous with either α3N or α5. Amino acid residues consisting of α3N and α5 sites come from two protomers of a dimer, thus all identified SmtB/ArsR proteins should form a homodimeric protein for proper functioning. For example, the α3N site of a Zn(II)- and Co(II)- responsive SmtB protein (from *S. elongatus* PCC 7942 strain) consists of Cys14, His18, Cys61′, and Asp64′, while the α5 site at the dimer interface has Asp104, His106, His117′, and Cys121′ (the apostrophes indicate that the amino acid residues come from another protomer here and hereinafter) [26] (Table 2). A pair of α3N sites are located at opposite ends of the dimer, while two α5 sites are found at the dimer interface [26, 27]. The binding of metal ions to the metal-binding sites does not affect the stable dimer formation in most cases [25, 26, 28, 29]. However, the role of bound metal in the stabilizing dimers has been suggested because a properly folded dimer of *Thiomonas arsenitoxydans* AioF can be obtained only in the presence of As(III), As(V), or Sb(III) in vitro [30, 31].

α3N and α5 sites are distinguished not only spatially but also functionally. The α3N site of CadC is thiolate-rich composed of Cys7, Cys11, Cys58′, and Cys60′ and preferentially binds to larger metals such as Cd(II), Pb(II), and Bi(II), while the α5 site contains nitrogen and oxygen ligands and binds preferentially to smaller metal ions such as Co(II) and Zn(II) [32, 33]. Even though both types of site can bind metal ions, only the α3N site of CadC was associated with allosteric regulatory functionality which was shown in the abrogated DNA-binding ability of the CadC (Cys60Gly) mutant protein, the binding of Zn(II) to which did not recover the regulatory functioning [32]. It

Table 1. Representative transcriptional regulators of SmtB/ArsR and MerR family.

Regulator	Strain	Responsive heavy metals	Target genes	References
SmtB/ArsR family				
AioF	*Thiomonas arsenitoxydans*	As(III), As(V)	*aioB* (small arsenite oxidase subunit) *aioA* (large arsenite oxidase subunit); transcriptional activator unlike other SmtB/ArsR family proteins	[31]
ArsR	*Escherichia coli* R773	As(III), Sb(III)	ATPase exporter (*arsA*) Diffusion transporter (*arsB*) Arsenate reductase (*arsC*)	[110]
AztR	*Anabaena* sp.	Cd(II), Pb(II), Zn(II)	ATPase efflux pump (*aztA*)	[111]
BxmR	*Oscillatoria brevis*	Ag(I), Cu(I), Zn(II), Cd(II)	*bxa1* (CPx-ATPase metal transporter) *bmtA* (metallothionein)	[24]
CadC	*Staphylococcus aureus* pI258	Cd(II), Pb(II), Bi(III), Zn(II), Co(II), Hg(II)	P-type ATPase metal efflux pump (*cadA*)	[36, 112–115]
CmtR	*Mycobacterium tuberculosis* *Streptomyces coelicolor*	Cd(II), Pb(II)	*cmtA* (P-type ATPase efflux pump)	[43, 116]
CzrA	*S. aureus* 912 *Bacillus subtilis*	Zn(II), Co(II), Ni(II)	Diffusion transporter (*czrB*)	[44, 45, 117]
KmtR	*M. tuberculosis*	NI(II), Co(II)	*Rv2025c* (CDF-family metal exporter)	[118]
NmtR	*M. tuberculosis*	Ni(II), Co(II)	ATPase exporter (*nmtA*)	[43]
SmtB	*Synechococcus elongatus* PCC 7942	Zn(II), Co(II), Cd(II), Cu(II), Hg(II), Ni(II), Au(II), Ag(I)	Metallothionein (*smtA*)	[21, 119, 120]
ZiaR	*Synechocystis* sp. PCC 6803	Zn(II)	P-type ATPase metal efflux pump (*ziaA*)	[42]
MerR family				
CueR	*E. coli*	Cu(I), Ag(I), Au(I)	P-type ATPase (*copA*) Multi-copper oxidase (*cueO*)	[121]
GolS	*Salmonella bongori* *S. enterica*	Au(I)	Metal exporter (*golT*) CBA efflux system (*gesABC*) Metal-binding protein (*golB*)	[122]
MerR	Tn*21* transposon	Hg(II)	Inner-membrane protein (*merT*) Periplasmic mercury binding protein (*merP*) Mercuric reductase (*merA*) Organomercurial lyase (*merB*) Antagonistic regulator (*merD*) Transmembrane protein for Hg(II) uptake (*merC*, *merE*, *merF*)	[123, 124]
PbrR	*Cupriavidus metallidurans* CH34	Pb(II)	Pb(II) uptake protein (*pbrT*) P-type efflux ATPase (*pbrA*) Inner-membrane protein (*pbrB*) Prelipoprotein signal peptidase (*pbrC*) Pb(II) binding protein (*pbrD*)	[125]
ZntR	*E. coli*	Zn(II), Cd(II)	Zn(II)/Cd(II) exporter (*zntA*)	[119]

has been shown that the CadC heterodimer containing a wild-type monomer and a cysteine-substituted monomer can bind to the DNA operator but cannot dissociate from the DNA upon binding of metal ions [34]. In contrast to CadC, the binding of Zn(II) to the α5 site is required for the allosteric regulation of the SmtB regulator from *S. elongatus* PCC 7941 [27]. This is consistent with another mutational study in that the disruption of His105 and His106 at the α5 site resulted in a loss of derepression in response to Zn(II), while Cys14Ser, Cys61Ser, and Cys121Ser comprising the

Table 2. Essential residues of the SmtB/ArsR and MerR family proteins.

Protein	Metal-binding Residues	Function or description	References
SmtB/ArsR family			
AioF (*Thiomonas arsenitoxydans*)	Cys53, Cys111, Cys115	AioF is a transcriptional activator	[30]
ArsR (*Escherichia coli* pR773)	Cys32, Cys34, Cys37	Metal-binding site; trigonal coordination; mutants of either of Cys32 Cys34 do not response to inducers while maintaining DNA binding.	[54]
	His50	Located at the DNA-binding domain; H50Y substitution results in constitutive expression of the *ars* operon.	[54]
ArsR (*Acidithiobacillus ferrooxidans*)	Cys95, Cys96, Cys102	Metal-binding site	[56]
ArsR (*Corynebacterium glutamicum*)	Cys15, Cys16, Cys55	This metal-binding site is not aligned with the other metal-binding sites of the SmtB/ArsR family proteins.	[55]
CadC (*Staphylococcus aureus* pI258)	Cys7, Cys11, Cys58′, Cys 60′	Metal-binding site; tetrahedral or trigonal; Cys11 is not absolutely necessary.	[113]
	Asp101, His103, His114′, Glu117′	Non-essential metal-binding site preferentially binds to Zn(II) over Cd(II); D101G and H103A substitution abrogates binding to Zn(II)	[22]
CmtR (*Mycobacterium tuberculosis*)	Cys57, Cys61, Cys102′	Metal-binding site; C102S substitution significantly reduces the affinity with Pb(II) by ~1000-fold and disables the dissociation of the Cmt-DNA complex.	[126]
CmtR (*Streptomyces coelicolor*)	Cys57, Cys61, Cys102′	Metal-binding site 1 is identical with *M. tuberculosis* CmtR	[116]
	Cys24, Cys110, Cys111	Metal-binding site 2; mutation in site 2 causes Cd(II) responsiveness but not Pb(II).	
CzrA (*S. aureus*)	Asp84, His86, and His97′, His100′	Metal-binding site; mutation of Asp84 and His97 results in a deleterious effect on allosteric regulation; His86 and His100 are readily substituted.	[28, 127]
KmtR (*M. tuberculosis*)	His88, Glu101, His102, His110, His111	Metal-binding site	[118]
NmtR (*M. tuberculosis*)	Asp91, His93, His104, His107, His109, and His116	Metal-binding site; Gly2-His-3-Gly4 can form an alternate site, replacing His109 and His116.	[43]
SmtB (*Synechococcus elongatus* PCC 7942)	Cys14, His18, Cys61′, Asp64′	α3N metal-binding site; non-regulatory binding site; the substitution of cysteines does not have a negative effect on allosteric regulation.	[26, 52]
	Asp104, His106, His117′, Glu120′	α5 metal-binding site; regulatory site; H106Q substitution is defective in the disassembly of SmtB-DNA.	[26, 52]
	His105, His106	Disruption of His105 and His106 cause loss of derepression	[35]
MerR family			
CueR (*E. coli*)	Cys112, Cys120	Metal-binding site; mutation to serine represses transcription activity.	[71, 77]
	Ars75	Ars75 is at the hinge region connecting the metal-binding loop and the DNA-binding domain; mutation of R75A decreases transcriptional activation.	[77]
	Ser77	CueR mutant, S77C becomes responsive to both +1 and +2 ions.	[128]
GolS (*Salmonella enterica*)	Met16, Tyr19	Provides selectivity on promoter sequences	[129]
	Ser77	GolS mutant, S77C becomes responsive to both +1 and +2 ions.	[128]
	Ala113, Pro118	Substitution of A113 or P118 hampers the selectivity toward Au(I) and Cu(I).	[130]

Table 2. Continued.

Protein	Metal-binding Residues	Function or description	References
MerR (Tn*501*)	Ala89, Ser131	Substitution of Ala89 or Ser131 results in constitutive expression of the *mer* operon	[57]
	Cys82, Cys117, Cys126	Metal-binding site; mutation in cysteines dramatically reduces the affinity with Hg(II); C82Y mutation interferes with MerR dimerization.	[131, 132]
	Pro127, His118	Mutation of P127L or H118A impairs allosteric regulation.	[133, 134]
	Arg53, Leu76, Ala85, Lys99, Ser125, Ser131, Glu72, Leu74, Ala89, Lys99, Met106	A single mutation in these residues makes repressing defective, causing leaky or constitutive expression of the *mer* operon; most of these residues are located in the dimerization domain.	[134]
	Multiple mutations (12 to 22)	Preference of MerR for metal ions changes to Cd(II); the combined effect of many residues for metal selectivity has been suggested.	[135]
PbrR (*Cupriavidus metallidurans*)	Cys14, Cys79, Cys134	Cysteine mutants are defective in Pb(II)-induced activation of P*pbrA*	[125]
SoxR (*E. coli*)	Gly15, Tyr31, Leu36, Ile62, Ala63, Gln64, Ile66, Ile73, His84, Leu86, Leu94, Ser95, Ser96, Ile106, Glu115, Asp117, Cys124, Arg127	These mutations are dispersed throughout a protein; they are defective in DNA-binding ability and transcriptional activation.	[136]
ZntR (*E. coli*)	Cys114, Cys124	Metal-binding site 1	[71, 137]
	Cys79, Cys115, His119	Metal-binding site 2	

α3N site retained metal-responsive regulation [35].

For bacterial cells to maintain cellular homeostasis, it is an important task for metalloregulatory proteins to discriminate for a specific metal ion among a number of different ones, and to achieve this , they adopt different coordination geometries between the metal ions and the ligands. Coordination geometry is characterized by the type of ligand, coordination number, bond length between the metal ion and the ligand, and the dihedral angles of the ligand-metal-ligand [25]. Chelate structures contain sulfur (cysteine and methionine), nitrogen (histidine), and oxygen (aspartate and glutamate) and the coordination number ranges from 3 (trigonal) to 6 (octahedral). The use of two types of coordination geometry by one metalloregulatory protein is exemplified by CadC; the α3N site exhibits tetrahedral geometry to bind Cd(II) and Bi(III) but Cys11 does not participate in trigonal geometry to chelate Pb(II) [32, 36]. The CzrA protein in *S. aureus* discriminates between Co(II) and Zn(II) from Ni(II) by coordinating them in a tetrahedral and octahedral complex, respectively. In contrast, the NmtR protein binds preferentially to metal ions in the order Ni(II)>Co(II)>Zn(II) to form an octahedral complex with Ni(II) and a tetrahedral complex with Co(II) and Zn(II) [25].

