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Modulating the Properties of Metal-Sensing Whole-Cell Bioreporters by Interfering with *Escherichia coli* Metal Homeostasis

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In *Escherichia coli*, the transcription of genes related to metal homeostasis is activated by the presence of target metals. The promoter regions of those genes can be fused with reporter genes to generate whole-cell bioreporters (WCBs); these organisms sense the presence of target metals through reporter gene expression. However, the limited number of available promoters for sensing domains restricts the number of WCB targets. In this study, we have demonstrated an alternative method to generate novel WCBs, based on the notion that since the sensing mechanisms of WCBs are related to metal transportation systems, their properties can be modulated by disrupting metal homeostasis. Mutant *E. coli* strains were generated by deleting the *znt*-operon genes *zntA*, which encodes a zinc-export protein, and *zntR*, which encodes a *znt*-operon regulatory protein, to investigate the effects on the metal-sensing properties of WCBs. Deletion of *zntA* increased the sensitivity but abolished the selectivity of cadmium-sensing WCBs, whereas arsenic-sensing WCBs gained sensitivity toward cadmium. When *zntR* was deleted, cadmium-sensing WCBs lost the ability to detect cadmium, and this was recovered by introducing exogenous *zntR*. In addition, the metal-binding site of ZntR was genetically engineered to modulate metal selectivity. This study provides a valuable platform for the development of novel *E. coli*-based WCBs.

Keywords: Arsenic, *Escherichia coli*, cadmium, metal homeostasis, whole-cell bioreporter, *znt*-operon

Introduction

Heavy metal(oid)s are essential for life, but accumulation can lead to stress and adverse effects. To maintain proper cellular metal levels, many living organisms, including animals, plants, and microbes, possess metal homeostasis systems [1–4]. These systems are activated for both the uptake of essential metals and the export of excess metals. Thus, the fusion of promoter regions controlling the transcription of these genes with reporter genes such as fluorescent proteins and enzymes has been employed to generate cell-based biosensors, called whole-cell bioreporters (WCBs), to sense target heavy metals in diverse environments [5, 6]. Despite that heavy metals such as arsenic, cadmium, and mercury can be hazardous when released into environmental systems, quantifying their amounts by traditional instrumental analysis is still a difficult and time-consuming process. Therefore, bacterial cell-based biosensors have been intensively developed [7–9], as they are easily applied to diverse environmental systems and are much more economical than instrumental analysis. In addition, the risks of heavy metals are typically assessed on the basis of the total amount, as determined by instrumental analysis. However, this is inappropriate, as not all of the metal present will impact living organisms. Importantly, the heavy metal levels detected by WCBs are not the total

amounts, but the bioavailable amounts in the samples, making WCBs even more valuable.

The bacterial cell-based WCBs were rapidly developed upon completion of bacterial whole-genome sequencing. A series of genes that respond to diverse harmful materials, such as antibiotics and heavy metals, were identified, and the promoters were employed as sensing domains for target materials. For examples, the *ars*, *znt*, *mer*, and *nik* operons are induced by the presence of arsenic, zinc, mercury, and nickel, respectively, and their promoters have been employed to generate WCBs to detect target metals [9−11]. However, the selectivity and sensitivity of these biosensors vary depending on the nature of the promoters and regulatory proteins. For example, although the *E. coli znt*-operon promoter responds to zinc, a WCB harboring *znt*-promoter*::egfp* (encoding enhanced green fluorescent protein (EGFP)) responded to cadmium best, followed by mercury [12]. In addition, although it is known to be copper-responsive, an *E. coli cop*-operon promoter-based WBC had insufficient sensitivity to detect copper. The activation of WCB reporter gene expression is controlled by regulatory proteins that act as repressors in the absence of target metals, and are released from promoter regions upon metal ion binding, inducing reporter gene transcription [7, 13]. Therefore, it may be possible to modulate the selectivity of WCBs by engineering changes in the properties of the regulatory protein. If the metal selectivity of a regulatory protein was changed through molecular engineering, existing WCBs could be improved, and new WCBs could be generated. For example, the specificity of WCBs can be enhanced by interrupting the metal-export system, resulting in the accumulation of the metal inside cells [14]. This report demonstrated an over 50-fold increase in metal sensitivity by deleting a gene encoding a zinc transporter in *Pseudomonas putida*.

