The Connection between Czc and Cad Systems Involved in Cadmium Resistance in Pseudomonas putida

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Abstract: Heavy metal pollution is widespread and persistent, and causes serious harm to the environment. Pseudomonas putida, a representative environmental microorganism, has strong resistance to heavy metals due to its multiple efflux systems. Although the functions of many efflux systems have been well-studied, the relationship between them remains unclear. Here, the relationship between the Czc and Cad systems that are predominantly responsible for cadmium efflux in P. putida KT2440 is identified. The results demonstrated that CzcR3, the response regulator of two-component system CzcRS3 in the Czc system, activates the expression of efflux pump genes czcCBA1 and czcCBA2 by directly binding to their promoters, thereby helping the strain resist cadmium stress. CzcR3 can also bind to its own promoter, but it has only a weak regulatory effect. The high-level expression of czcRS3 needs to be induced by Cd\(^{2+}\), and this relies on the regulation of CadR, a key regulator in the Cad system, which showed affinity to czcRS3 promoter. Our study indicates that the Cad system is involved in the regulation of the Czc system, and this relationship is important for maintaining the considerable resistance to cadmium in P. putida.

Keywords: P. putida KT2440; CzcRS two-component system; CadR regulator; gene regulation; cadmium resistance

1. Introduction

Anthropogenic and geological activities release heavy metals into the environment, making heavy metal pollution a significant threat to human and ecosystem health [1,2]. With the rapid development of industries such as mining [3], plating [4], nano-metallic materials [5], and electronics [6], this problem is increasing. Common hazardous heavy metal pollutants include As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, etc. Some of these metal ions (e.g., Co\(^{2+}\), Cu\(^{2+}\), and Mn\(^{2+}\)) are essential for biological processes at trace concentration [7]. To obtain these important metal ions, bacteria encode a variety of uptake transporters. These transporters are usually low-specific and driven by the chemiosmotic gradient across the cytoplasmic membrane [7]. For instance, the chemiosmotic transporter CorA mediates the influx of Mg\(^{2+}\), Co\(^{2+}\), and Ni\(^{2+}\) [8], and the Mn\(^{2+}\) and Zn\(^{2+}\) transporters allow Cd\(^{2+}\) to enter the cell [9,10]. This mode of transportation easily causes bacteria to uptake excessive metal ions and some toxic heavy metals in the environment contaminated by heavy metals. Excessive heavy metals in organisms can disturb metabolic functions through the denaturing of proteins, generating reactive oxygen species, and disrupting the intracellular ion balance [11–13].

It has been reported that the soil in some regions contains multiple heavy metals, such as Cd (0.1–3.6 mg/kg), Cr (51–207 mg/kg), Cu (14–109 mg/kg), and Pb (9.6–100 mg/kg) [14,15]. Bacteria in the environment are often exposed to heavy metals, thus they have evolved resistance systems to protect themselves from these hazardous substances. The sequestration of heavy metal ions by extracellular polymeric substances and metallothionein confers bacteria a certain degree of heavy metal tolerance [16,17]. Some bacteria can also transform heavy
Metal ions into non-toxic forms, for instance, Cd$^{2+}$ can be immobilized by biosynthesizing CdS quantum dots in *Pseudomonas frogi* and Cd-containing nanoparticle inclusions in *Cupriavidus* species [18,19]. Alternatively, expulsion through efflux systems is a more common and effective way to protect bacteria from heavy metals. The transportation of different heavy metals is generally driven by one or several specific efflux pumps; therefore, bacteria usually encode many different types of efflux pump systems in the genome and plasmid [20,21]. The diversity of heavy metal resistant genes in a bacterial strain, which is partly promoted by horizontal gene transfer, results in multiple heavy metal resistant phenotypes [22].