Phylogenetic analysis of the amino acid sequences of SmtB/ArsR family shows that similar proteins are grouped with each other while ArsR proteins form two separate branches (Fig. 1). We have arbitrarily named the group of ArsR proteins that the model ArsR of *E. coli* belongs to as class 1 and another group of ArsR for which the biochemical data are scarce as class 2. Both classes predominantly contain Proteobacteria strains, but the ArsR proteins of Actinobacteria and Firmicutes belong to class 1 whereas those of Bacteroidetes are associated with class 2. Furthermore, a comparison of the metal-binding sites from the amino acid alignment clearly shows that Cys32 and Cys34 (plus Cys37) are conserved only in class 1 while Cys95 and Cys96 (plus Cys102 or 103) are conserved in class 2 (Table 2, Figs. 1 and 2). Interestingly, we found the conserved ELCVCDL motif for metal-binding sites in class 1 ArsR, SmtB, CadC, and CzrA but not in class 2 ArsR (Fig. 2A). The authors of a previous review article on SmtB/ArsR came to a similar conclusion that a $CxCx_2C$ motif is predominantly present in α3N sites while the phylogenetically unrelated α5 ArsR family proteins did not contain the motif [20]. Until recently, biochemical data primarily obtained from the model ArsR of *E. coli* have provided a paradigm for understanding the entire ArsR protein family. However, these data may not be applicable to class 2 ArsR, which actually accounts for a large

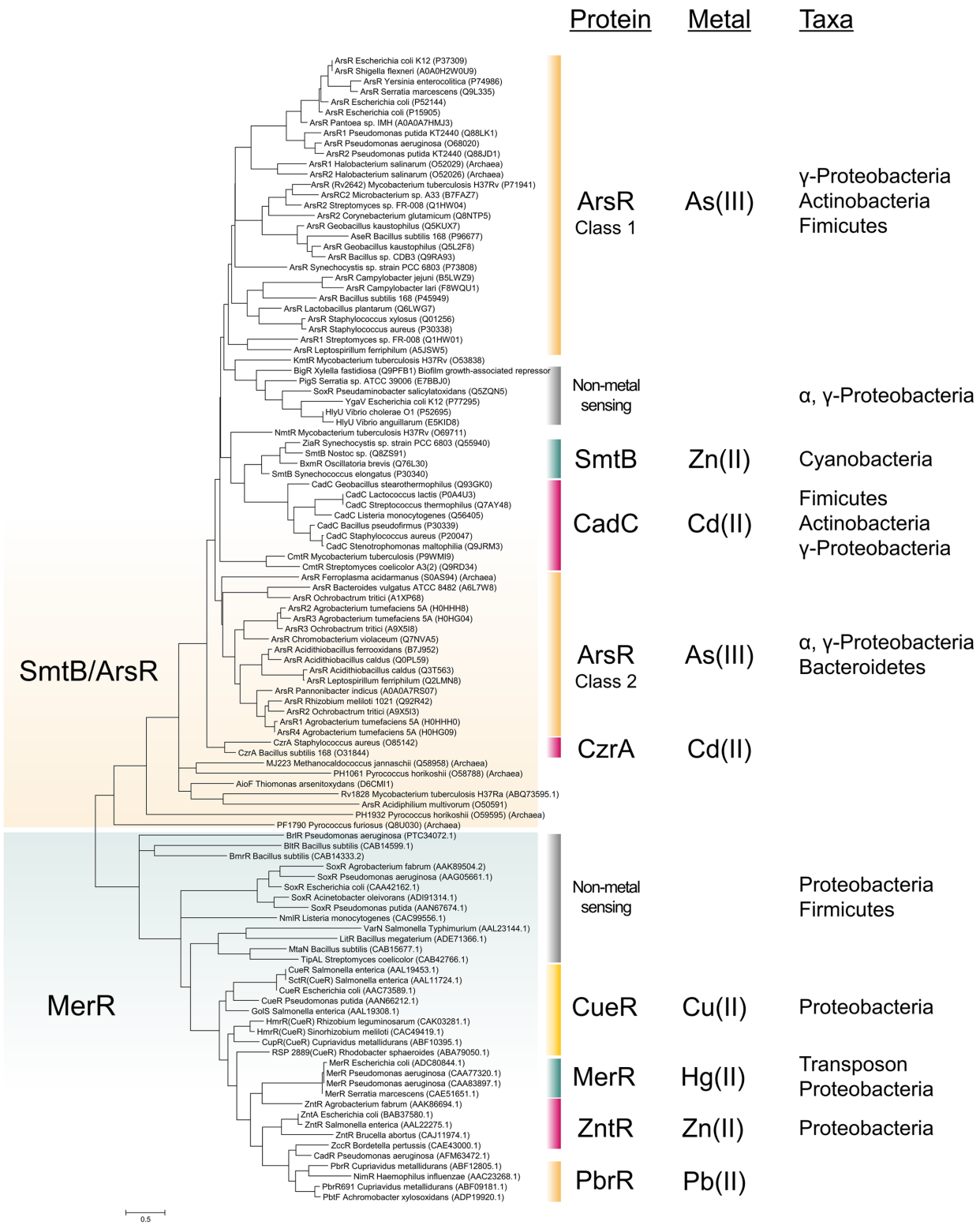

Fig. 1. A maximum-likelihood phylogenetic tree built from amino acid sequences of the experimentally characterized SmtB/ArsR and MerR family proteins.

Sequences were aligned by ClustalW algorithm and trees were constructed using MEGA 6.0. Accession numbers of GenBank or UniProt are in parentheses.

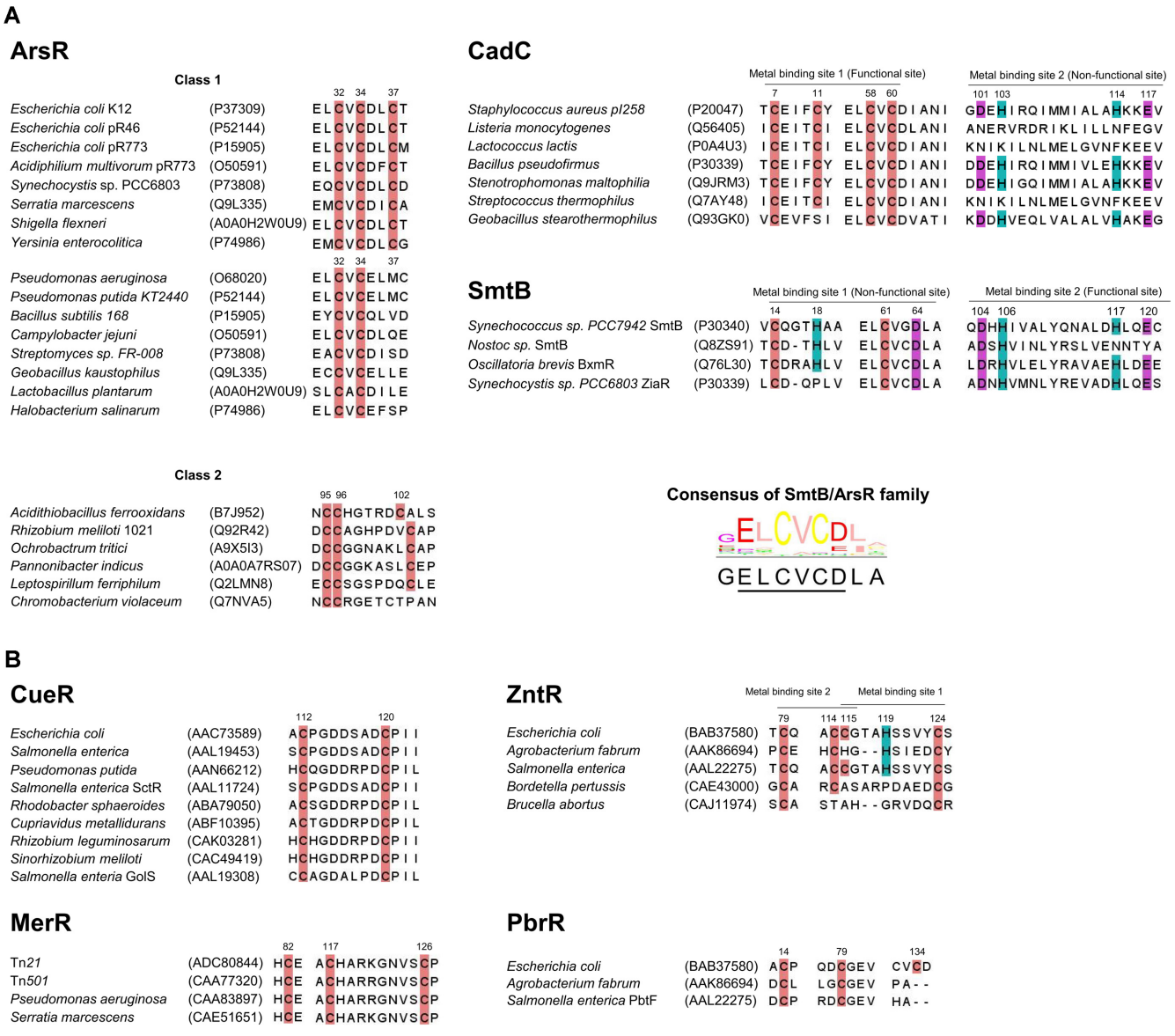

Fig. 2. Metal-binding sites of the (**A**) SmtB/ArsR and (**B**) MerR family transcriptional regulators.
Amino acid residues responsible for metal binding listed in Table 2 are marked in colors. Class 1 and 2 in ArsR correspond to those of the phylogenetic tree in Fig. 1. The consensus sequence of the SmtB/ArsR family was coined from class 1 ArsR, SmtB, and CadC, except for class 2 ArsR. The consensus sequence of MerR could not be found. Sequences were obtained from experimentally characterized proteins, and accession numbers of GenBank or UniProt are in parentheses. They were aligned with the ClustalW algorithm embedded in MEGA 6.0. The alignment was inspected and visualized in JalView.

proportion of the ArsR proteins. Therefore, further investigation is required to understand the regulatory mechanism of class 2 ArsR proteins.

Allosteric Regulation Via Conformational Change

The SmtB/ArsR family contains the winged HTH motif to bind to the operator DNA sequence [26, 37], which is also found in the DNA-binding domains of other transcriptional regulators such as LexA, LacI, and MerR [38–40]. The recognition helix of the HTH motif is known to be present in the center of the major groove in the DNA helix, and interaction between the HTH motif and DNA is mediated via polar sidechains directly or through bridging water molecules [41]. An early structural investigation of

SmtB suggests that DNA binding via Cys61 and His97 is disrupted upon the binding of Zn(II) to the metal-binding site, resulting in negative regulation of the *smtA* gene [22], while in CadC, α4-turn-α5 corresponds to the HTH motif. It has been suggested that recognition helix α5 interacts with the major groove of DNA, which is consistent with a previous report [41]. The positively charged Arg78 and Lys82 at the α5 site could interact with the phosphate groups of DNA, and it has been predicted that one of the winged motifs interacts with the adjacent minor groove [22].