In this study, we demonstrate approaches to enhance and modulate the selectivity and sensitivity of WCBs by interfering with the metal homeostasis system and engineering metal-binding regulatory proteins in *E. coli*. Genes in the *znt* operon, which are responsive to divalent metal cations such as Pb(II), Zn(II), and Cd(II), were deleted, and their effects on metal-sensing WCBs carrying plasmids harboring *zntAp::egfp* and *nikAp::egfp*, which are used to quantify bioavailable cadmium and arsenic in soils, respectively [10, 12], were investigated. Interruption of metal homeostasis by gene deletion and regulatory protein bioengineering changed the properties of the WCBs, providing an alternative method to enhance existing WCBs and generate novel WCBs targeting additional materials.

Materials and Methods

Materials

E. coli BL21 (DE3) was used as a host for WCB generation, and mutant strains were generated using the Quick & Easy *E. coli* Gene Deletion Kit (Gene Bridges, Germany). WCB fluorescence was measured on an FC-2 fluorescence spectrophotometer (Scinco, Korea) equipped with a xenon lamp as a light source to generate wavelengths of 300–800 nm excitation/emission, and 0.1–5 nm bandwidth filter sets. Heavy metal(loid)s used in the WCB assays were prepared as 1 mM stock solutions by dissolving $AsCl_3$, $CdCl_2$, $CrSO_4$, $NiCl_2$, $HgCl_2$, $PbSO_4$, $ZnCl_2$, and $CuSO_4$ in distilled water. HotStar Taq for PCR and a genomic DNA extraction kit were purchased from Qiagen (USA). Restriction enzymes and T4 DNA ligase were purchased from Takara (Takara Korea Biomedical). Primers were purchased from Macrogen (Korea)

Generation of Mutant *E. coli* Strains with Gene Deletions

The *zntA* and *zntR* genes in the *E. coli znt* operon were deleted using the Quick & Easy *E. coli* Gene Deletion Kit. Primers contained FRT-flanked PGK-gb2-neo cassette and target gene sequences (listed in Table 1). A pRedET plasmid carrying the recombinase gene was first transformed into *E. coli* BL21 (DE3), and then the FRT-flanked PGK-gb2-neo cassette targeting each gene was introduced by electroporation at 1,350 V, 10 μF, and 600 Ω using an Eppendorf Electroporator 2510. The target genes were replaced with the kanamycin resistance gene (*kan*) upon induction with 10% arabinose. Gene deletion was confirmed by PCR. The *E. coli* strains used in the study are summarized in Table 2.

Plasmid Construction

The plasmids pZntA-EGFP and pNik-EGFP, which possessed fusions between the promoter regions of the *znt* and *nik* operons, respectively, and *egfp,* have been previously described [12, 15]. The promoter regions were amplified from *E. coli* chromosomal DNA by PCR and inserted into pET-21(a) with BglII and XbaI; *egfp* was inserted downstream with BamHI and XhoI. The *zntR* gene was amplified from chromosomal DNA and then inserted into pCDF-Duet to generate pCDF-ZntR. Mutations in the metal-binding site of ZntR were generated by 2-step PCR to obtain pZntR-ΔCys115, pZntR-ΔHis119/Ser120, and pZntR-ΔCys115/His119. DNA sequencing was used to confirm all constructs (listed in Table 2).