The cadmium efflux system, which mainly consists of the Czc system and Cad system, is a well-characterized mechanism of heavy metal resistance in bacteria [23]. The Czc system is composed of CzcCBA, CzcRS, and CzcD. CzcCBA, a transporter across the inner and outer membranes, is assembled from the outer membrane protein CzcC, the membrane fusion protein CzcB, and the inner membrane protein CzcA [24]. It functions as a cation-proton antiporter to transport excess Cd$^{2+}$, Zn$^{2+}$, and Co$^{2+}$ in the cytoplasm and periplasm to outside the cell [25]. The expression of *czcCBA* operon is regulated by the two-component system CzcRS. The regulatory effect of CzcRS can be activated by Cd$^{2+}$, Zn$^{2+}$, and Co$^{2+}$ [26]. Structural and functional analysis showed that when the periplasmic sensor domain of the histidine kinase CzcS binds to Zn$^{2+}$, the intracellular kinase domain of CzcS will autophosphorylate and then transmits the phosphate group to the intracellular response regulator CzcR [27]. Subsequently, the phosphorylated CzcR promotes the transcription of *czcCBA*. CzcD is a cation diffusion facilitator (CDF) protein family transporter located in the cytoplasmic membrane. It can also reduce Cd$^{2+}$, Zn$^{2+}$, and Co$^{2+}$ accumulation in the cytoplasm, but only provides a small degree of resistance to metal ions compared to CzcCBA [28,29]. The Cad system contains the P-type ATPase superfamily efflux pump CadA, the ArsR/SmtB family regulator CadC, and the MerR family transcriptional regulator CadR. CadR has a high affinity to Cd$^{2+}$ and a relatively weak affinity to Zn$^{2+}$ and Pb$^{2+}$ [30]. When the CadR dimer binds Cd$^{2+}$, it can distort the targeted promoter, leading to transcription activation of the targeted genes [30]. CadA, which is expressed under the control of CadR, is an effective transporter for expelling Cd$^{2+}$ in the cytoplasm to the periplasm by utilizing the energy from ATP decomposition [30,31]. CadC, which is encoded immediately downstream of *cadA* in *Staphylococcus aureus*, acts as a transcriptional repressor of *cadA-cadC* operon [32]. In some bacteria, such as *Ralstonia metallidurans* and *Pseudomonas putida*, the Czc and Cad systems are both important to confer full resistance to several heavy metals: the elimination of either system would significantly reduce resistance to Cd$^{2+}$ and Zn$^{2+}$ [33,34].

*P. putida* is a class of beneficial bacteria that exists widely in terrestrial and aquatic environments [35]. It can efficiently degrade various organic pollutants and promote plant growth [36,37]. Due to the development of genetic tools designed for genome editing, and the deep understanding of metabolic pathways, *P. putida* strains have been engineered as bacterial platforms for the biosynthesis of industrially relevant compounds such as cis,cis-muconic acid [38,39]. Some *P. putida* strains isolated from areas contaminated with heavy metal contaminants, such as *P. putida* CD2 and *P. putida* X4, show strong resistance to some hazardous heavy metals, especially cadmium [40,41]. These resistances are inseparable from the contribution of the Czc and Cad systems encoded in their genomes. However, so far, few studies have focused on the connection that evolved under heavy metal stress between these two types of efflux systems. In this study, the representative environmental model strain *P. putida* KT2440 [42] was used to explore the relationship between the Czc and Cad systems. The results provide insights into the regulatory connection between CadR, CzcRS3, and CzcCBA at a molecular level, and their roles in cadmium resistance of *P. putida*. 
2. Results and Discussion

2.1. Involvement of Two-Component System CzcRS3 in Heavy Metal Resistance

Among the three czcR genes predicted in the *P. putida* KT2440 genome (*Pseudomonas* Genome Database version 20.2, https://www.pseudomonas.com, accessed on 3 September 2021), czcR1 (PP_0029) and czcR3 (PP_1438) are immediately followed by a cognate histidine kinase encoding gene (czcS), while czcR2 (PP_0047) is orphaned [43]. In *P. putida* X4, the homologous operon of *czcRS1* could maintain a considerable expression level without metal ion inducers and is even repressed by Zn$^{2+}$ and Cd$^{2+}$, whereas the homologous operon of *czcRS3* needs to be induced by Cd$^{2+}$ for expression [41]. This implies that *czcRS3* of *P. putida* KT2440 may be more related to cadmium resistance. Therefore, the role of this two-component system in heavy metal resistance, and the regulation of its expression, was the focus of this study. Firstly, the *czcRS3* was inactivated by homologous recombination, and the modifications in heavy metal resistance were tested. The MIC of Cd$^{2+}$ in LB medium was reduced by four-fold in the Δ*czcRS3* deletion mutant (Δ*czcRS3*), while no change was observed in the MICs of other heavy metals (Co$^{2+}$, Cr$^{3+}$, Cu$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Pb$^{2+}$, and Zn$^{2+}$), compared to that in the wild-type (Table 1). When the cloned *czcRS3* was expressed under the control of the inducible *tac* promoter in Δ*czcRS3* (Δ*czcRS3*(czcRS3oe)), cadmium resistance was significantly increased, to a level that exceeded the wild-type level (Figure 1). These results indicate that CzcRS3 plays an important role in cadmium resistance of *P. putida* KT2440.

