Introduction

Owing to anthropogenic activities, environmental contamination with various pollutants, including hazardous chemicals, antibiotics, and heavy metals, is accelerating. Since these toxic pollutants threaten human health, effective remediation strategies have become increasingly important. However, such processes are often implemented long after the fact, emphasizing the importance of the early detection of environmental contaminants. Environmental pollutants are generally quantified by using analytical instruments, but instrumental analyses are limited by high costs, time-consuming processes, and technical requirements.

Bacterial cell-based biosensors, or whole-cell bioreporters (WCBs), are an alternative tool for the quantification of hazardous materials. Most WCBs share similar working mechanisms. In brief, the recognition of a target by sensing domains induces a biological event, such as changes in protein conformation or gene expression, providing a basis for quantification. WCBs targeting heavy metal(loid)s employ metalloregulators as sensing domains and control the expression of genes in the presence of target metal(loid) ions, but the diversity of targets, specificity, and sensitivity of these WCBs are limited. In this study, we genetically engineered the metal-binding loop (MBL) of ZntR, which controls the znt-operon in Escherichia coli. In the MBL of ZntR, three Cys sites interact with metal ions. Based on the crystal structure of ZntR, MBL sequences were modified by site-directed mutagenesis. As a result, the metal-sensing properties of WCBs differed depending on amino acid sequences and the new selectivity to Cr or Pb was observed. Although there is room for improvement, our results support the use of currently available WCBs as a platform to generate new WCBs to target other environmental pollutants including metal(loid)s.

Keywords: Heavy metals, protein engineering, whole-cell bioreporter, ZntR, metal-binding loop
Materials and Methods

Materials

*Escherichia coli* DH5α was used as the host strain for WCBs. The Quick & Easy *E. coli* Gene Deletion Kit (Gene Bridges, Germany) was employed to generate mutant *E. coli* DH5α strains. pET-21(a) and pCDF-Duet (Novagen, USA) were used to construct the plasmid carrying the reporter gene with the promoter of the znt operon and carrying zntR and its mutants, respectively. Heavy metal(loid)s AsCl3, SbCl2, CdCl2, CrSO4, NiCl2, HgCl2, PbSO4, ZnCl2, CuSO4, and AuCl, were purchased from Sigma-Aldrich (USA) and used as 1 mM stock in distilled water. HotStarTaq and Turbo Pfu were used for amplifying genes and site-directed mutagenesis, respectively, and were purchased from Qiagen (Germany). Restriction enzymes and T4 DNA ligase were purchased from Takara Biomedical (Korea). Primers used in this study were synthesized and purchased from Macrogen (Korea).

Deletion of Endogenous Genes in *E. coli*

The deletion of genes was performed following a previously described protocol [6]. Briefly, the FRT-flanked PGK-gb2-neo cassette and target gene sequences were amplified by PCR and introduced into *E. coli* harboring the pRedET plasmid carrying the gene encoding recombinase by electroporation at 1,350 V, 10 μF, and 600 Ω using an Eppendorf Electroporator 2510. Eventually, target genes were replaced with the kanamycin resistance gene (kan) upon induction with 10% arabinose. The deletion of target genes was further confirmed by PCR. For double-gene deletion mutants, *kan* replaced zntR was removed by expressing FLP recombinase using the 708-FLPp plasmid, and then *copA* was replaced with *kan*. In this study, two mutant strains of *E. coli*, *zntR::kan* and Δ*zntR/copA::kan*, were generated and used as hosts for WCBs.

Plasmid Construction

The plasmid carrying zntA::egfp generated in our previous studies was used as a reporter construct [7]. The zntR gene and its mutants encoding ZntR and its derivatives were cloned into the modified pCDF-Duet vector. Since the original vector has the T7 promoter to control gene expression, isopropyl β-D-1-thiogalactopyranoside (IPTG) induction is required in *E. coli* BL21. For simplicity, the T7 promoter was replaced with the Ptac promoter for constitutive gene expression in *E. coli*. The zntR gene and mutants were inserted into the modified pCDF-Duet with BamHI/XhoI restriction sites. Some of the mutants were generated by 2-step overlap PCR and others were obtained by site-directed mutagenesis with Turbo Pfu. All mutants generated in this study are listed in Table 1 with the amino acid sequences of MBLs.