WCB Assay

Wild-type and mutant *E. coli* BL21 (DE3) strains were used as host cells, and the pZntA-EGFP and pNik-EGFP plasmids were introduced to generate WCBs sensing cadmium and arsenic, respectively. For the assay, WCB cells grown overnight at 37°C were inoculated into fresh lysogeny broth (LB) containing 50 μg/ml ampicillin. The cells were grown at 37°C in a shaking incubator until an optical density at 600 (OD_{600}) of 0.3–0.4, and then the cells were exposed to each metal. After 1 and 2 h of exposure, 1 ml of

Table 1. List of primers used in this study.

No	Target gene	Restriction enzyme site	Primer sequence (5′ to 3′)
1[a]	*zntR*	BamHI	GTG**GGATCC**GATGTATCGCATTGGTGAG
2[a]		XhoI	GTG**CTCGAG**TCAACAACCACTCTTAACG
3	ΔCys115	-	ACTGCTATGAGCAGTCCCACAGGCATCGTTAAGGCG
4		-	CGCCTTAACGATGCCTGTGGGACTGCTCATAGCAGT
5	ΔHis119/	-	ACAATAAACACTAGCAGTCCCACAACAGGC
6	Ser120	-	TGTGGGACTGCTAGTGTTTATTGTTCGATTCTTG
7	ΔCys115/	-	ATAAACACTGCTAGCAGTCCCACAGGCATC
8	His119	-	TGTGGGACTGCTAGCAGTGTTTATTGTTCG
9[b]	*zntA*	-	ATCGCGCTCAATGTTGCGATCGGTTTGCCTTATCTCCTGCGCAACAATCTaattaaccctcactaaagggcg
10[b]	Deletion	-	AGAGTGTATCCTTCGGTTAATGAGAAAAAACTTAACCGGAGGATGCCATGtaatacgactcactatagggctc
11[b]	*zntR*	-	TGCTGACAAGTTGTTGGACAAAATCAACGATAACTAGTGGAGTATGTATGaattaaccctcactaaagggcg
12[b]	Deletion	-	CGCGAGTGTAATCCTGCCAGTGCAAAAAATCAACAACCACTCTTAACGCCtaatacgactcactatagggctc

[a]The restriction enzyme sites are indicated in bold letters.

[b]Sequences that served as PCR primers to amplify the FRT-PGK-gb2-neo-FRT cassette are underlined.

WCB cells was collected to measure the EGFP fluorescence induced by metal exposure on a fluorescence spectrophotometer. The excitation wavelength was 480 nm, and emission was scanned from 500 to 600 nm. The arbitrary units of EGFP intensity were converted to an induction coefficient, defined as [EGFP intensity with metal exposure]/[EGFP intensity without metal exposure].

Recovery of ZntR Activity

To investigate the effects of ZntR on WCBs, wild-type and *zntR*-deleted *E. coli* strains were used as host cells to generate three WCBs: wild type with pZntA-EGFP, Δ*zntR* with pZntA-EGFP, and Δ*zntR* with pZntA-EGFP/pCDF-ZntR. To test the recovery ability of exogenous ZntR in *zntR*-deleted *E. coli*, 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at an OD_{600} of approximately 0.3 to induce ZntR expression, and metal treatment was performed after 1 h of further incubation. The fluorescent signals of the WCBs were measured using 1 ml of WCB after 1 and 2 h incubations. IPTG was added to all three WCBs for consistency. The ZntR mutants pZntR-ΔCys115, pZntR-ΔHis119/Ser120, and pZntR-ΔCys115/His119 were transformed into *zntR*-deleted *E. coli* with pZntA-EGFP, and then treated with IPTG to induce protein expression. The metal selectivity of the WCBs was tested using 1 μM As(III), Cd(II), Cr(II), Ni(II), Hg(II), Pb(II), and Cu(II).

Results and Discussion

Deletion of *zntA* Abolishes the Metal Selectivity of WCBs Harboring pZntA-EGFP

Previously, we used WCBs harboring pZntA-EGFP as a biosensor to quantify bioavailable cadmium in contaminated

Table 2. Plasmids and *E. coli* strains used in this study.