Table 1. Minimum inhibitory concentration (MIC) of heavy metals for wild-type *P. putida* KT2440 and Δ*czcRS3* in LB medium.

| Metal Ion (mM) | Cd$^{2+}$ | Co$^{2+}$ | Cr$^{3+}$ | Cu$^{2+}$ | Mn$^{2+}$ | Ni$^{2+}$ | Pb$^{2+}$ | Zn$^{2+}$ |
|---------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| wild-type     | 1         | 1         | 4         | 8         | 16        | 8         | 8         | 8         |
| Δ*czcRS3*     | 0.25      | 1         | 4         | 8         | 16        | 8         | 8         | 8         |

Figure 1. The role of *czcRS3* in *Pseudomonas putida* cadmium resistance. The wild-type *P. putida* KT2440 (WT, black), deletion Δ*czcRS3* (orange), and overexpressed Δ*czcRS3*(czcRS3oe) (blue, harboring pB403-czcRS3) cells were incubated in LB medium supplemented with CdCl$_2$ at concentrations ranging from 0 to 1 mM. The absorbance at 600 nm ($A_{600}$) was measured to evaluate the bacterial growth under cadmium stress. The data represent the mean ± standard deviation of three replicates.

2.2. CzcRS3 Controls the Expression of Two CzcCBA Efflux Pumps

The genome of KT2440 encodes several efflux pumps (czcD, cadA1, cadA2, cadA3, czcBA1, czcBA2, and czcC-cusBA) that have a clear or putative relationship with cadmium
resistance [34,43,44]. To elucidate the decreased cadmium resistance of ΔczcRS3, the expression of these genes in ΔczcRS3 was detected by promoter-fused lacZ (β-galactosidase encoding gene) reporter plasmids. As shown in Figure 2A, the expression of cadA1 and czcC-cusBA was not detected in either wild-type or ΔczcRS3, regardless of exposure to Cd\(^{2+}\). Their expression might need to be induced by other factors. This was supported by the fact that cadA1 maintained a very low or undetectable expression level without a divalent metal inducer, while Zn\(^{2+}\), but not Cd\(^{2+}\), was able to induce its expression [34]. In contrast, czcD, cadA2, and cadA3 maintained significant expression levels in both the wild-type and ΔczcRS3, with or without Cd\(^{2+}\) (Figure 2A). There was no visible difference in the expression of these five genes between the wild-type and ΔczcRS3, suggesting that CzcRS3 did not regulate the expression of these efflux pumps.

![Figure 2. Role of CzcRS3 in the expression of efflux pump encoding genes.](image)

The activation of czcCBA1 and czcCBA2 promoters in the wild-type could not be detected under the cadmium-free condition, but increased significantly upon exposure to Cd\(^{2+}\) (Figure 2A). However, when czcRS3 was knocked out, these two operons were no longer induced by Cd\(^{2+}\), indicating that expression of czcCBA1 and czcCBA2 relied on CzcRS3 and Cd\(^{2+}\) (Figure 2A). To further verify this, czcRS3 was supplemented to the mutant by a plasmid carrying the cloned czcRS3 driven by the inducible tac promoter, which could avoid the interference from the regulators involved in czcRS3 expression. As expected, the expression of czcCBA1 and czcCBA2 in ΔczcRS3 was triggered by the expression of czcRS3 (Figure 2B). Interestingly, the cells of ΔczcRS3 with overexpressed czcRS3 (ΔczcRS3(czcRS3oe)) on the cadmium-free side could also activate the expression of these two efflux pumps (Figure 2B). These results imply that CzcRS3 is the key regulator.
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2.3. CzcR3 Directly Binds to the Promoters of czcCBA1 and czcCBA2