To perform the allosteric regulatory function of metalloregulators, coordination of metal ions in a chelate structure should be transduced into the DNA-binding/dissociation ability. Upon binding of Zn(II) with CzrA via a tetrahedral chelate structure, hydrogen bond networks initiate from the non-ligating face of essential amino acid His97 to the carbonyl of Leu63′ at the recognition helix, resulting in the stabilization of the low DNA-binding affinity conformation [28]. The solution structure of CzrA bound to DNA has provided insight into the allosteric regulatory function via the transduction of metal-ion binding to bring about the conformational change [37]. Comparison of the DNA-bound and Zn(II)-bound states of CzrA has revealed that the wing and recognition domain move like a pendulum to interact with the major groove of DNA, resulting in significant rotation of one protomer relative to the other. α5 metal-binding sites show loosely packed inter-protomer packing in the DNA-bound state (the "open" state), while conversely, binding of Zn(II) to the α5 site forms a tight chelate structure (the "closed" state) which is unable to interact with the major groove of DNA [37].

It is noteworthy that the binding of metal ions to the regulatory binding sites is important for causing conformational changes since the currently recognized model for metalloregulatory proteins has only one regulatory binding site (either α3N or α5), while the role of the other binding site, if present, has not yet been elucidated either functionally or structurally. Structural comparison between Zn(II)-bound wild-type CadC and mutant CadC lacking the α5 site without Zn(II) has shown that there is no overall difference [33], which is consistent with a report stating that only α3N in CadC and α5 in SmtB have regulatory functions [28]. Formation of a correct chelate structure has also been found to be important for the structural switch; amino acid substitution of His86 and His100 in CzrA retains the tetrahedral coordination and the regulatory function is unaffected. However, Asp84Asn, His97Asn, or His97Asp in CzrA disrupts the tetrahedral coordination, which has a detrimental effect on the conformational change linked to allosteric regulation [25].

Regulatory DNA Region

Promoter region analysis of *S. elongatus* PCC 7942 *smtA*, *Synechocystis* sp. PCC 6803 *ziaA*, *S. aureus* NCTC 8325-4 *zntA*, *S. aureus* 912 *czrA*, and *M. tuberculosis nmtA* has shown the presence of 12-2-12 imperfect inverted repeats [27, 42–45]. Meanwhile, the core motif 5′-TGAAxx-xx-xxTTCA sequence can be recognized by the putative recognition of the α-helices of the SmtB, ZiaR, CzrA, and NmtR metalloregulators [27]. Electrophoretic mobility shift assay (EMSA) data on the metalloregulators from various microbial species has shown that micromolar concentration of metal ions is required to dissociate SmtB/ArsR family proteins from the regulatory DNA region [46–48]. However, examination of the optical spectroscopic data has indicated that metal binding to the metalloprotein occurs at picomolar concentrations. For example, the metal-binding affinities of Zn(II) with the α3N and α5 sites of SmtB are $K_{Zn} \geq 10^{13}\ M^{-1}$ and $K_{Zn} \sim 5 \times 10^{11}\ M^{-1}$, respectively. The affinities of a less potent inducer of Co(II) were similar for both sites ($K_{Co} \sim 2–5 \times 10^{9}\ M^{-1}$) [27]. Even though the intracellular concentrations and *in vivo* binding affinities of heavy metals have not been well characterized, the picomolar binding affinity indicates that metal recognition by the sites is very sensitive. It is of note that growth inhibition by micronutrient metals such as Zn(II), Cu(II), and Co(II) occurs at concentrations of several hundred micromoles whereas heavy metals Cd(II) and Hg(II) are toxic at the micromolar level [49, 50]. In addition, an obligate anaerobic bacterium *Bacteroides vulgatus* ATCC 8482 exhibits resistance against 100 mM of pentavalent arsenate and methylarsenate, although its growth is inhibited by 100 μM trivalent methylarsenate [51]. Therefore, diverse mechanisms for the recognition of cognate metals and the regulation of toxic metal resistance are expected within this family.

EMSA experiments performed on SmtB and the promoter region of the *smtA* gene have shown that multiple band shifts occur, suggesting more than one SmtB is bound to the promoter region [27]. Indeed, four SmtB homodimers can bind to a 40 bp DNA fragment containing a single 12-2-12 imperfect inverted repeat: two dimers per inverted repeat and additional two more dimers bound to the adjacent region. The binding affinities of the first two dimers are very high, while those of the third and fourth dimers are reduced by ~10 and ~30-fold, respectively. The

binding of Zn(II), Cd(II), and Co(II) with a monomer at a ratio of 1:1 results in dramatically reduced affinity with DNA by ~500–2000-fold, thereby leading to dissociation from the promoter region of DNA [52].

Evolution of Metal-Binding Sites in the SmtB/ArsR Family

As discussed previously, the SmtB/ArsR family shows overall similarity in sequences and structures by sharing winged HTH motifs located at the end of an elongated dimer. CadC and SmtB have a 48.4% sequence similarity and a 79% structural similarity, and the conserved DNA sequence motif at the promoter region where SmtB/ArsR binds has been identified in different genes for resistance to metal toxicity [27, 53]. Hence, SmtB/ArsR proteins could have evolved from a common ancestor even though the metal-binding sites in the family of proteins are functionally and structurally diverse: the cognate metal, coordination geometry, binding affinity, and preference for metal species are all different. Due to the diversity of the metal-binding sites in structurally similar proteins, the question of whether they are the result of convergent evolution has arisen.

The evidence of convergent evolution supports that the metal-binding sites of the proteins are different from each other. ArsR contains an As(III) binding site consisting of three cysteine residues at the DNA-binding site [54], while the α3N site of CadC is composed of four cysteine residues, and Cys58 and Cys60 of CadC correspond to Cys32 and Cys34 of ArsR, respectively. Moreover, α5 of CadC for Zn(II) is a non-regulatory site composed of non-thiolate residues ($DXHX_{10}HX_2E$) and is identical to the regulatory site of SmtB. Conversely, there are several exceptions, such as *Acidithiobacillus ferroxidans* ArsR and *Corynebacterium glutamicum* ArsR, as their conserved metal-binding sites are not related to the other SmtB/ArsR proteins that have been extensively investigated as models [55, 56].

There is a different view on the evolutionary history based on the ligand structure of ArsR, CadC, and SmtB. Giedroc and colleagues suggested that ArsR could be an ancient form of this family and evolution proceeded in the order ArsR, CadC, and SmtB, because the complexity of the ligand structure increases in that sequence [22]. In addition, the spatial location also became complex. The two metal-binding sites of CadC require amino acid residues from two protomers: the α3N site has a regulatory function and corresponds to that of ArsR while the α5 site is non-regulatory. SmtB also contains two metal-binding sites requiring two protomers, but only the α5 site has a regulatory function. Saha et al. suggested the order of evolution of the SmtB/ArsR family as ArsR (α3)-AztR

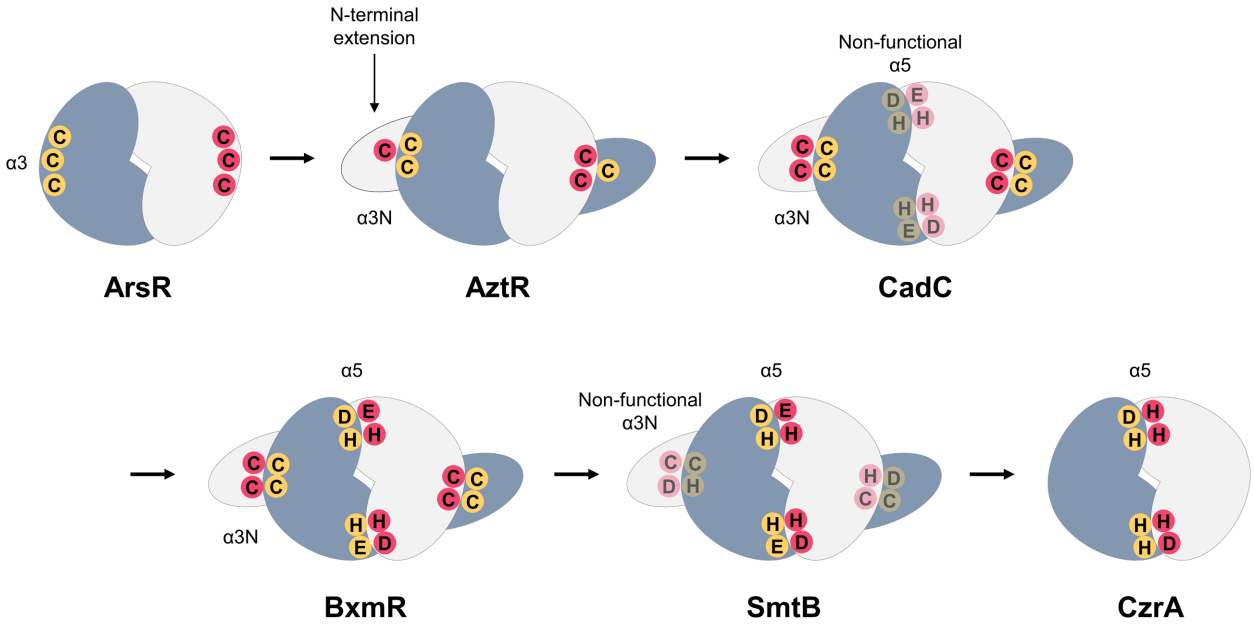

Fig. 3. Evolution of the metal-binding sites of the SmtB/ArsR family proteins.
The protein structures are simplified by showing schematic drawings of two protomers and N-terminal extension. Amino acid residues of the metal-binding sites are marked in one-letter amino acid codes. The faint color of the amino acids indicates that the metal-binding site is non-functional; a metal ion can bind to the ligands, however, it does not cause conformational change and transcriptional regulation. Yellow and red colors of the amino acids indicate that they are from different protomers.

(α3N)-CadC (functional α3N and non-functional α5 site)-BxmR (α3N and α5 are both functional sites)-SmtB (functional α5 and non-functional α3N)-CzrA (α5) (Fig. 3) [20]. However, our comparison of the metal-binding sites from diverse metalloregulatory proteins determined that the hypothesis on the evolutionary order does not cover all the SmtB/ArsR family proteins (Fig. 2). Because alignment of amino acids constituting the metal-binding sites of CadC and SmtB revealed the liganding residues are not strictly conserved in various CadC and SmtB proteins. There are literally thousands of SmtB/ArsR family proteins in the database but only a handful of them have been experimentally investigated, thus for many, their mechanisms, structures, and evolutionary history still remain unexplored.

The MerR Family

The MerR family is another major metalloregulatory protein family. At the time of writing, the NCBI Gene database gave approximately 4,600 and 9,000 genes when searching for "MerR regulator" and "ArsR regulator", respectively. The MerR family contains MerR, CueR, CadR, PbrR, and ZntR which sense Hg(II), Cu(I), Cd(II), Pb(II), and Zn(II), respectively. The most contrasting features of the MerR family compared to the SmtB/ArsR family is that proteins from the former function as both transcription repressors and activators [57], and they also sustain the protein-DNA-binding complex regardless of the presence of inducer metal ions, which represses the transcription of their own genes [58]. The mechanism of how they perform their role as a transcriptional regulator has long been a question posed and intensively investigated, probably due to concerns over the extreme toxicity of Hg and the unprecedented regulatory mechanism at the time of discovery [59]. The common aspects of the MerR and SmtB/ArsR families are their extremely high selectivity and sensitivity toward cognate metal ions. A substantial number of structural, biochemical, and genetic investigations has been performed to address such questions, and we summarize those works in the following sections to provide insight into the MerR family proteins.

