AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

Genetics and Molecular Biology | Full-Length Text

Activation of CzcS/CzcR during zinc excess regulates copper tolerance and pyochelin biosynthesis of *Pseudomonas aeruginosa*

Ting Li,[1] Huiluo Cao,[2] Cheng Duan,[1] Shuzhen Chen,[1] Zeling Xu[1]

AUTHOR AFFILIATIONS See affiliation list on p. 13.

ABSTRACT Zinc is an important transition metal that is essential for numerous physiological processes while excessive zinc is cytotoxic. *Pseudomonas aeruginosa* is a ubiquitous opportunistic human pathogen equipped with an exquisite zinc homeostatic system, and the two-component system CzcS/CzcR plays a key role in zinc detoxification. Although an increasing number of studies have shown the versatility of CzcS/CzcR, its physiological functions are still not fully understood. In this study, transcriptome analysis was performed, which revealed that CzcS/CzcR is silenced in the absence of the zinc signal but modulates global gene expression when the pathogen encounters zinc excess. CzcR was demonstrated to positively regulate the copper tolerance gene *ptrA* and negatively regulate the pyochelin biosynthesis regulatory gene *pchR* through direct binding to their promoters. Remarkably, the upregulation of *ptrA* and downregulation of *pchR* were shown to rescue the impaired capacity of copper tolerance and prevent pyochelin overproduction, respectively, caused by zinc excess. This study not only advances our understanding of the regulatory spectrum of CzcS/CzcR but also provides new insights into stress adaptation mediated by two-component systems in bacteria to balance the cellular processes that are disturbed by their signals.

IMPORTANCE CzcS/CzcR is a two-component system that has been found to modulate zinc homeostasis, quorum sensing, and antibiotic resistance in *Pseudomonas aeruginosa*. To fully understand the physiological functions of CzcS/CzcR, we performed a comparative transcriptome analysis in this study and discovered that CzcS/CzcR controls global gene expression when it is activated during zinc excess. In particular, we demonstrated that CzcS/CzcR is critical for maintaining copper tolerance and iron homeostasis, which are disrupted during zinc excess, by inducing the expression of the copper tolerance gene *ptrA* and repressing the pyochelin biosynthesis genes through *pchR*. This study revealed the global regulatory functions of CzcS/CzcR and described a new and intricate adaptive mechanism in response to zinc excess in *P. aeruginosa*. The findings of this study have important implications for novel anti-infective interventions by incorporating metal-based drugs.

KEYWORDS *Pseudomonas aeruginosa*, two-component system, CzcS/CzcR, zinc excess, copper tolerance, pyochelin biosynthesis

Transition metal ions are present in almost all types of environments and they are essential for bacterial growth and pathogenesis owing that they play critical roles in numerous biological processes (1). However, variations in environmental concentrations of transition metals are ubiquitous, often causing stress to bacterial physiology. Metals are also toxic and even lethal to bacterial cells when they are depleted or in excess. Therefore, the administration of ion contents at the host-pathogen interface is

Editor Pablo Ivan Nikel, Danmarks Tekniske Universitet The Novo Nordisk Foundation Center for Biosustainability, Kgs. Lyngby, Denmark

Address correspondence to Zeling Xu, zelingxu@scau.edu.cn.

Ting Li and Huiluo Cao contributed equally to this article. Author's order was determined by who drafted the manuscript.

The authors declare no conflict of interest.

See the funding table on p. 13.

Received 21 December 2023
Accepted 24 January 2024
Published 20 February 2024

an important strategy employed by animal hosts to defend against invading pathogens. For instance, iron (Fe) sequestration and copper (Cu) intoxication are commonly found in host immune cells such as macrophages and neutrophils (2).