Table 1. The list of recombinant ZntR wild type and mutants tested in this study with amino acid sequences of metal-binding loops (MBLs).

| No. | Name   | Amino acid sequences of MBLs | Order of responding strength |
|-----|--------|------------------------------|-----------------------------|
|     |        |                              | *E. coli-zntR* | *E. coli-zntR/copA* |
| 1   | ZntR-WT| CCGTAHSSVYCS                 | Cd>Hg                  | Cd>Hg                  |
| 2   | ZntR-HJ1| CCGTARSSVYCS                | Cd>Hg>Pb               | Hg>Cd>Pb               |
| 3   | ZntR-HJ3| CCGTAHSSVYCS                | None                   | -                      |
| 4   | ZntR-HJ4| CCGTARSSVYCS                | Hg>Cd                  | -                      |
| 5   | ZntR-HJ6| CCGTARSSVYCS                | Hg>Cd>Pb               | Hg>Cd>Pb               |
| 6   | ZntR-HJ7| CCGTARSSVYCS                | Hg>Cd>Pb               | Hg>Cd>Pb               |
| 7   | ZntR-HJ8| CCGTARSSVYCS                | Hg>Cd>Pb               | Hg>Cd>Pb               |
| 8   | ZntR-HJ9| CCGTAHSSVYCS                | None                   | -                      |
| 9   | ZntR-HJ10| CCGTAHSSVYCS               | None                   | -                      |
| 10  | ZntR-HJ11| CCGTAHSSVYCS              | None                   | -                      |
| 11  | ZntR-HJ12| CCGTAHSSVYCS              | None                   | -                      |
| 12  | ZntR-HJ13| CCGTAHSSVYCS              | Hg>Cd                  | -                      |
| 13  | ZntR-HJ14| CCGTAHSSVYCS              | None                   | -                      |
| 14  | ZntR-HJ15| CCGTAHSSVYCS              | Hg>Cd                  | -                      |
| 15  | ZntR-HJ16| CCGTAHSSVYCS              | Hg>Cd>Pb               | -                      |
| 16  | ZntR-HJ17| CCGTAHSSVYCS              | Hg>Cd                  | -                      |
| 17  | ZntR-HJ18| CCGTAHSSVYCS              | None                   | -                      |
| 18  | ZntR-HJ19| CCGTAHSSVYCS              | None                   | -                      |
| 19  | ZntR-HJ20| CCGTAHSSVYCS              | Hg>Cd                  | -                      |
| 20  | ZntR-HJ21| CCGTAHSSVYCS              | Hg>Cd                  | -                      |
| 21  | ZntR-HJ22| CCGTAHSSVYCS              | Cd>Hg>Pb               | Cd>Hg>Pb               |
| 22  | ZntR-HJ23| CCGTAHSSVYCS              | Cd>Hg                  | Cd>Hg>Pb               |
| 23  | ZntR-HJ24| CCGTAHSSVYCS              | Hg>Cd>Pb               | Cd>Hg>Pb               |
| 24  | ZntR-HJ25| CCGTAHSSVYCS              | Hg>Cd>Pb               | Cd>Hg>Pb               |

1The metal(loid) ions inducing over 2.0 of induction coefficient values were included, and the WCBs showing less than 2 of induction coefficient values were excluded for further tests.

2*E. coli-zntR* and *E. coli-zntR/copA* represented the genetically engineered *E. coli* strains with Δ*zntR::kan* and Δ*zntR/copA::kan*, respectively.