Response regulators function by binding to the targeted promoters and activating or inhibiting transcription events, after being phosphorylated by the cognate histidine kinase [45]. Although CzcR has a significant regulatory role in gene expression, its binding site on promoter has rarely been identified. Thus, the binding of CzcR3 to the promoter of czcCBA1 and czcCBA2 was explored. Electrophoresis analysis showed that purified CzcR3 was able to form a homodimer in vitro (Figure S1), which was expected since its cognate histidine kinase CzcS could also occur dimerization [27]. Since the response regulators displayed a much higher DNA-binding affinity after phosphorylation [26,46], carbamyl phosphate was used as a phosphate group donor, which could be used for in vitro phosphorylation of response regulators, such as ArcA [47] and NtrC [48]. The EMSA results showed that, in the presence of carbamyl phosphate, CzcR3 had an affinity for the promoters of czcC1 and czcC2 (Figure 3A,C). To further identify the binding site of CzcR3 on the promoters, a DNase I footprinting assay was performed. The results showed that, relative to the translation start site, CzcR3 bound to the regions from −120 to −144 bp for czcC1 and −45 to −69 bp for czcC2 (Figure 3B,D). There was a high degree of similarity between these two sequences, and they displayed a common inverted repeat sequence (ATTAC-N6-GTAAAT) internally, in which the 6 bp spacer was likely to be rich in A/T (Figure 3E). This feature was also found in the DNA-binding sequence of CzcR in Pseudomonas stutzeri [49].

![Figure 3](image-url)

**Figure 3.** The binding of CzcR3 to the promoters of two czcCBA operons. (A,C) The FAM-tagged promoter probes of czcC1 (A) and czcC2 (C) were subjected to EMSA with the purified CzcR3 and carbamyl phosphate (CP). The hysteretic bands indicate the complex of CzcR3 and the probes. (B,D) The binding sites of CzcR3 on promoters of czcC1 (B) and czcC2 (D) were identified by Dnase I footprinting assay, in which the probe was incubated with CzcR3 (blue peak) or BSA (red peak, as control). The region in the dashed box, which displays the blue peak weaker than the red control, represents the binding region of CzcR3 on the promoter. The arrows indicate the translation start sites of czcC1 (B) and czcC2 (D). (E) The binding sequences of CzcR3 on czcC1 and czcC2 promoters show a high degree of similarity.
2.4. Identification of the Transcription Pattern of czcRS3

To determine the role of CzcRS3 in cadmium resistance, the regulation of the czcRS3 operon was studied. The expression level of czcRS3 in the wild-type strain was evaluated by the reporter plasmid for czcR3. When heavy metals were absent, the czcRS3 promoter exhibited low transcription activity (Figure 4A). Several heavy metals, including Cd$^{2+}$, Co$^{2+}$, Cr$^{3+}$, Cu$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Pb$^{2+}$, and Zn$^{2+}$, were used as inducers, at a concentration of 1/5 MIC. Among these inducers, only Cd$^{2+}$ could strongly induce czcRS3 expression, reflecting the important relationship between CzcRS3 and cadmium resistance. Pb$^{2+}$ and Zn$^{2+}$ also displayed an inducing effect on czcRS3 expression, but much weaker than Cd$^{2+}$ (Figure 4A).

![Figure 4](image-url)

**Figure 4.** The regulation of czcRS3 expression. (A) Induction of heavy metals on czcRS3 expression. The expression level of czcRS3 in wild-type KT2440 was detected by reporter plasmid pBRTZ-czcR3 after treatment with heavy metals at a concentration of 1/5 MIC for 4 h. (B) Identification of the transcription start site of czcRS3 operon by 5'-RACE assay. The underlined −10 and −35 elements show similarity to the recognition sequence of σ$^{D}$ (* indicates the matched DNA bases). (C) The affinity of CzcR3 for czcRS3 promoter in presence of carbamyl phosphate (CP). (D) Identification of the CzcR3-binding site on czcRS3 promoter. The promoter probes were incubated with CzcR3 (blue peak) or BSA (red peak, as control) in DNase I footprinting assay. The region in the dashed box represents the binding region of CzcR3 on the promoter. (E) The regulatory role of CzcRS3 in self-expression. The data represent the mean ± standard deviation of three replicates. The significant difference was determined by Student’s t-test (** p < 0.01; ns, non-significant, p > 0.05).

Subsequently, the transcription start site of czcRS3 was identified by a 5'-RACE assay, which showed that it was located 39 bp upstream of the translation start site of czcR3 (Figure 4B). The −10 and −35 elements of this transcription start site displayed similarity to the recognition sequence of sigma factor σ$^{D}$ [50]. However, there were several mismatched nucleotides in the critical −10 element, suggesting that efficient transcription of czcRS3 might require the assistance of a positive regulator [51].