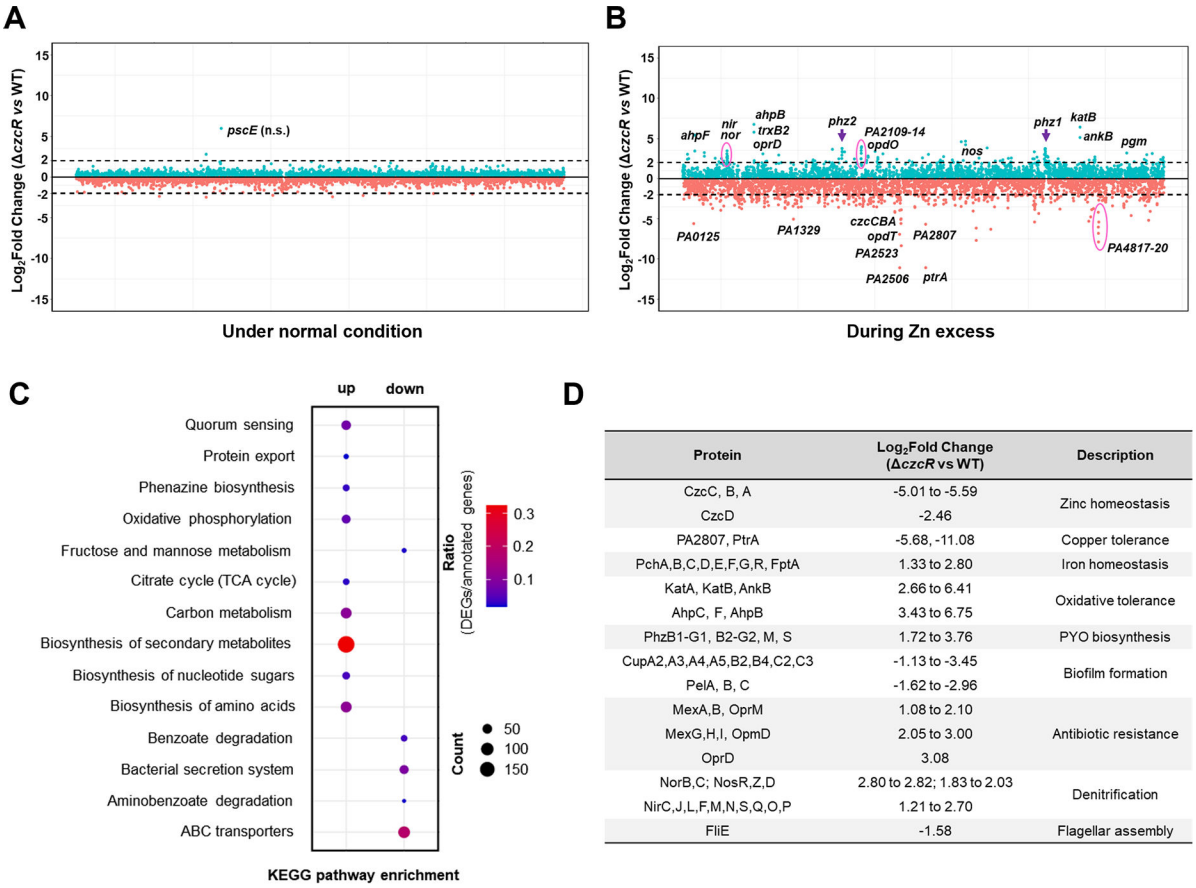
Zinc (Zn) is the second most abundant transition metal which is utilized by a large percentage of proteins in bacteria and is an important factor for enzymatic catalysis and gene regulation (3). It is also detrimental when present in excess. *Pseudomonas aeruginosa* is a Gram-negative bacterium that is prevalent in the environment. It is also an important opportunistic human pathogen and a leading cause of hospital-acquired infections (4). *P. aeruginosa* is known to be a metabolically versatile pathogen that is capable of adapting to and thriving under different environmental stresses with the help of its abundant signaling systems (5). To adapt to conditions with different Zn concentrations, the pathogen has developed several sophisticated signaling systems to stabilize intracellular Zn homeostasis by modulating the uptake, storage, and efflux of Zn ions (6). Among these, the two-component system (TCS) CzcS/CzcR is an essential one that employs CzcS to sense the extracellular signal Zn and subsequently activates CzcR to control the expression of Zn efflux proteins CzcCBA and CzcD, thereby alleviating intracellular Zn accumulation and toxicity (7–9).

In recent years, an increasing number of studies have revealed the role of CzcS/CzcR in the regulation of various virulence-related traits, such as biofilm formation, quorum sensing activity, swimming motility, as well as antibiotic resistance (10–13). These findings showed the versatility of CzcS/CzcR in addition to modulating Zn detoxification and implied that the physiological functions of the TCS may be greatly underestimated. Thus, a comprehensive investigation of the regulon of the Zn-responsive CzcS/CzcR and its physiological functions is desirable, which might broaden our understanding of the adaptive mechanism of *P. aeruginosa* and provide new implications for targeted anti-infective interventions. To this end, in this study, we performed a comparative transcriptome analysis between the PAO1 wild-type (WT) strain and the TCS mutant Δ*czcR* to explore the physiological roles of CzcS/CzcR in *P. aeruginosa*. In addition to genes related to Zn efflux and previously reported functions, it is surprisingly revealed that Zn activates CzcS/CzcR to control thousands of genes including those involved in other metal-associated cellular processes such as Cu tolerance and Fe uptake.