The Mechanism for Hg Resistance

Because of environmental abundance, extreme toxicity and the absence of biological function of Hg, it is important for bacteria to have a repertoire for Hg resistance. Efforts to isolate mercury-resistant bacteria have shown that the minimum inhibitory concentration of Hg does not exceed 10 μM [60–62]. With this in mind, the presence of mercury-resistance genes in mobile genetic elements like transposons and their widespread presence among bacterial strains may prove beneficial for survival in toxic environments [63]. The best-studied *mer* operons originate in Tn*21* of the IncFII plasmid in *E. coli* and Tn*501* in *Pseudomonas aeruginosa* [64, 65]. The *merR* gene is divergently located upstream of the *mer* operon, and the target genes of MerR regulation in most microorganisms commonly encode for an inner-membrane protein (*merT*), a periplasmic mercury binding protein (*merP*), a mercuric reductase (*merA*), and an antagonistic regulator (*merD*). The presence of other resistance genes such as an organomercurial lyase (*merB*), a transmembrane protein for Hg(II) uptake (*merC*, *merE*, or *merF*), vary by microbial strain (Table 1) [66, 67]. The expression of MerC, MerE, or MerF increases Hg(II) susceptibility in resistance gene-negative cells [67]. When Hg(II) ions enter the cytoplasm by diffusion, they bind to MerR and activate the transcription of *merPTAD* genes. Hg(II) ions bind to periplasmic protein MerP and are subsequently transferred to the cytoplasm via inner-membrane protein MerT. In the cytoplasm, mercuric reductase MerA converts toxic Hg(II) ions to non-toxic and volatile Hg(0), and then Hg(0) vapor diffuses out from the cell [68]. It is interesting that MerR regulates four or more genes in an operon, while other members of the MerR family such as ZnrR or CueR are involved in the regulation of one or two resistance genes, *i.e.* an efflux pump and an oxidase (Table 1). Possessing genes involved in sequestration, transportation, and detoxification under transcriptional regulation could be attributed to the extreme toxicity of Hg(II) without any biological role.

Regulation by Distortion and Bending of DNA

The MerR family proteins function as repressors or activator while maintaining a complex quaternary structure with DNA and RNA polymerase (RNAP) regardless of the presence of inducer metal ions. A DNase footprint assay has shown that both MerR-DNA and Hg(II)-MerR-DNA complex bind to the spacer region between -35 and -10 [59]. To understand the sophisticated mechanism, a considerable number of genetic, biochemical, and structural investigations have been performed over many decades.

MerR of *Bacillus megaterium* possesses a winged HTH DNA-binding domain at the N-terminal (α1-α2-β1-β2-α3-α4), a dimerization helix (α5), and a metal-binding motif at the C-terminal (α6-α7) [69]. Like the SmtB/ArsR family

proteins, the MerR family proteins function in homodimer form, but unlike the former, the latter possess metal-binding sites and a DNA-binding domain at the C- and N-terminals, respectively. Two protomers are associated via antiparallel coiled-coil packing between two α5 helices. The homodimeric form is essential for proper functioning because the DNA-binding domains should a pair up comprising both protomers and the metal-binding sites are composed of residues from each protomer. Other members of MerR, such as CueR, ZntR, and SoxR, share structural similarity with subtle differences [70, 71].

The homodimer of MerR of *E. coli* binds to its cognate DNA-binding sequence between the -35 and -10 RNAP recognition sites, which are located 26 bp upstream of the *merT* gene [72]. The promoter regions of *merR* and *merT* overlap, thus when MerR binds to the promoter region, it prevents transcription of *merR* and *merT* at the same time. Unlike the SmtB/ArsR family, MerR is bound to the promoter with RNAP during both repression and activation [59]. The spacing between -35 and -10 of *merT* is 19 bp and 20 bp in Gram-negative and Gram-positive bacteria, respectively, which is longer than the usual 17±1 bp of *E. coli* promoters [73, 74]. Mutant strains having 20 or 21 bp spacer exhibit inhibition in the induction of the *mer* operon, even in the presence of Hg(II) ions, whereas mutants with a 17–18 bp spacer show constitutive *mer* expression regardless of the presence of the inducer, implying that the 19 bp spacer length is essential for MerR-mediated repression and activation [75]. MerR family transcriptional regulators need to overcome this suboptimal promoter to activate gene expression. Structural comparison of apo-MerR and Hg(II)-MerR of *B. megaterium* has shown that the distance between two DNA-binding domains (α2 and α2′) is shortened from 34 to 29 Å upon binding of Hg(II) [69]. Meanwhile, structural analysis of CueR in solution has also demonstrated that the distance between two DNA-binding domains of CueR becomes shorter by 1.7 nm (from 6.3 nm to 4.6 nm) upon Cu(I) binding [76]. To achieve structural flexibility, the hinge region connecting the Cu(I)-binding site to the DNA-binding domain experiences the largest change upon Cu(I) binding. Because the DNA-binding domains dock to the major groove of B-form DNA, the binding of metal ions eventually results in the bending of the bound DNA. Cu(I)-CueR shortens the distance between -35 and -10 of the *copA* gene from 58 to 52 Å, which is closer to the distance of 54 Å of the 17 bp *E. coli* consensus, thereby promoting gene expression [77].

Longer spacing also results in different dihedral angles from regular spacing (17 ± 1 bp). Approximately 70° distortion between -35 and -10 hinders the binding of RNAP and the formation of an open complex for transcriptional initiation [78–80]. In the apo-MerR state, only the -35 region is associated with the σ factor of RNAP, while the -10 region is twisted away and transcription cannot occur. Apo-MerR alone twists the promoter DNA by 19° and the binding of an Hg(II) ion results in the distortion of DNA by an additional 33° [80]. Underwinding of the 19 bp DNA spacer by 52° realigns the -10 and -35 elements on the face of the DNA helix to resemble the cylindrical orientation of these elements as if they are found in a promoter with a spacer length of 18 bp. Reorientation by DNA underwinding allows the σ factor to bind to the -35 and -10 regions and RNAP to initiate transcription. This optimization of the promoter configuration by allosteric DNA distortion is the key step for transcriptional activation by MerR [66], and similar mechanisms have been found from a Cu(I)- and Ag(I)-responsive CueR, [77]. Three-dimensional modeling of a ternary complex containing Cu(I)-CueR-DNA-RNAP has also shown that apo-CueR bends the promoter DNA away from RNAP to prevent recognition of the -10 region by the σ2 subunit of RNAP [77]. The MerR-like repression-activation mechanism has been found in other members of the MerR family, such as ZntR and SoxR [71, 81], suggesting that longer spacing between RNAP binding sites and activation by modulating the DNA dihedral angular structure is the conserved mechanism of the MerR family proteins.

Hypersensitivity and Selectivity

Like the SmtB/ArsR family proteins, the MerR exhibit extremely high sensitivity and selectivity toward cognate metal ions. For example, a competition assay between L-cysteine and MerR has shown that the association constant of Tn*501* MerR protein for Hg(II) ion is in the order of 10 [57]. Meanwhile, the results of a *mer-lux* transcriptional fusion and transcription run-off assay has determined that the $K_{0.5}$ (Hg concentration required for half-maximal luminescence intensity) is 9.3×10^{-8} and 5×10^{-8} M, respectively [59, 82]. Other members of MerR family such as CueR and ZntR also showed extremely low $K_{0.5}$ that is 2×10^{-21} M and 1.15×10^{-15} M, respectively. expression level of the *mer* operon from 10% to 90% has been achieved by around a 7-fold change in Hg(II) concentration with this sharp response to the signal corresponding to the response coefficient (R_s) between 3 and 9, which is called the threshold phenomenon [83]. The conformational change

from the inactive apo-CueR state to the active Cu(I)-CueR-DNA state has also been observed for a 4-fold change in Cu(I) concentration [76], while MerR shows high selectivity by recognizing nanomolar Hg(II) even in the presence of millimolar concentrations of thiol-competing ligands [84]. Interestingly, the $K_{0.5}$ of CueR is far less than one atom per cell and it is not clear how a prokaryotic cell obtains Cu(I) required for cofactors of many enzymes. Such high sensitivity and selectivity can be understood based on the fact that in most environments, the concentration of Hg(II) is generally in the pico- to femtomolar range, including where bioaccumulation occurs [66].

The order of ligand affinity is known to be $Hg(SH)_2$ > $Hg(OH)_2$ > $HgBr_2$ ≥ Hg(OH)Cl > $HgCl_2$ [85], which makes sense because the MerR family proteins use cysteine as ligands. The Hg(II)-binding sites in the MerR of *B. megaterium* are composed of Cys79, Cys114′, and Cys123′, which are strictly conserved in the other MerR from various microbial strains. In the inactive state, Cys79 and Cys114′ are closely spaced, which means they could be responsible for the initial association with an Hg(II) ion by transient bidentate binding. Planar trigonal coordination seems to be accomplished by recruiting Cys123′ in the chelating structure [69].

The valance state, ionic radius, and charge-accepting ability of the metal ion, along with the net charge, charge-donating ability, dipole moment, polarizability, and the number of metal-ligating atoms, are considered to be physical and chemical factors affecting the affinity between the metal ions and the ligands [86]. In terms of protein structure, the number of liganding residues, the length of the metal-binding motif, and the environment of the binding site determine the binding specificity [1, 71, 87]. In this regard, the preference of MerR for Hg(II) can be understood because the exposed metal-binding site of apo-MerR is buried upon the binding of Hg(II), resulting in an overall conformational change to activate transcription. On the contrary, Cu(I) cannot achieve tight packing with the metal-binding site of MerR [69], thus a higher concentration of Cu(I) only results in the minor induction of transcriptional activity [84]. Besides, the number of conserved ligands and coordination geometry are different for each cognate metal ion. Analysis of amino acid sequences and cognate metal ions of the MerR family proteins has shown that two cysteine residues are conserved in the +1 ion (Ag(I), Au(I), and Cu(I))-binding to CueR, HmrR, and PmtR, respectively, while three cysteines are conserved in the +2 ion (Cd(II), Co(II), Pb(II), and Zn(II))-binding to CadR, MerR, PbrR, ZccR, and ZntR, respectively. One of the cysteine residues is present in all MerR family proteins binding +2 ions (Cys79 in ZntR), but this is replaced by a serine in the MerR family proteins binding +1 ions (Ser77 in CueR). Therefore, Cu(I) and Zn(II) form bidentate and binuclear binding with CueR and ZntR, respectively [70, 71].

Heavy Metal Biosensors

The detection of heavy metal ions is of utmost importance from an ecotoxicology perspective because they can cause extreme toxicity, even at very low concentrations. In the case of As, WHO standard for drinking water is < 10 μg/l (or ppb), but the concentration of As from groundwater often exceeds this limits in many places around the world [88]. Analytical techniques including UV-vis spectrometry, electrothermal atomic absorption spectrometry, and inductively coupled plasma-atomic emission spectrometry are usually used in the measurement of heavy metal ion concentrations. Although these techniques provide accurate concentration measurements with the low limit of detection, they frequently suffer from disadvantages such as difficult sample preparation, high cost, and non-specific sensing due to interference by other ions and impurities [89]. Biosensors have attracted a great deal of attention as an alternative approach because of their superiority over chemical and instrumental methods. They are generally cheaper to construct, operate, and maintain than expensive analytic devices and are portable to remote areas or can be used under field conditions where *in situ* measuring is impossible. However, whole-cell biosensors, which can even replicate themselves, can only detect the bioavailable fraction to assess the impact of target molecules on the environment, but the development of biosensors combined with electrical devices is expected [90]. Heavy metal transcriptional regulators are very attractive biological parts for the construction of biosensors because of the structural and biochemical data that has been accumulated over decades. With this in mind, we summarize how heavy metal biosensors have been developed and improved and also present the efforts made to overcome their limitations and their future perspectives.