Analysis of the czcRS3 promoter indicated a potential CzcR3 binding site in the −116 to −101 bp region, relative to the first codon of czcR3, which showed a high similarity to the CzcR3-binding sites on the czcC1 and czcC2 promoters. EMSA verified the binding ability of CzcR3 to its own promoter (Figure 4C), while DNase I footprinting assay showed that CzcR3 protected the region between −98 and −133 bp, relative to the first codon of czcR3.
CdCl$_2$ treatment. It should be noticed that this 36 bp protected region was much longer than the CzcR3-protected region on czcC1 and czcC2 promoters, which were both approximately 25 bp (Figure 3B,D). Interestingly, this long protected region contained three putative CzcR3 recognition motifs (ATAAC, ATTAC, and GTTAT). Whether the binding complex contained two or three CzcR3 monomers could not be determined, since the structure of CzcR3 or its interaction with DNA has not yet been elucidated. To test the regulatory role of CzcRS3 on its encoding operon, the reporter plasmid pBRTZ-czcR3 was introduced into ∆czcRS3. Surprisingly, the activity of czcR3 promoter in ∆czcRS3 was not significantly different from that in the wild-type (Figure 4E). Under induction by Cd$^{2+}$, both the wild-type and ∆czcRS3 could effectively activate the czcR3 promoter (Figure 4E), suggesting that the unconventional binding of CzcR3 to its promoter had no obvious regulatory effect. This also implies that there are other regulators involved in czcRS3 expression.

2.5. Cd$^{2+}$ Induces czcRS3 Expression through CadR

CadR is a well-characterized transcriptional regulator that responds to Cd$^{2+}$, Zn$^{2+}$, and Pb$^{2+}$ in P. putida KT2440 [30,52]. Along with the fact that Cd$^{2+}$, Zn$^{2+}$, and Pb$^{2+}$ could induce czcRS3 expression (Figure 4A), it is possible that CadR participates in czcRS3 regulation. To confirm this hypothesis, the cadR deletion mutant (∆cadR) was constructed, and the expression of czcRS3 was determined in this mutant. As expected, the promoter of czcRS3, regardless of the presence of Cd$^{2+}$, could not be activated in ∆cadR (Figure 5A). When ∆cadR was complemented with cadR (∆cadR(cadRoe)), the activity of czcRS3 promoter was restored to the wild-type level, and increased significantly upon exposure of 0.1 mM Cd$^{2+}$ (Figure 5A). This indicates that CadR is the key regulator in response to Cd$^{2+}$ to activate the expression of czcRS3.

Figure 5. Involvement of CadR in czcRS3 expression. (A) The expression level of czcRS3 in wild-type KT2440 (WT), deletion ∆cadR, and overexpressed ∆cadR(cadRoe) which harbors pB403-cadR. (B) The expression level of czcRS3 in KT2440 strains with overexpressed czcRS3. The activity of β-galactosidase expressed from pBRTZ-czcR3 was measured after treatment with or without CdCl$_2$ for 4 h. (C) The growth of ∆cadR with overexpressed cadR (cadRoe) or czcRS3 (czcRS3oe) in LB medium supplemented with CdCl$_2$ at the indicated concentrations. The data represent the mean ± standard deviation of three replicates. The significant difference was determined by Student’s t-test (*** p < 0.01; ns, non-significant, p > 0.05).
To test whether the binding of CzcR3 on its own promoter had physiological significance in the context of knocking out cadR, the plasmid carrying the inducible czcRS3 was introduced into ΔcadR, as well as the wild-type. Overexpression of czcRS3 slightly activated the czcR3 promoter in ΔcadR, and this activation was not dependent on supplementation with Cd\[^{2+}\] (Figure 5B). In comparison, it could not cause a significant change in the expression level of czcRS3 in the wild-type (Figure 5B). The weak activation effect of the overexpressed CzcRS3 in czcRS3 expression is possibly masked by CadR in the wild-type. The effect of overexpressed CzcRS3 on cadmium resistance was also tested under the background of ΔcadR. The cadmium resistance was significantly reduced after cadR was knocked out (Figure 5C), because cadR knockout would block the activation of cadA, which encodes a major cadmium efflux pump [34,52]. Complementation with cadR increased the cadmium resistance of ΔcadR to the wild-type level, while overexpression of czcRS3 could also restore the cadmium resistance of ΔcadR to a certain extent (Figure 5C). This indicates that the regulatory effect of CadR on cadmium resistance partly depends on CzcRS3. In other words, CzcRS3 is a downstream regulator of CadR, and together they endow P. putida KT2440 with strong resistance to cadmium.