RESULTS

Zn activates CzcS/CzcR to modulate global gene expression

To comprehensively understand the regulatory roles of the Zn-responsive TCS CzcS/CzcR in *P. aeruginosa*, we first conducted a comparative transcriptome analysis between the PAO1 WT and Δ*czcR* strains after they were cultured in the LB medium. However, we did not observe any genes with significantly different expression levels between the two strains (Fig. 1A), suggesting that disruption of the TCS CzcS/CzcR did not affect the transcriptome of *P. aeruginosa* under normal conditions. This was consistent with previous studies showing that expression and activation of CzcS/CzcR require the presence of the inducing signal Zn (9, 14). We then monitored the growth of the WT and Δ*czcR* strains and the activity of P*czcC*, a promoter under the direct control of CzcR, in the WT and Δ*czcR* strains when both strains were cultured in the presence of Zn at different concentrations. It was shown that the final biomasses between the WT and Δ*czcR* strains were not significantly different under Zn treatment until the concentration of Zn was higher than 0.5 mM (Fig. S1A). Notably, the activity of P*czcC* was continuously induced in the WT strain with the increasing concentration of Zn up to 0.5 mM (Fig. S1B). Therefore, we repeated the comparative transcriptome analysis by culturing the WT and Δ*czcR* strains with supplementation of 0.5 mM Zn in the medium. It was shown that the transcriptome profiles of these two strains were dramatically different during Zn excess (Fig. 1B). A total of 2,653 genes were differentially expressed in the Δ*czcR* mutant compared to that in the WT strain with P value < 0.05 (Table S1). Among them, 1,371 genes were upregulated and 1,282 genes were downregulated by the deletion of *czcR*. Enrichment analysis based on the Kyoto Encyclopedia of Genes and Genomes



Protein	Log_2Fold Change (Δ*czcR* vs WT)	Description
CzcC, B, A	-5.01 to -5.59	Zinc homeostasis
CzcD	-2.46	
PA2807, PtrA	-5.68, -11.08	Copper tolerance
PchA,B,C,D,E,F,G,R, FptA	1.33 to 2.80	Iron homeostasis
KatA, KatB, AnkB	2.66 to 6.41	Oxidative tolerance
AhpC, F, AhpB	3.43 to 6.75	
PhzB1-G1, B2-G2, M, S	1.72 to 3.76	PYO biosynthesis
CupA2,A3,A4,A5,B2,B4,C2,C3	-1.13 to -3.45	Biofilm formation
PelA, B, C	-1.62 to -2.96	
MexA,B, OprM	1.08 to 2.10	Antibiotic resistance
MexG,H,I, OpmD	2.05 to 3.00	
OprD	3.08	
NorB,C; NosR,Z,D	2.80 to 2.82; 1.83 to 2.03	Denitrification
NirC,J,L,F,M,N,S,Q,O,P	1.21 to 2.70	
FliE	-1.58	Flagellar assembly

FIG 1 CzcS/CzcR modulates global gene expression during Zn excess. (A and B) Genome-wide transcriptomic profiles of the PAO1 WT strain and the Δ*czcR* mutant when they were cultured under the normal condition (A) or with the supplementation of 0.5 mM $ZnSO_4$ (B). Green dots were the genes upregulated in the Δ*czcR* mutant, and orange dots were the genes downregulated in the Δ*czcR* mutant. The black dot lines represented 2-Log_2Fold expression changes. Genes with dramatic differences in expression were selectively labeled. (C) KEGG pathway enrichment of genes that were upregulated and downregulated in the Δ*czcR* mutant. The size of the dot represented the number of genes in the pathways. (D) Representative upregulated and downregulated genes in the Δ*czcR* mutant with different biological functions were displayed.