Classical Heavy Metal Biosensors

A biosensor is typically composed of sensing, regulatory, and output modules. For heavy metal biosensors, a metalloregulator and a promoter/operator region containing a DNA-binding sequence for a regulatory

protein are the sensing and regulatory part, respectively. The output module is usually inserted downstream of the promoter to replace the resistance genes. Luciferase, green fluorescent protein (GFP), and β-galactosidase are the most frequently used output reporters because they do not require additional components to produce output signals and the luminescence, fluorescence, and electrons are easily detected by spectrophotometers and potentiometers. The general principles of a heavy metal biosensor are almost identical to a heavy metal resistance system. In the absence of cognate metal ions, a metalloregulator binds to DNA-binding sequences, *i.e.* operators, to prevent the transcription of the reporter gene. Upon binding of the metal ion to the regulatory protein, the protein-metal-ion complex dissociates from the operator (the SmtB/ArsR family) or switches from a repressor to an activator (the MerR family) to allow the transcription of the reporter genes instead of the resistance genes. Most assay methods are performed by mixing samples with biosensor cells grown in liquid media and incubating them for a certain amount of time to allow the cells to produce the output signal, which is measured at a single time-point or at the rate of the signal increment. The amount of signal production is proportional to the amount of inducer, thus extrapolating the intensity of the output signal provides a reading of the concentration of the heavy metal ions. For accurate measurement, output signals per cell, *i.e.* specific signal change, can be obtained by dividing the change in reporter signals by the change in cellular optical density [91].

A simple example is in the form of an arsenite and antimonite biosensor composed of *arsR* and *lacZ* genes encoding for β-galactosidase. In this simplest gene circuit, the native *ars* fragment containing the *arsR* gene and *ars* operator/promoter sequence is cloned with *lacZ* encoding β-galactosidase [106] and *p*-aminophenyl β-D-galactopyranoside is provided as a substrate and is cleaved by β-galactosidase to produces *p*-aminophenol, protons, and electrons which are measured with a potentiometer. This simple biosensor transduces the concentration of heavy metal ions into electric current, and its limit of detection is 1×10^{-7} M of arsenite.

Even after a long history of microbial biosensors, most of their genetic circuits have not varied much from their antecedents. They usually contain a well-characterized transcriptional regulator, an operator/promoter DNA sequence, and an output reporter from a well-characterized source. *E. coli* has been used as the host cell for most whole-cell biosensors, while *lac*, *lux*, or *gfp* are the most used output genes. Summaries of heavy metal-sensing biosensors in other review papers indicate that only well-characterized metalloregulators from a small number of strains have been used [91, 92]. This limitation on simple biosensors may be attributed to the lack of sufficient biochemical data for a wider variety of transcriptional regulators. The design and improvement of a biosensor require an understanding of each biopart as a prerequisite, but the comprehension of most transcriptional regulatory mechanisms has come from a small number of model proteins. As we mentioned previously concerning the functional and structural differences in metal-binding sites of various metalloregulators, it is risky to apply the current knowledge of model proteins shown in Fig. 1 to uncharacterized proteins. Furthermore, since a satisfactory limit of detection has often been achieved with simple circuits, some researchers might not have felt the need to devise novel and better biosensors.

It has often been said that commercialized biosensors are rare even though the construction of proof-of-concept circuits have prevailed. In an effort to develop heavy metal biosensors, various detection platforms could be verified in an attempt to apply lab-scale biosensor systems in the field. Because liquid culture assays performed in a lab require the preparation of cells during the exponential growth phase, it is not always easy to reproduce the biosensor performance in a field trial. The short shelf-life of biosensors is another problem for commercialization, and to solve this, spore-forming *B. subtilis* and *B. megaterium* have been used as host cells [93]. Once the strain had been genetically engineered to function as a Zn-biosensor, spore formation was induced for long-term storage, and germination in the presence of a sample matrix and biosensor functioning were proved. Meanwhile, Stocker et al. used a paper strip as a biosensor platform containing dried reporter cells [94], and exposure to an aqueous test solution for 30 min allowed the development of GFP. In summary for the heavy metal biosensors constructed so far, scientists have not readily employed biological parts from various sources due to the absence of biochemical data, but various detection methods have been tested for application of biosensors in the field.

Improving the Performance of Biosensors

Even though limits of detection beyond safety guidelines have often been achieved with a simple genetic circuit made of native biological components, there are several issues hampering the development of a novel biosensors with superior functionality. In a native gene arrangement,

a transcriptional regulator and resistant genes are usually under the control of a single promoter regulated by a transcriptional regulator [95]. Because the output module substitutes for the resistance genes, a certain amount of leaky expression of reporter genes concomitantly occurs when the basal level of a transcriptional regulator is expressed to repress its own expression. Insufficient repression due to low DNA-binding affinity also causes leaky expression of reporter genes, which is a problem, especially for SmtB/ArsR and MerR family regulators. Because transcriptional activation from the uninduced status to the maximal level occurs in a narrow concentration range [83], this approach can lead to false-positive interpretation and a higher limit of detection in biosensors with transcription factors.

A strategy called insulation has successfully improved the signal-to-noise ratio by adding P_{ars} upstream of the reporter *lac*, *lux*, and *gfp* genes, resulting in the detection capability of 5 μg/l of As(III) [94, 96]. Merulla et al. systematically assessed the effect of auxiliary repressor binding sites on the reduction of background noise by placing an additional operator up- or downstream of the promoter, adjusting the distance between the operator and 5′-UTR, and inverting the direction of the operator sequences [97]. They concluded that ArsR bound to the second operators provides a physical obstruction for RNAP, which is called a transcriptional roadblock [98]. Hence, the chance of background expression is substantially lowered because the expression of the reporter gene only occurs when ArsR is dissociated from both operators. The signal-to-noise ratio can also be enhanced by uncoupling the native ArsR-P_{ars} regulatory system in which ArsR is placed under the control of P_{T7} or P_{lac} while maintaining the expression of *gfp* under P_{ars}. This system contributes toward minimizing the signal-to-noise ratio from 20 to 7.5 μg l^{-1} As(III) [99]. Both the insulation and the uncoupling strategy are aimed at lowering the leaky expression of the reporter gene to enhance the signal-to-noise ratio.

Instead of lowering the noise, increasing the output signal is another approach to promoting the signal-to-noise ratio. Nistala et al. constructed a positive feedback-based gene amplifier combining P_{luxI}-*gfp*-constitutive LuxR variant (cLuxR) [100] that functions as a positive feedback loop that binds to the P_{luxI} promoter to activate the expression of *gfp* and *luxR* again. The function of this feedback loop was successfully validated in a tetracycline sensor, demonstrating the modular functionality of the signal amplifier. The CadC-T7 circuit uses a different approach in that *egfp* is placed under the control of the *cadO* operator and the T7 promoter (P_{T7}), while the expression of T7 RNAP is controlled by the *cadO* operator and CadC repressors. In this circuit, the eGFP signal is only produced when CadC is released from two *cadO* upstream of *cadC*-T7 RNAP and *egfp* in response to Cd(II), followed by T7 RNAP expression [101]. Due to tight transcriptional control and microfluidic detection system, this CadC-T7 circuit achieved the lower limit of detection of Cd(II) and Pb(II).

A toggle switch is another genetic circuit that enhances the signal-to-noise ratio by reducing the background noise and increasing the output signal simultaneously. A characteristic of a toggle switch is the clear separation of the two stable phases: the uninduced "Off" state and the induced "On" state [102]. A toggle switch to detect Cd(II) has been constructed by using divergently transcribed P_{cadR}-*lacI*^{q}-*gfp* and P_{tac}-*cadR*: the "Off" state contains isopropyl β-D-1-thiogalactopyranoside to activate the P_{tac} promoter, express *cadR*, and repress the *gfp* signal, while the "On" state containing Cd(II) and the dissociation of CadR from P_{cadR} allows the expression of LacI^{q} and GFP. LacI^{q} represses the P_{tac} promoter, and thereby maximal induction of GFP is achieved [103].

The biological logic gate is quite useful when detecting multiple metals simultaneously or for the selective detection of a single species in a mixture of multiple metals because it can integrate input signals into output ones. Siuti et al. made a set of 16 logic gates that can be made from two input signals and one output [104] by modifying biological components such as a promoter, a terminator, and the *gfp* gene by introducing recombination target sites *attB* and *attP* in the flanking region. For example, an AND gate was built by placing a promoter and the *gfp* gene in the reverse direction. The GFP signal is produced only when both phiC31 and Bxb1 recombinases are induced and flipped the promoter and the *gfp* gene, respectively. Because the gene flipping reaction by recombinase and integrase is unidirectional, the logic gates act as an irreversible switch: once it turned on, it hardly turns off. The irreversibility and the digital-like output signal of the recombinase-based logic gate are very useful because we often want to determine that the concentration is over the threshold or not at a given moment.

The construction of sophisticated genetic circuits requires an efficient construction method. In a simple gene arrangement, one may implement trial-and-improvement iteratively. However, this approach may not exploit the optimal combination of hundreds of biological parts, for

many of which the mechanisms and kinetics are not currently available. Based on such information, *in silico* modeling can help to foretell the behavior of a biosensor before development. When modeling parameters, one should consider transcription, translation, the turnover rates of mRNAs and proteins, binding affinities, the copy number of plasmids, promoter strength, cell division, transcriptional regulators, and any other procedures affecting reporter functions. Modeling and experimental validation of ArsR biosensors have demonstrated that sophisticated parameterization of the models has helped to predict functionality that is largely compatible with the experimental data and has also helped the development of a variant circuit with a steeper response to lower concentrations of As [105].

During the development of 2,4-dinitrotoluene biosensor, Yagur-Kroll *et al*. demonstrated the screening of a previously constructed 2,000 *E. coli* promoter library [106]. The high throughput screening approach is of interest since an overwhelming number of biological parts are generated from sequencing data. Given that the characterization of individual components takes enormous time and effort, high throughput screening may leap several research procedures and provide candidates for further optimization based on native performance. Synthetic biological techniques have culminated in automated genetic circuit design. Programming code input by the user is parsed into a logic gates diagram and the automation platform selects the standardized parts to produce the DNA sequence encoding of the desired circuit. Considering that the most manually-designed genetic circuits have not increased complexity with more than three regulators, it is impressive that automated design has produced a complex circuit contained 10 regulators and 55 parts [107]. We might also employ artificial intelligence to design novel genetic circuits in the future.

Perspectives

Characterization of heavy metal-sensing transcriptional regulators has provided fundamental knowledge on metal resistance mechanisms, specific metal chelation by proteins, and tightly regulated transcription. Sophisticated biochemical data has made the metalloregulators standardized bioparts that have been used as sensory components in numerous biosensors and has helped pioneer proof-of-concept studies [108]. However, most understanding of metal sensory proteins has been disclosed from a small number of model proteins. Our phylogenetic analysis and sequence alignments with a tremendous amount of the genome database suggest that there is still unexploited diversity of metal-sensing transcriptional regulators with unknown mechanisms (Fig. 1). AioF, an SmtB/ArsR family transcriptional activator in *Thimonas arsenitoxydans*, is an exemplary exception to the traditional paradigm "the SmtB/ArsR family comprises transcriptional repressors" and indicates a lack in our understanding [30]. Cultivation of a novel strain with high resistance to heavy metal could be a good source for the discovery of novel metalloregulators [109]. The prediction from sequencing data and the experimental characterization of new transcriptional regulators should be followed to provide fundamental information for our understanding and application of metalloregulators.