2.6. CadR Binds to czcRS3 Promoter Directly

To investigate whether CadR regulated czcRS3 expression directly, the His-tagged CadR was purified and its affinity for czcRS3 promoter was tested. The EMSA result showed that the purified CadR was able to bind to the DNA fragment containing the czcRS3 promoter (Figure 6A). This binding process did not require the participation of Cd\[^{2+}\], in agreement with previous work [30]. Furthermore, the DNase I footprinting assay identified the region protected by CadR from DNase I, which was located at −35 to −59 bp, relative to the first codon of czcR3 (Figure 6C). An obvious inverted repeat sequence (CTTGACCCTG-N9-CAGGGTCAAG) was found over this region. The sequence of CadR-binding region on czcRS3 promoter is highly similar to that on the cadA promoter [30], further supporting this result. To verify the CadR-binding region on czcRS3 promoter, several base mutations were introduced in the promoter fragment (TTGACAAAG-N9-CAAAGTC) for EMSA. As expected, the point-mutated fragment could not interact with CadR (Figure 6B). These results indicate that CadR regulates czcRS3 by directly binding its promoter.

Since CadR functions in a homodimer form, its binding site on DNA shows the characteristic of an inverted repeat. The binding of CadR to the promoter does not require metal ions; however, the CadR structure would change after binding to Cd\[^{2+}\], resulting in a distortion of the DNA and a reduced distance between the −10 and −35 elements of the promoter. This further leads the promoter to change from a repressed state to an activated state [30]. Although CadR can interact with Cd\[^{2+}\], Pb\[^{2+}\], and Zn\[^{2+}\], its binding affinity for Pb\[^{2+}\] and Zn\[^{2+}\] is much weaker than for Cd\[^{2+}\] [30]. Therefore, Cd\[^{2+}\] can strongly induce expression of the CadR-regulated genes, whereas Pb\[^{2+}\] and Zn\[^{2+}\] have weaker effects [52]. This also explains the differences in degrees of induction in czcRS3 expression by Cd\[^{2+}\], Pb\[^{2+}\], and Zn\[^{2+}\] (Figure 4A).
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3.2. Construction of Strains and Plasmids
Primers for mutant construction are listed in Table S2. To construct the knockout
promoter in Δ
3.1. Bacterial Strains and Growth Conditions
All bacterial strains and plasmids used in this study are listed in Table S1. Throughout
the study, the P. putida strains were grown in Lysogeny broth (LB) medium at 28 °C, and
the E. coli strains were grown in LB medium at 37 °C. When required, antibiotics (pur-
chased from Sangon Biotech, Shanghai, China) were used at the following concentrations: chloramphenicol (25 mg/L), gentamycin (20 mg/L), tetracycline (20 mg/L), kanamycin (50 mg/L) and ampicillin (150 mg/L). Analytical-grade salts of CdCl₂·2.5H₂O, CoCl₂·6H₂O, CrCl₃·6H₂O, CuCl₂·2H₂O, MnCl₂·4H₂O, NiCl₂·6H₂O, Pb(NO₃)₂, and ZnCl₂ (purchased from Macklin, Shanghai, China) were used to prepare 1 M stock solutions, which were sterilized by filtration.

3.2. Construction of Strains and Plasmids
Primers for mutant construction are listed in Table S2. To construct the knockout
plasmids, the upstream and downstream regions of czcRS3 and cadR, which were amplified
from KT2440 genome by polymerase chain reaction (PCR), were fused into Sacl-digested
pDS3.0 [53] using the ClonExpress II one step cloning kit (Vazyme, Nanjing, China), yielding
pDS-czcRS3 and pDS-cadR, respectively. The plasmid in E. coli S17-1 was transferred to wild-type P. putida KT2440 for allelic exchange, and sacB counter-selection was used to select the mutants as described in detail previously [53,54]. Finally, ΔczcRS3 and ΔcadR were obtained.