(KEGG) pathways showed that these differentially expressed genes were mainly enriched in 14 pathways (Fig. 1C). Genes upregulated in the Δ*czcR* mutant were mainly enriched in quorum sensing, protein export, phenazine biosynthesis, oxidative phosphorylation, citrate cycle (TCA cycle), carbon metabolism, biosynthesis of secondary metabolites, biosynthesis of nucleotide sugars, biosynthesis of amino acids, and genes downregulated in the Δ*czcR* mutant were mainly enriched in fructose and mannose metabolism, benzoate degradation, bacterial secretion system, aminobenzoate degradation, and ABC transporters. As selectively listed in Fig. 1D, in addition to the previously reported functions of CzcS/CzcR in regulating Zn efflux, pyocyanin (PYO) biosynthesis, biofilm formation, flagellar assembly, and antibiotic resistance, CzcS/CzcR was also shown to modulate many other physiological processes, such as Cu tolerance, Fe homeostasis, oxidative tolerance, and denitrification.

Expression of Cu tolerance genes *ptrA* and *PA2807* is directly controlled by CzcS/CzcR

PtrA is a small periplasmic protein that is involved in Cu tolerance (15). Consistent with previous observations (8, 11), our transcriptome result showed that *ptrA* was one of the most downregulated genes in the Δ*czcR* mutant during Zn excess with even higher fold changes than genes involved in Zn efflux (Fig. 1B). A cupredoxin-like protein

PA2807, which has also been reported to be involved in Cu tolerance (8, 16), was also expressed at a dramatically lower level in the Δ*czcR* mutant than that in the WT strain, suggesting that CzcS/CzcR may play an important role in Cu tolerance. We performed reverse transcription-quantitative PCR (RT-qPCR) to verify the expression of *ptrA* and *PA2807* in the WT strain and the Δ*czcR* mutant. As shown in Fig. 2A, there was no significant difference in the expression levels of *ptrA* and *PA2807* between the two strains when they were cultured without the presence of Zn. Similar to the transcriptome result, the expression of both genes in the Δ*czcR* mutant was substantially lower than that in the WT strain when the strains were cultured in the presence of Zn and the reduced expression of two genes in the Δ*czcR* mutant could be fully restored to the WT level with