3The bold and underlined letters indicated the point mutations on MBLs.
Whole-Cell Bioreporter Assay for Metal(loid) Selectivity

WCBs were generated by introducing plasmid pairs carrying zntR mutants and zntAp::egfp into E. coli DH5α-zntR, -zntR/copA, and -zntR/copA. To evaluate metal(loid) selectivity, WCBs were grown in LB broth overnight and then the cultures were transferred to fresh LB and grown at 37°C until the optical density at 600 nm (OD$_{600}$) reached around 0.4. Then, 5-ml aliquots were added to test tubes and a metal(loid) solution (5 μM) prepared as a 1 mM stock solution of AsCl$_3$, SbCl$_5$, CdCl$_2$, CrSO$_4$, NiCl$_2$, HgCl$_2$, PbSO$_4$, ZnCl$_2$, CuSO$_4$, or AuCl$_3$ was added for exposure. After 1.5 h of incubation, E. coli cells were harvested, and the cell pellet was resuspended in Tris buffer (50 mM Tris-Cl, 160 mM KCl, pH 7.4) before measuring the intensity of eGFP. All experiments were replicated at least 3 times.

Fluorescence Measurements

The fluorescence of eGFP induced by metal(loid) treatment was measured using an FS-2 fluorescence spectrophotometer (Scinco, Korea). Since LB broth interferes with data collection, it was replaced with 50 mM Tris-HCl (pH 7.4) by centrifugation and resuspension. Excitation/emission wavelengths of 470/510 nm were used. The fluorescent intensity induced by metal(loid) treatment was converted to an induction coefficient defined as the ratio of the eGFP intensity from exposure to metal(loid) ions to eGFP intensity without exposure. All experiments were replicated at least 3 times.

Data Analysis

The Dunnett Program (Ver 1.5) was used for statistical analysis of all data when the minimum difference between the control and the treatment means was statistically significant. The analysis was performed with a 95% significance level ($p < 0.05$).

Results

Rational Design of Metal-Binding Loops

The zinc-binding site of ZntR possesses 3 cysteines on metal-binding loops and 1 cysteine on another location, and they are known to play a pivotal role for interacting with cadmium, mercury, and zinc ions [7, 8, 12, 13]. Since cysteines in the MBL have this role in interactions with metal ions, cysteine residues were targeted for mutation to modulate the metal-binding properties of ZntR. Among 4 cysteines involved in metal ion binding in ZntR, 3 cysteines in the MBL were changed and Cys79 (outside of the MBL region) was maintained. Diverse combinations of amino acid sequences for MBLs of ZntR derivatives were generated and are summarized in Table 1.

To change the metal ion selectivity and specificity, the MBL of ZntR, i.e., amino acid residues 114 to 125 (CCGTAHSSVYCS), was targeted for mutagenesis. From ZntR-WT, 23 ZntR mutants were generated by 2-step overlap PCR and site-directed mutagenesis. First, ZntR-HJ1 was obtained by replacing His119 with Arg because it plays a critical role in PbrR691 to interact with Pb [14]. Then, HJ3 and HJ4 were obtained by additional mutations from Ser124 to Val to interfere with the activity of the hydroxyl group of Ser. Cys115, the second cysteine in the MBL, was altered to Asp, Ser, and Glu to generate ZntR HJ6, HJ7, and HJ8 from HJ4, respectively. The replacement of cysteines with negatively charged residues and hydroxyl groups was expected to change the metal-binding properties. Additionally, Cys114 was changed to Asp, Gly, Ser, and Pro from ZntR WT to generate HJ9–HJ12. Next, ZntR HJ13 was obtained by Ser125Gly mutation and then HJ14 to HJ16 were generated by replacing C115 with Asp, Ser, and Glu on HJ13. HJ17 to HJ20 were obtained by the His119Arg mutation on HJ9 to HJ12. Five additional mutants were generated based on experimental data for previous mutants. To increase the metal-binding affinity, negatively charged residues, such as Glu and Asp, were inserted into MBL regions to generate ZntR HJ21–25.