Sensitivity and selectivity of native metalloregulators have surpassed state-of-the-art technology and how we bridge the gap between the extreme native sensitivity of metalloregulators and the limit of detection of artificial biosensors is an important task. Nanomolar sensitivity seems to be easily within the grasp of artificial gene circuits, while ZntR and CueR have shown femto- and zeptomolar sensitivity, respectively [10, 71]. We have reviewed several strategies to improve the sensitivity of biosensors, such as 1) lowering the background noise by insulating and uncoupling the expression of the output signal from the basal expression of sensory modules, 2)

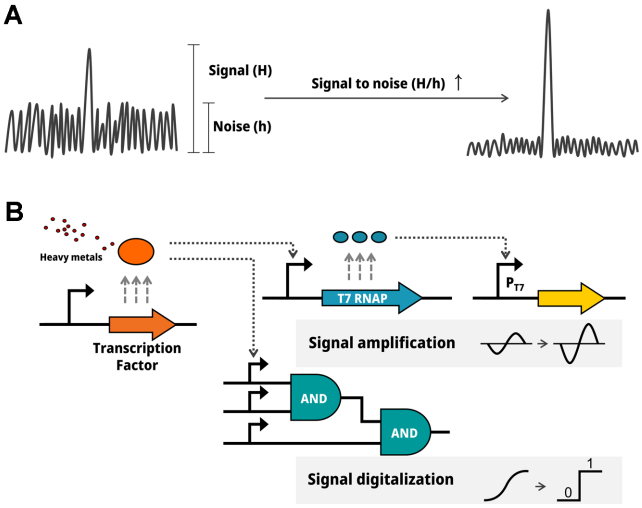

Fig. 4. Strategies to enhance the performance of a biosensor. (**A**) Enhancing the signal-to-noise ratio is the primary method for lowering the limit of detection. Native characteristics of a heavy metal-sensing transcriptional regulator largely determine the overall functionality of a biosensor. (**B**) Novel genetic circuit design and logic gates can result in signal amplification and signal digitalization.

increasing the output by signal amplification, and 3) signal digitalization by toggle switches and logic gates (Fig. 4). Improvement of these methods or devising novel approaches may utilize the full potential of metal-sensing transcriptional regulators.

Over many years of research, we have increased our understanding of prokaryotic metal homeostasis and detoxification by transcriptional regulation, and we have taken advantage of their capability to help us cope with our environmental problems. The future direction for the development of heavy metal biosensors sounds simple; they need to be small, portable, easily applied for assaying, accurate, sensitive, specific, and reproducible. Many biosensors have shown notable results such as a limit of detection low enough to sense nanomolar heavy metal ions and a portable platform for *in situ* detection. However, providing easily interpretable signals is an unfinished task that synthetic biology needs to accomplish. As we have summarized, the characterization of novel transcriptional regulators will bring about superior heavy metal biosensors in coordination with the novel design of genetic circuits.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Reyes-Caballero H, Campanello GC, Giedroc DP. 2011. Metalloregulatory proteins: metal selectivity and allosteric switching. *Biophys. Chem.* **156:** 103-114.
2. Andreini C, Bertini I, Cavallaro G, Holliday GL, Thornton JM. 2008. Metal ions in biological catalysis: from enzyme databases to general principles. *J. Biol. Inorg. Chem.* **13:** 1205-1218.
3. Arredondo M, Núñez MT. 2005. Iron and copper metabolism. *Mol. Aspects Med.* **26:** 313-327.
4. Solioz M, Odermatt A, Krapf R. 1994. Copper pumping ATPases: common concepts in bacteria and man. *FEBS Lett.* **346:** 44-47.
5. Ferguson AD, Deisenhofer J. 2004. Metal import through microbial membranes. *Cell* **116:** 15-24.
6. Capdevila DA, Edmonds KA, Giedroc DP. 2017. Metallochaperones and metalloregulation in bacteria. *Essays Biochem.* **61:** 177-200.
7. Lloyd JR, Lovley DR. 2001. Microbial detoxification of metals and radionuclides. *Curr. Opin. Biotechnol.* **12:** 248-253.
8. Blindauer CA, Harrison MD, Robinson AK, Parkinson JA, Bowness PW, Sadler PJ, *et al*. 2002. Multiple bacteria encode metallothioneins and SmtA-like zinc fingers. *Mol. Microbiol.* **45:** 1421-1432.
9. Jacobsen FE, Kazmierczak KM, Lisher JP, Winkler ME, Giedroc DP. 2011. Interplay between manganese and zinc homeostasis in the human pathogen *Streptococcus pneumoniae*. *Metallomics* **3:** 38-41.
10. Outten CE, O'Halloran T V. 2001. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science* **292:** 2488-2492.
11. Irving H, Williams RJP. 1948. Order of stability of metal complexes. *Nature* **162:** 746-747.
12. Jaishankar M, Tseten T, Anbalagan N, Mathew BB, Beeregowda KN. 2014. Toxicity, mechanism and health effects of some heavy metals. *Interdiscip. Toxicol.* **7:** 60-72.
13. Chowdhury UK, Biswas BK, Chowdhury TR, Samanta G, Mandal BK, Basu GC, *et al*. 2000. Groundwater arsenic contamination in Bangladesh and West Bengal, India. *Environ. Health Perspect.* **108:** 393-397.
14. Williams PN, Islam MR, Adomako EE, Raab A, Hossain SA, Zhu YG, *et al.* 2006. Increase in rice grain arsenic for regions of Bangladesh irrigating paddies with elevated arsenic in groundwaters. *Environ. Sci. Technol.* **40:** 4903-4908.
15. Williams PN, Lei M, Sun G, Huang Q, Lu Y, Deacon C, *et al.* 2009. Occurrence and partitioning of cadmium, arsenic and lead in mine impacted paddy rice: Hunan, China. *Environ. Sci. Technol.* **43:** 637-642.
16. Kaur H, Kumar R, Babu JN, Mittal S. 2015. Advances in arsenic biosensor development – A comprehensive review. *Biosens. Bioelectron.* **63:** 533-545.
17. Baumann B, van der Meer JR. 2007. Analysis of bioavailable arsenic in rice with whole cell living bioreporter bacteria. *J. Agric. Food Chem.* **55:** 2115-2120.
18. Waldron KJ, Rutherford JC, Ford D, Robinson NJ. 2009. Metalloproteins and metal sensing. *Nature* **460:** 823-830.
19. Ma Z, Jacobsen FE, Giedroc DP. 2009. Coordination chemistry of bacterial metal transport and sensing. *Chem. Rev.* **109:** 4644-4681.
20. Saha RP, Samanta S, Patra S, Sarkar D, Saha A, Singh MK. 2017. Metal homeostasis in bacteria: the role of ArsR–SmtB family of transcriptional repressors in combating varying metal concentrations in the environment. *BioMetals* **30:** 459-503.
21. Wu J, Rosen BP. 1993. Metalloregulated expression of the *ars* operon. *J. Biol. Chem.* **268:** 52-58.
22. Ye J, Kandegedara A, Martin P, Rosen BP. 2005. Crystal structure of the *Staphylococcus aureus* pI258 CadC Cd(II)/

Pb(II)/Zn(II)-responsive repressor. *J. Bacteriol.* **187:** 4214-4221.
23. Reyes-Caballero H, Lee CW, Giedroc DP. 2011. *Mycobacterium tuberculosis* NmtR harbors a nickel sensing site with parallels to *Escherichia coli* RcnR. *Biochemistry* **50:** 7941-7952.
24. Liu T, Nakashima S, Hirose K, Shibasaka M, Katsuhara M, Ezaki B, *et al*. 2004. A novel cyanobacterial SmtB/ArsR family repressor regulates the expression of a CPx-ATPase and a metallothionein in response to both Cu(I)/Ag(I) and Zn(II)/Cd(II). *J. Biol. Chem.* **279:** 17810-17818.
25. Pennella MA, Shokes JE, Cosper NJ, Scott RA, Giedroc DP. 2003. Structural elements of metal selectivity in metal sensor proteins. *Proc. Natl. Acad. Sci. USA* **100:** 3713-3718.
26. Cook WJ, Kar SR, Taylor KB, Hall LM. 1998. Crystal structure of the cyanobacterial metallothionein repressor SmtB: a model for metalloregulatory proteins. *J. Mol. Biol.* **275:** 337-346.
27. VanZile ML, Chen X, Giedroc DP. 2002. Structural characterization of distinct α3N and α5 metal sites in the cyanobacterial zinc sensor SmtB. *Biochemistry* **41:** 9765-9775.
28. Eicken C, Pennella MA, Chen X, Koshlap KM, VanZile ML, Sacchettini JC, *et al*. 2003. A metal–ligand-mediated intersubunit allosteric switch in related SmtB/ArsR zinc sensor proteins. *J. Mol. Biol.* **333:** 683-695.
29. Fernández M, Morel B, Ramos JL, Krell T. 2016. Paralogous regulators ArsR1 and ArsR2 of *Pseudomonas putida* KT2440 as a basis for arsenic biosensor development. *Appl. Environ. Microbiol.* **82:** 4133-4144.
30. Moinier D, Slyemi D, Byrne D, Lignon S, Lebrun R, Talla E, *et al*. 2014. An ArsR/SmtB family member is involved in the regulation by arsenic of the arsenite oxidase operon in *Thiomonas arsenitoxydans*. *Appl. Environ. Microbiol.* **80:** 6413-6426.
31. Slyemi D, Moinier D, Talla E, Bonnefoy V. 2013. Organization and regulation of the arsenite oxidase operon of the moderately acidophilic and facultative chemoautotrophic *Thiomonas arsenitoxydans*. *Extremophiles* **17:** 911-920.
32. Busenlehner LS, Weng TC, Penner-Hahn JE, Giedroc DP. 2002. Elucidation of primary (α3N) and vestigial (α5) heavy metal-binding sites in *Staphylococcus aureus* pI258 CadC: evolutionary implications for metal ion selectivity of ArsR/SmtB metal sensor proteins. *J. Mol. Biol.* **319:** 685-701.
33. Kandegedara A, Thiyagarajan S, Kondapalli KC, Stemmler TL, Rosen BP. 2009. Role of bound Zn(II) in the CadC Cd(II)/Pb(II)/Zn(II)-responsive repressor. *J. Biol. Chem.* **284:** 14958-14965.
34. Sun Y, Wong MD, Rosen BP. 2002. Both metal binding sites in the homodimer are required for metalloregulation by the CadC repressor. *Mol. Microbiol.* **44:** 1323-1329.
35. Turner JS, Glands PD, Samson ACR, Robinson NJ. 1996. Zn^{2+}-sensing by the cyanobacterial metallothionein repressor SmtB: different motifs mediate metal-induced protein-DNA dissociation. *Nucleic Acids Res.* **24:** 3714-3721.
36. Busenlehner LS, Cosper NJ, Scott RA, Rosen BP, Wong MD, Giedroc DP. 2001. Spectroscopic properties of the metalloregulatory Cd (II) and Pb (II) sites of *S. aureus* pI258 CadC. *Biochemistry* **40:** 4426-4436.
37. Arunkumar AI, Campanello GC, Giedroc DP. 2009. Solution structure of a paradigm ArsR family zinc sensor in the DNA-bound state. *Proc. Natl. Acad. Sci. USA* **106:** 18177-18182.
38. Zhang APP, Pigli YZ, Rice PA. 2010. Structure of the LexA–DNA complex and implications for SOS box measurement. *Nature* **466:** 883.
39. Lewis M, Chang G, Horton NC, Kercher MA, Pace HC, Schumacher MA, *et al*. 1996. Crystal structure of the lactose operon repressor and its complexes with DNA and inducer. *Science* **271(5253):** 1247-1254.
40. Wang D, Huang S, Liu P, Liu X, He Y, Chen W, *et al*. 2016. Structural analysis of the Hg(II)-regulatory protein Tn*501* MerR from *Pseudomonas aeruginosa*. *Sci. Rep.* **6:** 33391.
41. Gajiwala KS, Burley SK. 2000. Winged helix proteins. *Curr. Opin. Struct. Biol.* **10:** 110-116.
42. Thelwell C, Robinson NJ, Turner-Cavet JS. 1998. An SmtB-like repressor from *Synechocystis* PCC 6803 regulates a zinc exporter. *Proc. Natl. Acad. Sci. USA* **95:** 10728-10733.
43. Cavet JS, Meng W, Pennella MA, Appelhoff RJ, Giedroc DP, Robinson NJ. 2002. A nickel-cobalt-sensing ArsR-SmtB family repressor contributions of cytosol and effector binding sites to metal selectivity. *J. Biol. Chem.* **277:** 38441-38448.
44. Kuroda M, Hayashi H, Ohta T. 1999. Chromosome-determined zinc-responsible operon *czr* in *Staphylococcus aureus* strain 912. *Microbiol. Immunol.* **43:** 115-125.
45. Singh VK, Xiong A, Usgaard TR, Chakrabarti S, Deora R, Misra TK, *et al*. 1999. ZntR is an autoregulatory protein and negatively regulates the chromosomal zinc resistance operon *znt* of *Staphylococcus aureus*. *Mol. Microbiol.* **33:** 200-207.
46. Murphy JN, Saltikov CW. 2009. The ArsR repressor mediates arsenite-dependent regulation of arsenate respiration and detoxification operons of *Shewanella* sp. strain ANA-3. *J. Bacteriol.* **191:** 6722-6731.
47. Antonucci I, Gallo G, Limauro D, Contursi P, Ribeiro AL, Blesa A, *et al*. 2017. An ArsR/SmtB family member regulates arsenic resistance genes unusually arranged in *Thermus thermophilus* HB27. *Microb. Biotechnol.* **10:** 1690-1701.
48. Gao C, Yang M, He Z-G. 2012. Characterization of a novel ArsR-like regulator encoded by Rv2034 in *Mycobacterium tuberculosis*. *PLoS One* **7:** e36255.
49. Blundell MR, Wild DG. 1969. Inhibition of bacterial growth by metal salts. A survey of effects on the synthesis of ribonucleic acid and protein. *Biochem. J.* **115:** 207-212.
50. Gupta A, Whitton BA, Morby AP, Huckle JW, Robinson NJ. 1992. Amplification and rearrangement of a prokaryotic metallothionein locus *smt* in *Synechococcus* PCC 6301