To construct the reporter plasmids, the 300–600 bp DNA fragments containing the
promoter region of the tested genes were amplified using the primers for promoter in
Table S2, and the fragments were ligated to Xbal- and PstI-digested pBRTZ, which contains
the promoter-less β-galactosidase encoding gene lacZ [55], yielding the reporter plasmids for czcD, cadA1, czcC1, cadA2, czcR3, czcC2, cadA3, and czcC (Table S1), respectively. To construct the overexpression plasmids, the DNA fragments containing czcRS3 or cadR encoding sequence were amplified using the primers for gene cloning in Table S2, and the fragments were ligated to EcoRI- and BamHI-digested pBBR1-403 [56] to yield pB403-

Figure 6: CadR directly binds to the czcRS3 promoter. The native (A) and mutated (B) probes of czcRS3 promoter were subjected to EMSA with the purified CadR. (C) The binding site of CadR on czcRS3 promoter was identified by DNase I footprinting assay, in which the native probe was incubated with CadR (blue peak) or BSA (red peak, as control). The region in the dashed box represents the binding region of CadR on czcRS3 promoter.
czcRS3 and pB403-cadR, respectively. When needed, isopropyl β-D-1-thiogalactopyranoside (IPTG) was used as the inducer of tac promoter at a concentration of 0.1 mM. The plasmids above were hosted in *E. coli* S17-1, and they were transferred to derivative strains of *P. putida* KT2440 by biparental mating. To construct the plasmids for protein purification, the encoding sequence of czcR3 and cadR were amplified using the primers for protein purification in Table S2, and the fragments were ligated to NcoI- and XhoI-digested pET28a to yield pET28a-czcR3 and pET28a-cadR, respectively. These plasmids were hosted in *E. coli* BL21(DE3).

### 3.3. Test for Susceptibility to Heavy Metals

The overnight cultures of *P. putida* strains were diluted with fresh LB medium to an OD$_{600}$ of 0.005, and the metal salts were also serially diluted by 2-fold in LB medium. The diluted culture (75 µL) was mixed with an equal volume of diluted metal salts or antibiotics in a 96-well plate. The mixture was incubated at 28 °C for 24 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic with no visible bacterial growth [57]. In the growth assays, the diluted cultures containing Cd$^{2+}$ were incubated in Spark 20M microplate reader (Tecan, Männedorf, Switzerland) and the absorbance at 600 nm (A$_{600}$) was measured at intervals of 1 h.

### 3.4. Measurement of β-Galactosidase Activity

An overnight culture of KT2440 strains harboring the reporter plasmid was inoculated in fresh LB medium (1:100). To reduce the effect of the hazardous heavy metals on growth, the cultures were pre-incubated for 8 h, and then treated with heavy metal ions for 4 h. The β-galactosidase activity was measured according to the procedures described previously [54,58]. Briefly, the reaction system contained 50 µL culture samples, 450 µL Z buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$ and 50 mM β-mercaptoethanol), 25 µL of 1 mg/mL sodium dodecyl sulfate (SDS), 50 µL chloroform, and 100 µL of 4 mg/mL 2-nitrophenyl-β-D-galactopyranoside (ONPG). The reaction proceeded at 28 °C and was terminated by 250 µL of 1 M Na$_2$CO$_3$. After centrifugation, Absorbance at 420 nm (A$_{420}$) of the supernatant was measured, and A$_{600}$ of the bacterial culture before lysis was also measured. The β-galactosidase activity was calculated as: Miller units = 1000 × (A$_{420}$/A$_{600}$/volume/time).

### 3.5. Identification of Transcription Start Site

Total RNA was isolated from wild-type KT2440 culture after treatment with 0.2 mM Cd$^{2+}$ for 4 h. The 5′-rapid amplification of cDNA ends (5′-RACE) was performed using the vaccinia capping enzyme (VCE, NewEnglandBiolabs, Ipswich, MA, USA) to add a 5′ end cap structure to the RNA sample [59]. The RNA sample was then treated by DNase I (Takara, Japan) to remove the residual genome DNA and purified by standard ethanol precipitation as described in the manual for DNase I. First-strand cDNA was synthesized using HiScript II reverse transcriptase (Vazyme, China) and primer czcRS3-RC (Table S2). Then, the template-switching oligonucleotide TSO-RNA (Table S2) was used as the template for reverse transcriptase to add the adapter sequence to the 3′ end of cDNA. The final cDNA was amplified using primers czcRS3-RC and TSO-DNA (Table S2), and the product was linked to pMD19-T (Takara, Japan). The transcription start site of czcRS3 operon was identified through sequencing.