Modulation of Metal-Sensing Properties by Genetic Engineering

Effects of recombinant ZntR. After generating E. coli with a zntR deletion, the restoration of ZntR function by inserting endogenous recombinant zntR was evaluated to test novel WCBs with alterations in the metal-binding properties of ZntR. The restoration of function has also been verified in our previous study [7]. However, it was necessary to verify the expression of ZntR under the Ptac promoter without IPTG treatment. Three WCB strains generated by inserting pZnt-eGFP into WT E. coli DH5α and inserting pZnt-eGFP and pZnt-eGFP/pCDF-ZntR WT into E. coli DH5α-zntR (zntR:kan) were exposed to various metal(loid) ions (Fig. 1). As shown in Fig. 1, WCBs based on WT E. coli and the mutant strain with recombinant ZntR showed similar responses toward metal(loid) ions, while WCB without zntR showed no fluorescent signals. Although the intensity was weaker for WCBs than for WT E. coli DH5α, the metal selectivity of WCBs was not changed, supporting the restoration of the metal-sensing properties of WCBs by recombinant ZntR.
Effects of charged amino acids. The responses of the first set of ZntR mutants with His119 and Ser125 mutations, denoted HJ1, HJ3, and HJ4, to various metal(loid) ions were evaluated (Fig. 2). The selectivity of HJ1 was similar and specificity was higher than those of ZntR-WT, as shown in Fig. 1. The responses toward metal(loid) ions were abolished by the Ser125Val mutation (ZntR-HJ3) and were restored by His119Arg/Ser125Val (ZntR-HJ4) (Figs. 2B and 2C). However, overall responses toward Cd and Hg decreased. These results clearly showed that the responses toward Pb were related to ZntR-HJ1 and HJ4 and responses toward Cd and Hg were decreased in HJ4.

Effects of cysteines. Although both ZntR-HJ1 and HJ4 showed responses to Pb, ZntR-HJ4 was selected as a template for further engineering to target Pb because of the decreased responses to Cd and Hg. Cys115 of ZntR-HJ4 was replaced with Asp, Ser, and Glu to generate another set of ZntR mutants, HJ6, 7, and 8, respectively. As shown in Figs. 2D–2F, the responses toward various metal(loid) ions were similar to those of HJ4. However, the responses to Pb increased slightly by replacing Cys115 with Asp, Ser, and Glu. In the case of ZntR-HJ8 (Cys115Glu/His119Arg/Ser125Val), the induction coefficient for Pb was greater than 2.0 (Fig. 2F). These results suggested that the mutation of Cys115 weakened the interactions of ZntR with Cd and Hg, the original ligands, and negatively charged residues promote the interaction of ZntR with other metal(loid) ions, such as Pb. Another key residue for metal ion sensing is Cys114, which is the first cysteine located on MBL. When it was replaced with Asp, Gly, Ser, and Pro from ZntR-WT to generate ZntR-HJ9 to 12, respectively, the metal(loid) specificities of WCBs were abolished, except for Cd and Hg (Fig. S1). However, the level of eGFP expression was low, with induction coefficients of < 2.0 for Cd and Hg. Accordingly, Cys114 was a pivotal residue in ZntR for interactions with metal(loid) ions.

Since ZntR-HJ4, 6, 7, and 8 with Cys115, His119, and Ser125 maintained metal-sensing properties with slight differences from those of ZntR-WT, we used these to generate additional mutants. We predicted that the flexibility of MBLs affects the metal-sensing properties of ZntR. In this regard, Gly was replaced by Val125 in ZntR-HJ4, 6, 7, to 8 to generate ZntR-HJ13–16 (Fig. 3). ZntR-HJ13 (His119Arg/Ser125Gly) showed similar responses to HJ4 (His119Arg/Ser125Val) and the others also showed similar metal(loid) selectivity to HJ4, 6, 7, and 8. Additionally,

**Fig. 1.** The metal(loid) specificity of WCBs were restored by recombinant ZntR. The induction coefficient values of the WCBs based on *E. coli* WT with pZntA-eGFP (white bar), *E. coli-zntR* with pZnt-eGFP (grey), and *E. coli-zntR* with pZnt-eGFP/pCDF-ZntR-WT (dark grey) were measured after 1.5 h of exposure to 5 μM of metal(loid) ions. The asterisk (*) means the data were significantly higher than the control (p < 0.05).