selected for tolerance to cadmium. *Proc. B Biol. Sci.* **248:** 273-281.
51. Li J, Mandal G, Rosen BP. 2016. Expression of arsenic resistance genes in the obligate anaerobe *Bacteroides vulgatus* ATCC 8482, a gut microbiome bacterium. *Anaerobe* **39:** 117-123.
52. VanZile ML, Chen X, Giedroc DP. 2002. Allosteric negative regulation of smt O/P binding of the zinc sensor, SmtB, by metal ions: a coupled equilibrium analysis. *Biochemistry* **41:** 9776-9786.
53. Teichert F, Bastolla U, Porto M. 2007. SABERTOOTH: protein structural alignment based on a vectorial structure representation. *BMC Bioinformatics* **8:** 425.
54. Shi W, Wu J, Rosen BP. 1994. Identification of a putative metal binding site in a new family of metalloregulatory proteins. *J. Biol. Chem.* **269:** 19826-19829.
55. Ordóñez E, Thiyagarajan S, Cook JD, Stemmler TL, Gil JA, Mateos LM, *et al.* 2008. Evolution of metal(loid) binding sites in transcriptional regulators. *J. Biol. Chem.* **283:** 25706-25714.
56. Qin J, Fu H-L, Ye J, Bencze KZ, Stemmler TL, Rawlings DE, *et al*. 2007. Convergent evolution of a new arsenic binding site in the ArsR/SmtB family of metalloregulators. *J. Biol. Chem.* **282:** 34346-34355.
57. Parkhill J, Ansari AZ, Wright JG, Brown NL, O'Halloran T V. 1993. Construction and characterization of a mercury-independent MerR activator (MerRAC): transcriptional activation in the absence of Hg(II) is accompanied by DNA distortion. *EMBO J.* **12:** 413-421.
58. Lund PA, Ford SJ, Brown NL. 1986. Transcriptional regulation of the mercury-resistance genes of transposon Tn*501*. *Microbiology* **132:** 465-480.
59. O'Halloran T V, Frantz B, Shin MK, Ralston DM, Wright JG. 1989. The MerR heavy metal receptor mediates positive activation in a topologically novel transcription complex. *Cell* **56:** 119-129.
60. Amin A, Latif Z. 2017. Screening of mercury-resistant and indole-3-acetic acid producing bacterial-consortium for growth promotion of *Cicer arietinum* L. *J. Basic Microbiol.* **57:** 204-217.
61. Naguib MM, Khairalla AS, El-Gendy AO, Elkhatib WF. 2019. Isolation and characterization of mercury-resistant bacteria from wastewater sources in Egypt. *Can. J. Microbiol.* **65:** 1-14.
62. Rafiq M, Hayat M, Anesio AM, Jamil SUU, Hassan N, Shah AA, *et al*. 2017. Recovery of metallo-tolerant and antibiotic resistant psychrophilic bacteria from Siachen glacier, Pakistan. *PLoS One* **12:** e0178180.
63. Matsui K, Yoshinami S, Narita M, Chien M-F, Phung LT, Silver S, *et al.* 2016. Mercury resistance transposons in Bacilli strains from different geographical regions. *FEMS Microbiol. Lett.* **363(5):** fnw013.
64. Barrineau P, Gilbert P, Jackson WJ, Jones CS, Summers AO, Wisdom S. 1984. The DNA sequence of the mercury resistance operon of the IncFII plasmid NR1. *J. Mol. Appl. Genet.* **2:** 601-619.
65. Stanisich VA, Bennett PM, Richmond MH. 1977. Characterization of a translocation unit encoding resistance to mercuric ions that occurs on a nonconjugative plasmid in *Pseudomonas aeruginosa*. *J. Bacteriol.* **129:** 1227-1233.
66. Barkay T, Miller SM, Summers AO. 2003. Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiol. Rev.* **27:** 355-384.
67. Sone Y, Nakamura R, Pan-Hou H, Itoh T, Kiyono M. 2013. Role of MerC, MerE, MerF, MerT, and/or MerP in resistance to mercurials and the transport of mercurials in *Escherichia coli*. *Biol. Pharm. Bull.* **36(11):** 1835-1841.
68. Hobman JL, Wilkie J, Brown NL. 2005. A design for life: prokaryotic metal-binding MerR family regulators. *Biometals* **18:** 429-436.
69. Chang CC, Lin LY, Zou XW, Huang CC, Chan NL. 2015. Structural basis of the mercury(II)-mediated conformational switching of the dual-function transcriptional regulator MerR. *Nucleic Acids Res.* **43:** 7612-7623.
70. Watanabe S, Miki K, Kita A, Kobayashi K. 2015. Crystal structure of the [2Fe-2S] transcriptional activator SoxR bound to DNA. *PNAS* **64:** C89-C89.
71. Changela A, Chen K, Xue Y, Holschen J, Outten CE, O'Halloran T, *et al*. 2003. Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. *Science* **301:** 1383-1387.
72. Park S-J, Wireman J, Summers AO. 1992. Genetic analysis of the Tn*21* mer operator-promoter. *J. Bacteriol.* **174:** 2160-2171.
73. Harley CB, Reynolds RP. 1987. Analysis of *Escherichia coli* promoter sequences. *Nucleic Acids Res.* **15:** 2343-2361.
74. Summers AO. 2009. Damage control: regulating defenses against toxic metals and metalloids. *Curr. Opin. Microbiol.* **12:** 138-144.
75. Parkhill J, Brown NL. 1990. Site-specific insertion and deletion mutants in the mer promoter-operator region of Tn*501*; the nineteen base-pair spacer is essential for normal induction of the promoter by MerR. *Nucleic Acids Res.* **18:** 5157-5162.
76. Sameach H, Narunsky A, Azoulay-Ginsburg S, Gevorkyan-Aiapetov L, Zehavi Y, Moskovitz Y, *et al.* 2017. Structural and dynamics characterization of the MerR family metalloregulator CueR in its repression and activation states. *Structure* **25:** 988-996.
77. Canalizo-Hernandez M, Schatz GC, Mondragon A, Philips SJ, O'Halloran T V, Yildirim I. 2015. Allosteric transcriptional regulation via changes in the overall topology of the core promoter. *Science* **349:** 877-881.
78. Browning DF, Busby SJW. 2004. The regulation of bacterial transcription initiation. *Nat. Rev. Microbiol.* **2:** 57.