	Name	Genetic properties	Reference
Plasmids	pET-21(a)	pBR322 ori, Amp^r	Novagen
	pCDF-Duet	CloDE13 ori, Str^r	
	pZntA-EGFP	pET-21(a) carrying *zntAp* from *E. coli* and *egfp* from pEGFP-N1	Yoon *et al.* [12]
	pNik-EGFP	pET-21(a) carrying *nik*-pr from *E. coli* and *egfp* from pEGFP-N1	Yoon *et al.* [12]
	pCDF-ZntR	pCDF-Duet carrying *zntR* from *E. coli*	This study
	pZntR-ΔC115	pCDF-Duet carrying mutant *zntR* ΔCys115	
	pZntR-ΔH119/S120	pCDF-Duet carrying mutant *zntR* ΔHis119/Ser120	
	pZntR-ΔC115/H119	pCDF-Duet carrying mutant *zntR* ΔCys115/His119	
Strains	BL21(DE3)	F^- *ompT hsdS*$_B$($r_B^- m_B^-$)*gal dcm lon* (DE3)	Stratagene
	BL21-*zntA*	BL21(DE3) Δ*zntA*	This study
	BL21-*zntR*	BL21(DE3) Δ*zntR*	

soils [12]. The WCBs employed the promoter region of the *E. coli znt* operon as a sensing domain and *egfp* as a reporter domain. Since this reporter is based on the regulation of a zinc-inducible operon, we hypothesized that the deletion of ZntA, which plays a key role in zinc export, could change its properties. To investigate this hypothesis, *zntA* was deleted from the chromosomal DNA of *E. coli* and replaced with a Δ*zntA*-FRT-PGK-gb2-neo-FRT cassette. Introduction of the pZntA-EGFP plasmid generated *zntA*-deleted WCBs, and their metal selectivity was compared with wild-type WCBs. The WCBs were exposed to 5 μM of arsenic, cadmium, chromium, nickel, mercury, lead, zinc, and copper, and EGFP induction was compared (Fig. 1A). With wild-type WCBs, EGFP induction was observed after cadmium and mercury treatments, consistent with our previous report [12]. However, the metal selectivity was abolished from Δ*zntA* WCBs. As shown in Fig. 1, no selective EGFP induction was observed in *zntA*-deleted WCBs. ZntA plays a role in the export of various divalent metal ions [16−18], suggesting that the mutant *E. coli* may have lost this function, resulting in the accumulation of metal ions inside the cells.

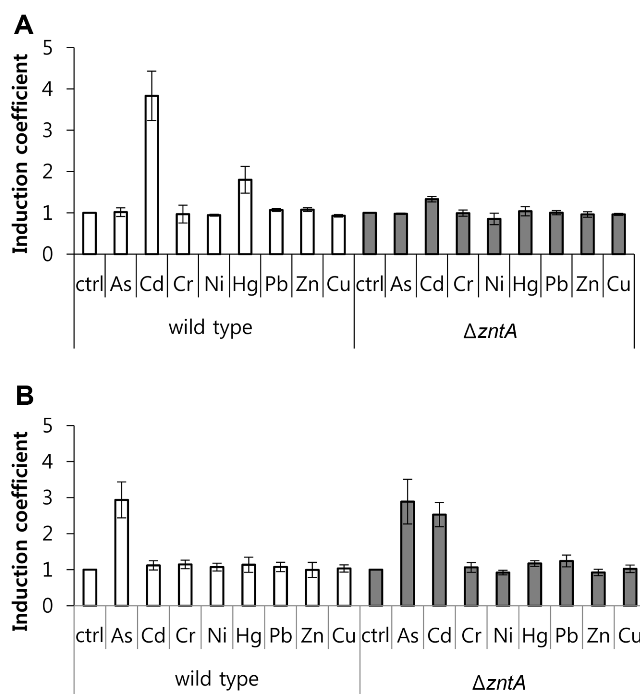

Fig. 1. Effects of *zntA* deletion on whole-cell bioreporters (WCBs) harboring *zntAp::egfp* and *nikAp::egfp*.