**Fig. 2.** The metal(loid) selectivity of WCBs based on *E. coli-zntR* with ZntR possessing mutations on Cys115, H119 and Ser124. (A) ZntR-HJ1 (His119Arg), (B) ZntR-HJ3 (Ser125Val), (C) ZntR-HJ4 (His119Arg/Ser125Val), (D) ZntR-HJ6 (Cys115Asp/His119Arg/Ser125Val), (E) ZntR-HJ7 (His119Ser/His119Arg/Ser125Val), (F) ZntR-HJ8 (Cys115Glu/His119Arg/Ser125Val). The asterisk (*) means the data were significantly higher than the control (p < 0.05).
the responses to Pb were observed while responses to Cd and Hg were decreased (Figs. 3B-3D). The selectivity toward Pb was enhanced by Val125Gly from ZntR-HJ6, HJ7, and HJ8.

The His119Arg mutation had a positive effect on ZntR interactions with Pb, irrespective of changes in the induction coefficient. To verify the effect of His119Arg on Cys114 mutants (ZntR-HJ9–12), ZntR-HJ17–20 were generated by replacing His119 with Arg. Interestingly, ZntR-HJ17 (Cys114Asp/His119Arg) and HJ20 (Cys114Pro/His119Arg) showed restored responses toward Cd and Hg (Fig. S2), while the others showed no response to any metalloid ions (data not shown). Compared to the metalloid responses of WCBs with ZntR-HJ9 and HJ12 (Figs. S1A and S1D), WCBs with ZntR-HJ17 and HJ20 harboring an additional His119Arg mutation showed responses toward Cd and Hg. Consequently, the metalloid specificity abolished by the Cys114 mutation was restored by an additional His119Arg mutation.

**Combined effects of charged amino acids and cysteines.** Our results indicated that His119Arg and the replacement of Cys115 with charged residues on ZntR altered the metal-sensing properties of WCBs. Accordingly, a set of ZntR loci possessing mutations at Cys115, G116, His119, Val122, and Ser125 were generated and are denoted ZntR-HJ21–HJ25 (Table 1). All 5 WCBs showed responses to Cd and Hg with various induction coefficients (Fig. 4). The WCB with ZntR-HJ21 (Gly116Asp/His119Arg) showed weaker responses to Cd and Hg.

![Fig. 3. Alteration of metalloid selectivity of WCBs upon Cys115 mutations on ZntR-His119Arg/Ser125Gly.](image)

![Fig. 4. The effects of cysteine mutation with the insertion of charged residues on MBLs on metalloid selectivity of WCBs.](image)
than those of ZntR-HJ1 (His119Arg) (Fig. 4A). Gly116Asp weakened the specificity of ZntR to Cd and Hg. The WCBs with ZntR-HJ22 (His119Arg/Val122Asp) and HJ23 (Gly116Asp/His119Arg/Val122Asp) showed similar selectivity toward metal(loid) ions to that of ZntR-HJ1 (His119Arg) (Figs. 4B and 4C). Based on these results, the addition of negatively charged residues, such as Asp, influenced the metal(loid) interactions. Two additional mutants, denoted ZntR-HJ24 and HJ25, were generated from ZntR-HJ14 and HJ16 by adding a negatively charged residue, Asp, to Ser120. Relative selectivity to Pb was enhanced because the overall responses to Cd and Hg were decreased (Figs. 4D and 4E).