79. Ansari AZ, Bradner JE, O'halloran T V. 1995. DNA-bend modulation in a repressor-to-activator switching mechanism. *Nature* **374:** 370.
80. Ansari AZ, Chael ML, O'Halloran T V. 1992. Allosteric underwinding of DNA is a critical step in positive control of transcription by Hg-MerR. *Nature* **355:** 87.
81. Outten CE, Outten FW, O'Halloran T V. 1999. DNA distortion mechanism for transcriptional activation by ZntR, a Zn (II)-responsive MerR homologue in *Escherichia coli*. *J. Biol. Chem.* **274:** 37517-37524.
82. Condee CW, Summers AO. 1992. A *mer-lux* transcriptional fusion for real-time examination of in vivo gene expression kinetics and promoter response to altered superhelicity. *J. Bacteriol.* **174:** 8094-8101.
83. Goldbeter A, Koshland DE. 1981. An amplified sensitivity arising from covalent modification in biological systems. *Proc. Natl. Acad. Sci. USA* **78:** 6840-6844.
84. Ralston DM, O'Halloran T V. 1990. Ultrasensitivity and heavy-metal selectivity of the allosterically modulated MerR transcription complex. *Proc. Natl. Acad. Sci. USA* **87:** 3846-3850.
85. Parks JM, Smith JC. 2016. Modeling mercury in proteins, pp. 103-122. *In* Methods in enzymology. Elsevier.
86. Dudev T, Lim C. 2014. Competition among metal ions for protein binding sites: determinants of metal ion selectivity in proteins. *Chem. Rev.* **114:** 538-556.
87. Chen PR, He C. 2008. Selective recognition of metal ions by metalloregulatory proteins. *Curr. Opin. Chem. Biol.* **12:** 214-221.
88. Nordstrom DK. 2017. Worldwide occurrences of arsenic in ground water. *Science* 2143-2144.
89. Kaur H, Kumar R, Babu JN, Mittal S. 2015. Advances in arsenic biosensor development–a comprehensive review. *Biosens. Bioelectron.* **63:** 533-545.
90. Siegfried K, Endes C, Bhuiyan AFMK, Kuppardt A, Mattusch J, van der Meer JR, *et al*. 2012. Field testing of arsenic in groundwater samples of Bangladesh using a test kit based on lyophilized bioreporter bacteria. *Environ. Sci. Technol.* **46:** 3281-3287.
91. Kim HJ, Jeong H, Lee SJ. 2017. Synthetic biology for microbial heavy metal biosensors. *J. Biotechnol.* **266:** 72-76.
92. Bereza-Malcolm LT, Mann G, Franks AE. 2015. Environmental sensing of heavy metals through whole cell microbial biosensors: a synthetic biology approach. *ACS Synth. Biol.* **4:** 535-546.
93. Date A, Pasini P, Sangal A, Daunert S. 2010. Packaging sensing cells in spores for long-term preservation of sensors: a tool for biomedical and environmental analysis. *Anal. Chem.* **82:** 6098-6103.
94. Stocker J, Balluch D. 2003. Development of a set of simple bacterial biosensors for quantitative and rapid measurements of arsenite and arsenate in potable water **37:** 4743-4750.
95. Scott DL, Ramanathan S, Shi W, Rosen BP, Daunert S. 1997. Genetically engineered bacteria: electrochemical sensing systems for antimonite and arsenite. *Anal. Chem.* **69:** 16-20.
96. Merulla D, Buffi N, Beggah S, Geiser M, Renaud P, Meer JR Van Der. 2013. Bioreporters and biosensors for arsenic detection. Biotechnological solutions for a world-wide pollution problem. *Curr. Opin. Biotechnol.* **24:** 534-541.
97. Merulla D, Meer JR Van Der. 2016. Regulatable and modulable background expression control in prokaryotic synthetic circuits by auxiliary repressor binding sites.
98. Lewis DEA, Adhya S. 2015. Molecular mechanisms of transcription initiation at gal promoters and their multi-level regulation by GalR, CRP and DNA loop. *Biomolecules* **5:** 2782-2807.
99. Tani C, Inoue K, Tani Y, Harun-ur-Rashid M, Azuma N, Ueda S, *et al.* 2009. Sensitive fluorescent microplate bioassay using recombinant *Escherichia coli* with multiple promoter-reporter units in tandem for detection of arsenic. *J. Biosci. Bioeng.* **108:** 414-420.
100. Nistala GJ, Wu K, Rao C V, Bhalerao KD. 2010. A modular positive feedback-based gene amplifier. *J. Biol. Eng.* **4:** 1-8.
101. Kim HJ, Lim JW, Jeong H, Lee SJ, Lee DW, Kim T, *et al.* 2016. Development of a highly specific and sensitive cadmium and lead microbial biosensor using synthetic CadC-T7 genetic circuitry. *Biosens. Bioelectron.* **79:** 701-708.
102. Gardner TS, Cantor CR, Collins JJ. 2000. Construction of a genetic toggle. *Nature* 1-4.
103. Wu CH, Le D, Mulchandani A, Chen W. 2009. Optimization of a whole-cell cadmium sensor with a toggle gene circuit. *Biotechnol. Prog.* **25:** 898-903.
104. Siuti P, Yazbek J, Lu TK. 2013. Synthetic circuits integrating logic and memory in living cells. *Nat. Biotechnol.* **31:** 448-452.
105. Berset Y, Merulla D, Hatzimanikatis V, Meer JR Van Der. 2017. Mechanistic modeling of genetic circuits for ArsR arsenic regulation. *ACS Synth. Biol.* **6:** 862-874.
106. Yagur-Kroll S, Lalush C, Rosen R, Bachar N, Moskovitz Y, Belkin S. 2014. *Escherichia coli* bioreporters for the detection of 2,4-dinitrotoluene and 2,4,6-trinitrotoluene. *Appl. Microbiol. Biotechnol.* **98:** 885-895.
107. Nielsen AAK, Der BS, Shin J, Vaidyanathan P, Paralanov V, Strychalski EA, *et al.* 2016. Genetic circuit design automation. *Science* **352(6281):** aac7341
108. Prindle A, Samayoa P, Razinkov I, Danino T, Tsimring LS, Hasty J. 2012. A sensing array of radically coupled genetic "biopixels." *Nature* **481:** 39-44.
109. Yu S, Teng C, Bai X, Liang J, Song T, Dong L, *et al.* 2017. Optimization of siderophore production by *Bacillus* sp. PZ-1 and its potential enhancement of phytoextration of Pb from soil. *J. Microbiol. Biotechnol.* **27:** 1500-1512.
110. Xu C, Shi W, Rosen BP. 1996. The chromosomal *arsR* gene of *Escherichia coli* encodes a trans-acting metalloregulatory protein. *J. Biol. Chem.* **271:** 2427-2432.

111. Liu T, Golden JW, Giedroc DP. 2005. A zinc(II)/lead(II)/cadmium(II)-inducible operon from the cyanobacterium *Anabaena* is regulated by AztR, an α3N ArsR/SmtB metalloregulator. *Biochemistry* **44:** 8673-8683.
112. Corbisier P, Ji G, Nuyts G, Mergeay M, Silver S. 1993. *luxAB* gene fusions with the arsenic and cadmium resistance operons of *Staphylococcus aureus* plasmid pI258. *FEMS Microbiol. Lett.* **110:** 231-238.
113. Sun Y, Wong MD, Rosen BP. 2001. Role of cysteinyl residues in sensing Pb (II), Cd (II), and Zn (II) by the plasmid pI258 CadC repressor. *J. Biol. Chem.* **276:** 14955-14960.
114. Nucifora G, Chu L, Misra TK, Silver S. 1989. Cadmium resistance from *Staphylococcus aureus* plasmid pI258 *cadA* gene results from a cadmium-efflux ATPase. *Proc. Natl. Acad. Sci. USA* **86:** 3544-3548.
115. Endo G, Silver S. 1995. CadC, the transcriptional regulatory protein of the cadmium resistance system of *Staphylococcus aureus* plasmid pI258. *J. Bacteriol.* **177:** 4437-4441.
116. Wang Y, Kendall J, Cavet JS, Giedroc DP. 2010. Elucidation of the functional metal binding profile of a Cd(II)/Pb(II) sensor CmtRSc from *Streptomyces coelicolor*. *Biochemistry* **49:** 6617-6626.
117. Harvie DR, Andreini C, Cavallaro G, Meng W, Connolly BA, Yoshida K, *et al.* 2006. Predicting metals sensed by ArsR-SmtB repressors: allosteric interference by a non-effector metal. *Mol. Microbiol.* **59:** 1341-1356.
118. Campbell DR, Chapman KE, Waldron KJ, Tottey S, Kendall S, Cavallaro G, *et al.* 2007. Mycobacterial cells have dual nickel-cobalt sensors sequence relationships and metal sites of metal-responsive repressors are not congruent. *J. Biol. Chem.* **282:** 32298-32310.
119. Brocklehurst KR, Hobman JL, Lawley B, Blank L, Marshall SJ, Brown NL, *et al.* 1999. ZntR is a Zn(II)-responsive MerR-like transcriptional regulator of *zntA* in *Escherichia coli*. *Mol. Microbiol.* **31:** 893-902.
120. Turner JS, Robinson NJ. 1995. Cyanobacterial metallothioneins: biochemistry and molecular genetics. *J. Ind. Microbiol.* **14:** 119-125.
121. Outten FW, Outten CE, Hale J, O'Halloran T V. 2000. Transcriptional activation of an *Escherichia coli* copper efflux regulon by the chromosomal MerR homologue, CueR. *J. Biol. Chem.* **275:** 31024-31029.
122. Checa SK, Espariz M, Audero MEP, Botta PE, Spinelli S V, Soncini FC. 2007. Bacterial sensing of and resistance to gold salts. *Mol. Microbiol.* **63:** 1307-1318.
123. Misra TK, Brown NL, Fritzinger DC, Pridmore RD, Barnes WM, Haberstroh L, *et al.* 1984. Mercuric ion-resistance operons of plasmid R100 and transposon Tn*501*: the beginning of the operon including the regulatory region and the first two structural genes. *Proc. Natl. Acad. Sci. USA* **81:** 5975-5979.
124. Moore MJ, Distefano MD, Zydowsky LD, Cummings RT, Walsh CT. 1990. Organomercurial lyase and mercuric ion reductase: nature's mercury detoxification catalysts. *Acc. Chem. Res.* **23:** 301-308.
125. Hobman JL, Julian DJ, Brown NL. 2012. Cysteine coordination of Pb(II) is involved in the PbrR-dependent activation of the lead-resistance promoter, P_{pbrA}, from *Cupriavidus metallidurans* CH34. *BMC Microbiol.* **12:** 109.
126. Wang Y, Hemmingsen L, Giedroc DP. 2005. Structural and functional characterization of *Mycobacterium tuberculosis* CmtR, a Pb(II)/Cd(II)-sensing SmtB/ArsR metalloregulatory repressor. *Biochemistry* **44:** 8976-8988.
127. Pennella MA, Arunkumar AI, Giedroc DP. 2006. Individual metal ligands play distinct functional roles in the zinc sensor *Staphylococcus aureus* CzrA. *J. Mol. Biol.* **356:** 1124-1136.
128. Ibáñez MM, Checa SK, Soncini FC. 2015. A single serine residue determines selectivity to monovalent metal ions in metalloregulators of the MerR family. *J. Bacteriol.* **197:** 1606-1613.
129. Humbert M V, Rasia RM, Checa SK, Soncini FC. 2013. Protein signatures that promote operator selectivity among paralog MerR monovalent metal ion regulators. *J. Biol. Chem.* **288:** 20510-20519.
130. Ibáñez MM, Cerminati S, Checa SK, Soncini FC. 2013. Dissecting the metal selectivity of MerR monovalent metal ion sensors in *Salmonella*. *J. Bacteriol.* **195:** 3084-3092.
131. Utschig LM, Bryson JW, O'halloran T V. 1995. Mercury-199 NMR of the metal receptor site in MerR and its protein-DNA complex. *Science* **268:** 380-385.
132. Shewchuk LM, Helmann JD, Ross W, Park SJ, Summers AO, Walsh CT. 1989. Transcriptional switching by the MerR protein: activation and repression mutants implicate distinct DNA and mercury (II) binding domains. *Biochemistry* **28:** 2340-2344.
133. Livrelli V, Lee Iw, Summers AO. 1993. In vivo DNA-protein interactions at the divergent mercury resistance (*mer*) promoters. I. Metalloregulatory protein MerR mutants. *J. Biol. Chem.* **268:** 2623-2631.
134. Caguiat JJ, Watson AL, Summers AO. 1999. Cd (II)-responsive and constitutive mutants implicate a novel domain in MerR. *J. Bacteriol.* **181:** 3462-3471.
135. Hakkila KM, Nikander PA, Junttila SM, Lamminmäki UJ, Virta MP. 2011. Cd-specific mutants of mercury-sensing regulatory protein MerR, generated by directed evolution. *Appl. Environ. Microbiol.* **77:** 6215-6224.
136. Chander M, Demple B. 2004. Functional analysis of SoxR residues affecting transduction of oxidative stress signals into gene expression. *J. Biol. Chem.* **279:** 41603-41610.
137. Yoon Y, Kang Y, Lee W, Oh K-C, Jang G, Kim B-G. 2018. Modulating the properties of metal-sensing whole-cell bioreporters by interfering with *Escherichia coli* metal homeostasis. *J. Microbiol. Biotechnol.* **28:** 323-329.