The heavy metal selectivity of cadmium- and arsenic-sensing WCBs based on wild-type and Δ*zntA* mutant *E. coli* BL21 (DE3) strains harboring *zntAp::egfp* (**A**) and *nikAp::egfp* (**B**), respectively, was compared after 1 h of heavy metal treatment. The EGFP intensity is represented as an induction coefficient, defined as [intensity with metal treatment]/[intensity without metal treatment].

Deletion of *zntA* Changes the Selectivity of WCBs Harboring pNik-EGFP

We have previously used WCBs based on the promoter region of the *nik* operon (*nik*-pr) fused to *egfp* as an arsenic biosensor [10]. To investigate the effects of *zntA* deletion on these WCBs, pNik-EGFP was transformed into both wild-type and Δ*zntA E. coli* BL21 (DE3) strains. As described above, both WCBs were exposed to metal ions and EGFP induction was measured. Unlike pZntA-EGFP WCBs, Δ*zntA* pNik-EGFP WCBs gained additional metal selectivity (Fig. 1B). Although the *nik* operon is known to be nickel responsive, wild-type WCBs displayed EGFP induction only after arsenic treatment, as observed in our previous report, whereas Δ*zntA* WCBs gained additional selectivity for cadmium.

Metal homeostasis in microorganisms is regulated by diverse metal transporting systems [19, 20]. The expression of ZntA, a Pb(II)/ Zn(II)/ Cd(II)-translocating ATPase, is activated by ZntR on the basis of intracellular free zinc levels, to decrease excess metal amounts [21−23]. Thus, it is possible that the deletion of *zntA* resulted in the accumulation of cadmium inside cells, resulting in EGFP expression in Δ*zntA* WCBs. Although it is unclear why the WCBs gained additional metal selectivity, it suggests that the properties of WCBs can be modulated by disrupting metal homeostasis systems in the host microorganism. In addition, this may represent a novel approach to generate new WCBs despite the limited number of sensing domains.

ZntR Regulates the Transcription of Genes Controlled by the *zntA* Promoter (*zntAp*)

To verify the role of ZntR, the mutant *E. coli* strain BL21-Δ*zntR* was generated. pZntA-EGFP and pCDF-ZntR plasmids were transformed into wild-type and Δ*zntR E. coli* BL21 (DE3) to generate three WCBs: wild-type *E. coli* BL21 (DE3) harboring pZntA-EGFP, BL21-Δ*zntR* harboring pZntA-EGFP, and BL21-Δ*zntR* harboring pZntA-EGFP and pCDF-ZntR. The three types of WCBs were tested against 0–5 μM of Cd(II). As shown in Fig. 2, EGFP induction increased in a concentration-dependent manner for the wild-type strain, whereas the Δ*zntR* strain harboring pZntA-EGFP did not respond to Cd(II). However, the response toward Cd(II) was recovered by introducing pCDF-ZntR, indicating that ZntR is a Cd(II) concentration-dependent critical regulator of the transcription of genes controlled by the *zntAp*. The transcription of genes controlled by the *zntAp* is activated

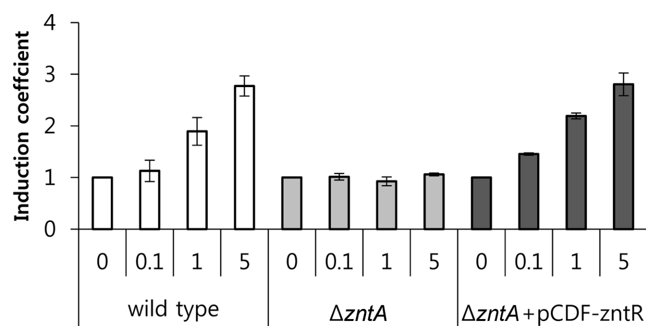

Fig. 2. Effects of *zntR* deletion on cadmium-sensing whole-cell bioreporters (WCBs) harboring *zntAp::egfp*.