Effects of Interfering Metal Homeostasis Systems

E. coli DH5α-zntR (copA::kan) was used as a host strain to test the effects of disrupting metal homeostasis on WCBs. Since copA encodes CopA, which is involved in the export of endogenous metal ions including copper, it plays a role in maintaining metal homeostasis in E. coli [16, 17]. Thus, the absence of CopA is expected to induce the accumulation of certain metal ions in cells, thereby enhancing the responses to metal ions. Based on the results obtained above, ZntR WT and derivatives, including HJ1, HJ6, HJ7, HJ8, HJ22, HJ24, and HJ25, were introduced into E. coli DH5α-zntR/copA with pZnt-eGFP to obtain WCBs. The metal(loid) selectivity of WCBs was tested in the same experimental conditions used in previous analyses. As shown in Fig. 5, the overall responses of WCBs were greater than those of the WCBs generated from E. coli DH5α-zntR, as expected. In the case of HJ1 and HJ22, the responses to Cd increased by 1.3- and 1.4-fold and the responses to Hg increased 3.0- and 1.8-fold. Additionally, the responses to Pb increased, with induction coefficients of 4.2 and 4.8, respectively (Figs. 5A and 5E). Others also showed greater signal intensities and some showed enhanced selectivity to Pb (Fig. 5). Although WCBs with HJ1 and HJ22 showed the best responses toward Pb based on induction coefficients, they were insufficient for Pb sensing due to the strong responses to Cd and Hg. The responses to Pb were relative to Hg or Cd for HJ7 and HJ24 were 0.73 and 0.86, respectively, while those for HJ1 and HJ22 were 0.25 and 0.35, respectively (Figs. 5C and 5F). Notably, a response toward Cr was newly observed with induction coefficients >2.0, although the signals were not significant (Fig. 5). However, the relative response to Cr was similar to those to other metal(loid)s, such as Cd, Hg, and Pb, in case of ZntR-HJ24 (Fig. 5F).

Discussion

WCBs are promising alternatives for the quantification of environmental pollutants owing to their simplicity, rapidity, and low cost [10, 18, 19]. WCBs share a sensing domain and reporter domains that recognize and indicate the presence of target contaminants, respectively. Sensing domains repress reporter gene expression by binding to the promoter region without targets. Interaction with targets induces a conformational change to recruit RNA polymerase to the promoter region to initiate the transcription of the reporter gene [20, 21]. Thus, targets are easily quantified by measuring the expression level of reporter genes. Owing to this simple process, WCBs have
been actively investigated in the past few decades. However, the number of genetic systems that could be used for WCBs were relatively limited to cover the environmental toxicants and pollutants, even though a large amount of genetic data on organisms has been accumulated these days. In this regard, it is important to modulate the sensitivity and selectivity of available WCBs by genetic engineering as well as to find out new genetic systems. In this study, we demonstrated the generation of WCBs with new targets by modulating the binding properties of sensing domains regarded as regulatory proteins in stress-inducible operons along with interfering with the function of genes involved in homeostasis systems in bacterial cells.

ZntR, a metalloregulator in the zinc-responsive operon in *E. coli*, senses Cd, Hg, and Zn and is used for bacterial cell-based biosensors to monitor those metal(loid) ions [22, 23]. Thus, WCBs employing ZntR could not be used to monitor specific target metal(loid) ions owing to the broad specificity of this regulator protein [24]. In fact, the attempts to modulate the properties of WCBs by introducing mutation on ZntR had been reported [15]. Even though studies were performed under different experimental conditions and systems, they showed the mutations on ZntR were able to change the properties. However, the metal selectivity of WCBs was presumed to Cd and Zn while the level of specificity was modulated. In concordance with this study, the new metal(loid)-sensing WCBs were not obtained by mutating the MBL region of ZntR in our previous studies, while single metal(loid) ion-specific WCBs (i.e., Cd-specific and Hg-specific WCBs) were obtained [5, 7]. Nonetheless, we thought the novel targets of WCBs could be obtained if the regulatory proteins possess the ability to interact with novel targets. To validate the idea, the mutations on the metal-binding site of ZntR were selected from the amino acid sequence alignment analysis with other metalloregulatory proteins.