### 3.6. Purification of His-Tagged CzcR3 and CadR

The *E. coli* BL21(DE3) strains harboring pET28a-czcR3 or pET28a-cadR were incubated in 500 mL LB medium with shaking at 220 rpm to an OD$_{600}$ of 0.5. The cultures were treated with 0.5 mM IPTG at 20 °C for 6 h. The cells were collected by centrifugation and resuspended in 20 mL of lysis buffer (10 mM Tris-HCl at pH 8.0, 50 mM NaCl, 10% v/v glycerol). After the cells were lysed by a high-pressure homogenizer, the His-tagged CzcR3 and CadR were purified using Ni-NTA spin columns (BBI Life Sciences, China), according
to the directions from the manufacturer. The His-tagged proteins were eluted with E250 buffer (10 mM Tris-HCl at pH 8.0, 250 mM NaCl, 10% v/v glycerol, 250 mM imidazole). The elution samples were dialyzed to remove imidazole.

3.7. Generation of Fluorescent Probes of Promoters

The primers for the generation of the FAM-tagged probes are listed in Table S2. Primers M13F-

3.8. Electrophoretic Mobility Shift Assay (EMSA)

In EMSA, 10 nM of FAM-tagged probe was mixed with increasing concentrations of the purified protein (CzcR3 and CadR) in a 20 µL binding buffer system (10 mM Tris-HCl at pH 8.0, 50 mM KCl, 5 mM MgCl₂, 5% v/v glycerol). In the binding system for CzcR3, the carbamyl phosphate was used as the donor of phosphate group. After 20 min of incubation at 28 °C, 15 µL of the sample was loaded onto a 5% (w/v) polyacrylamide gel and electrophoresed in TG buffer (12.5 mM Tris-HCl at pH 8.3, 96 mM glycine) at 100 V on ice for 90 min. The gels were photographed with ChemiDocXRS + (BioRad, Hercules, CA, USA).

3.9. DNase I Footprinting Assay

In DNase I footprinting assay, 40 nM of the FAM-tagged probe was mixed with about 2 µM of the purified protein or bovine serum albumin (BSA, negative control) in a 200 µL binding buffer system. After 20 min of incubation at 28 °C, the samples were treated with 0.3 U DNase I at room temperature for 5 min, and the reaction was quenched by treatment with 100 µL phenol-chloroform (1:1, v/v) and 80 °C heating. After centrifugation, the supernatant was mixed with 500 µL ethanol and 20 µL of 3 M sodium acetate (pH 5.2). The DNA was precipitated by centrifugation and dissolved in 20 µL ddH₂O. The DNA samples were analyzed by 3730XL DNA Sequencer and the data was processed by Peak Scanner Software v1.0 (Applied Biosystems, Waltham, MA, USA).

4. Conclusions

In this study, the role of a two-component system CzcRS3 in cadmium resistance of P. putida KT2440 was investigated and confirmed that CzcRS3 regulated the expression of two CzcCBA efflux pump operons by directly binding to their promoters. The regulation of czcRS3 expression was also explored and determined that CzcR3 bound to its own promoter in an atypical way, but this did not have a significant effect on transcription. Importantly, the expression of czcRS3 was directly regulated by CadR in response to Cd²⁺, Zn²⁺, and Pb²⁺, revealing the regulatory relationship between the Cad and Czc systems. Along with previous studies, these results support that these two systems essentially belong to a biological pathway (Figure 7). In this pathway, Cd²⁺ enters the cell and combines with CadR, and thereby CadR can promote the expression of the CadA3 efflux pump [30] and CzcRS3. CadA3 functions in the transport of Cd²⁺ from the cytoplasm to the periplasm [34], and CzcRS3 is responsible for activating the expression of two CzcCBA efflux pumps. CzcCBA can expel, not only part of the intracellular Cd²⁺, but also the Cd²⁺ in the periplasm that includes the Cd²⁺ exported from the cytoplasm by CadA, to the outside [33]. These two efflux systems, including their regulators, form effective cooperation to protect the P. putida from toxic cadmium in the environment.
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