Comparison of EGFP induction coefficients from wild-type *E. coli*, a Δ*zntR* mutant, and the Δ*zntR* mutant containing pCDF-Duet-ZntR. The WCBs were exposed to 0–5 μM cadmium for 1 h.

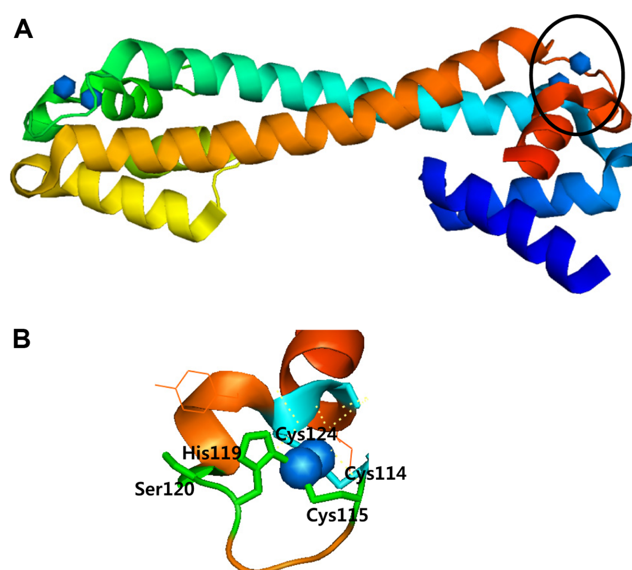

Fig. 3. Three-dimensional structure of *E. coli* ZntR.

(**A**) Structure of the ZntR dimer (PDB ID: 1Q08). The blue spheres represent two zinc ions, and the circle indicates the metal-binding loop. (**B**) Enlargement of the metal-binding loop. The residues involved in the metal interaction are shown in green and cyan.

by interactions between Cd(II) and ZntR [24, 25]. Therefore, it was expected that when *zntR* was deleted, WCBs would not respond to Cd(II). The structural conformation of ZntR changes upon Cd(II) binding, releasing it from the promoter region and activating transcription. Thus, modulation of the metal-binding properties of ZntR may represent an additional means to develop novel WCBs.

Modulating the Properties of WCBs Using Genetic Engineering of *zntR*

The three-dimensional structure of *E. coli* ZntR (Fig. 3; PDB ID: 1Q08) [26] was obtained from the Protein Data Bank (PDB) and visualized using PyMol software. ZntR acts as a dimer (Fig. 3A), and its metal-binding loops hold two zinc ions each. As shown in Fig. 3B, the zinc ions interact with Cys114, Cys115, His119, and Cys124 in the metal-binding loop. Interestingly, MerR family metalloregulators in microorganisms show homology at these residues [26, 27]. From sequence homology analysis, it was observed that monovalent metal ion-binding proteins, such as CueR and GolS, have shorter metal-binding loops and less cysteine residues, whereas divalent metal ion-binding proteins, including ZntR and MerR, have relatively longer loops with more cysteines. Thus, we decided to reduce the length of the metal-binding loop and the number of