PbrR691 is a Pb-binding metalloregulatory protein [14, 25, 26]. It belongs to the Mer family of proteins and interacts specifically with Pb ions. A pyramid is formed from the Pb ion and 3 cysteines in PbrR691 stabilize the interaction with Pb ions by forming a binding pocket and van der Waals interactions. We replaced His119 and Ser125 in MBL of ZntR with Arg and Gly/Val, respectively, and introduced additional mutations on Cys78, Cys114 and Cys124 known as key residues; they form a structural pocket for interactions with metal ions [13, 15]. In the case of ZntR-HJ1 (His119Arg), WCBs showed about 2-fold increased signals, when compared with that for the WT, and showed a slightly increased response to Pb (Fig. 2A). In the case of ZntR-HJ4, the responses toward Cd and Hg were decreased by the Ser125Val mutation. It was difficult to explain why the metal-binding properties were changed by these mutations, but it is possible that the structural coordination of MBLs and metal(loid) ions was affected by point mutations.

When Cys115 was replaced with Asp, Ser, and Glu, responses toward Pb increased. In particular, responses toward Pb were 2.2 and 1.5 times greater than those of ZntR-WT with Cys115Glu including ZntR-HJ8 and HJ16 (Figs. 3C and 4D). Interestingly, although mutations at Cys114 abolished metal(loid) responses (Fig. S1), the responses were restored by Cys114Asp and Cys114Pro with His119Arg (Fig. 5). Additionally, negatively charged residues, such as Asp and Glu, were introduced on MBL regions with His119Arg. The resulting ZntR-HJ21–HJ25 still showed Cd and Hg specificity but replacing Cys115 with other residues weakened the responses toward Cd and Hg, thereby increasing the specificity toward Pb (Figs. 4D and 4E).

ZntR mutants with Pb responses, such as HJ1, HJ6, HJ7, HJ8, HJ22, HJ24, and HJ25, were also introduced into *E. coli-zntR/copA* (*ΔzntR/copA::kan*) strains to evaluate the effects of *copA*, known to export copper ions out of cells [16, 27]. *copA* is classified into the P-type ATPase family, and P1B-type ATPases are known to transport Cu(I), Cu(II), Zn(II), Cd(II), Co(II), and Pb(II) across cell membranes [28]. Thus, we expected the responses toward metal(loid) ions to increase by deleting *copA*. The responses of WCBs to Pb were around 2 to 5 times greater; interestingly, increased responses toward Cr were observed (Figs. 5 and S2). Although speculative, our results suggest that *CopA* in *E. coli* is related to regulating the concentrations of Cr and Pb as well as Cu. Additionally, some of ZntR mutants showed new responses to Cr and Pb. Although they did not specifically respond to single metal ions, it would be possible to detect Pb or Cr in environmental systems. To validate the ability of the newly obtained WCBs to quantitatively detect Pb and Cr, we evaluated WCBs possessing ZntR-HJ7 and HJ24, respectively (Fig. S3). The responses of WCBs increased in concentration-dependent manners. Although we still observed responses toward Cd and Hg, these WCBs could be used for Cr and Pb sensing. Of course, it is true that using other genetic systems to detect Cr and Pb and the HJ7 and HJ24 were not good enough to sense those ions. Nonetheless, it would be valuable to generate Cr- and Pb-sensing WCBs from znt-operon system. In conclusion, we believed it would be important to show the possibility of generating new metal(loid)-sensing WCBs from existing WCB systems, while also demonstrating that this strategy has the potential to substantially expand the number of targets and corresponding WCBs.

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**Conflicts of Interest**

The authors have no financial conflicts of interest to declare.

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