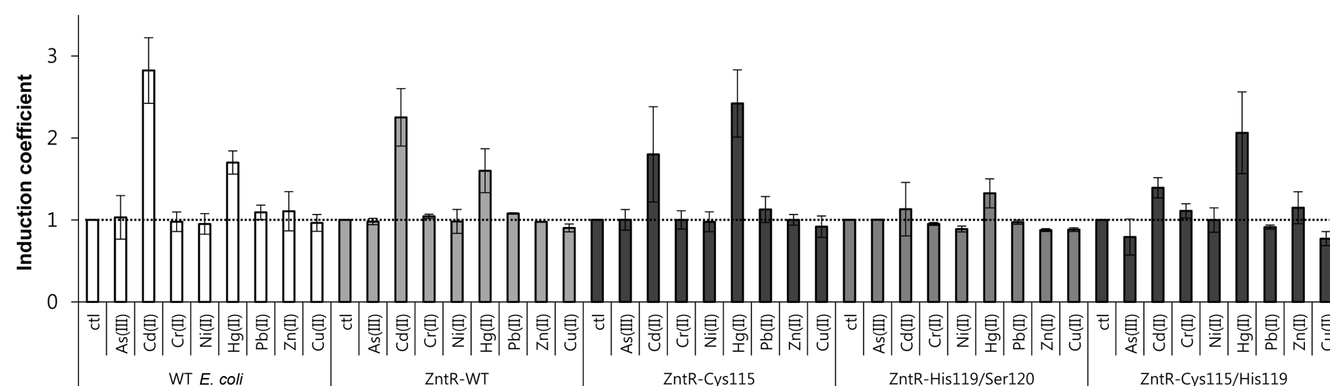

Fig. 4. Modulating the metal-sensing properties of whole-cell bioreporters (WCBs) harboring *zntAp::egfp* by introducing mutations to the ZntR metal-binding loop.

Wild-type *E. coli* BL21 (DE3)- and BL21-Δ*zntR*-based WCBs harboring *zntAp::egfp* were generated by introducing wild-type and mutant versions of the pCDF-ZntR plasmid. The WCBs were tested with 1 μM of eight heavy metals, and their metal selectivity and specificity are represented as induction coefficient values. The dotted line indicates the induction coefficient value of WCBs without metal treatment.

cysteines in ZntR to examine any changes in metal-binding properties.

We generated three ZntR mutants (ZntR-ΔCys115, ZntR-ΔHis119/Ser120, and ZntR-ΔCys115/His119), which were introduced in *E. coli* BL21-*zntR* with pZntA-EGFP, generating three mutant WCBs. To test the effects of the mutations on metal selectivity, the wild-type and mutant WCBs were used in selectivity tests with 1 μM As(III), Cd(II), Cr(II), Ni(II), Hg(II), Pb(II), Zn(II), and Cu(II). The expression of recombinant wild-type and mutant ZntR was induced, and metal ions were added 1 h later. EGFP intensity was measured after 0.5 and 1 h (Fig. 4). WCBs with wild-type ZntR showed the best response to Cd(II), similar to wild-type *E. coli* (BL21) WCBs, whereas the metal-sensing properties were altered in ZntR-mutated WCBs. The ΔCys115 mutation resulted in a higher response to Hg(II) than Cd(II). His119/Ser120 deletion weakened the sensitivity to Cd(II) and Hg(II), resulting in no metal selectivity under the tested experimental condition. With the ΔCys115/His119 mutation, the metal selectivity and sensitivity decreased toward Cd(II) and increased toward Hg(II), similar to ΔCys115. Taken together, these results suggest that ΔCys115 weakens Cd(II) specificity, whereas ΔHis119/Ser120, which results in a shorter metal-binding loop, abolishs metal interactions. As shown in Fig. 4, Cys115 and His 119 form coordinated bonds with metals. Thus, their deletion would disrupt the structural conformation of the region, resulting in changes in metal selectivity and specificity. This suggests that the sensitivity and selectivity of WCBs can be modulated by engineering regulatory proteins, indicating that existing WCB systems can be used as platforms for the development of novel biosensors.

Conclusively, bacterial cell-based WCBs are efficient and rapid biosensors for the detection of harmful materials in diverse environmental systems. The mechanism of WCBs is based on the activation of stress-responsive genes, and the detection of target materials is indicated by the stress-dependent expression of reporter genes. However, they cannot be applied to the majority of harmful materials owing to limited information regarding the stress-responsive genes induced by most target materials. In this study, we have used available WCB systems as a platform to develop novel WCBs. Two strategies were tested to modulate the selectivity and specificity of WCBs: (i) disruption of host metal homeostasis by deleting genes involved in metal transport, and (ii) modulation of target material specificity by introducing mutations into host regulatory proteins. The results indicate that novel WCBs can be generated from existing WCB systems through the biological engineering of host genes related to target material homeostasis.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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