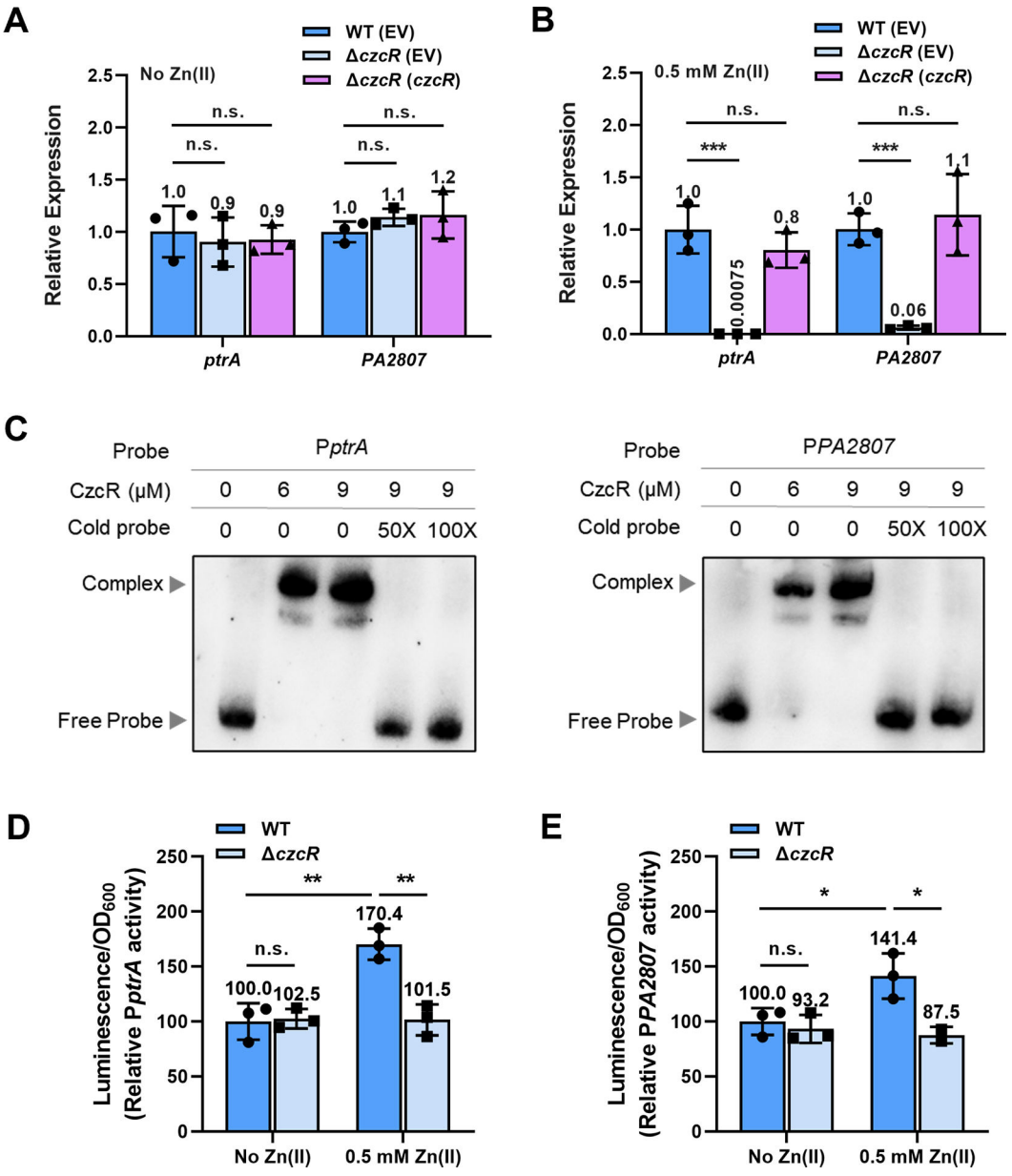


FIG 2 CzcS/CzcR induces the expression of *ptrA* and *PA2807* during Zn excess. (A and B) Relative expression of the *ptrA* and *PA2807* genes in the Δ*czcR* and Δ*czcR* (*czcR*) strains compared to the WT strain when they were cultured in the absence (A) or the presence (B) of Zn. (C) Electrophoretic mobility shift assays (EMSAs) showed the binding ability of CzcR at the promoters of *ptrA* and *PA2807*. (D and E) Expression of P*ptrA-lux* (D) and P*PA2807-lux* (E) was measured in the WT and Δ*czcR* strains when they were cultured in the absence or presence of Zn. Statistical significance was calculated compared to the WT group based on one-way analysis of variance (ANOVA) (A and B) or two-way ANOVA (D and E) (n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

the complementation of *czcR* (Fig. 2B), confirming that CzcS/CzcR positively regulates the expression of *ptrA* and *PA2807* in the presence of Zn. In addition to *ptrA* and *PA2807*, we also wondered if CzcS/CzcR regulates the expression of other genes involved in Cu tolerance. However, in the transcriptome result, we did not find obvious changes in the expression of other Cu tolerance genes (Table S1). Moreover, RT-qPCR also showed no obvious changes in the expression levels of several important Cu tolerance genes, such as *pcoA*, *pcoB*, *copA1*, and *copA2* (Fig. S2).

Previous semi-quantitative RT-PCR showed that *ptrA* (*PA2808*) and *PA2807* are located in the same operon which produces a polycistronic *ptrA-PA2807* mRNA (8). Since CzcR is a transcription factor that has been demonstrated to regulate PYO production, swimming motility, and antibiotic resistance by directly binding to promoters of target genes (10, 13, 14), we then purified CzcR and conducted electrophoretic mobility shift assay (EMSA) to explore if CzcR regulated *ptrA* and *PA2807* by binding to their promoters. CzcR was first shown to bind to the promoter of *czcCBA* (P*czcC*, positive control) but not to the coding region of *czcC* (negative control) (Fig. S3), suggesting the specificity of interactions between CzcR and target DNA sequences. Next, promoter regions of *ptrA* and *PA2807* genes were examined, and interestingly, EMSAs showed the binding of CzcR to both promoter regions (Fig. 2C). These results not only demonstrated that CzcR binds directly to the promoter of *ptrA* (P*ptrA*) to induce its transcription, but also indicated that *PA2807* can be transcribed from its own promoter P*PA2807* in addition to P*ptrA*. To further validate the presence of P*PA2807* which drives the transcription of *PA2807*, we generated a P*PA2807-lux* fusion to quantify its transcriptional activity. As with the promoter of *ptrA*, the activity of the P*PA2807* was significantly reduced by the deletion of *czcR* when strains were cultured in the presence of Zn (Fig. 2D and E), confirming that CzcR activates the transcription of a monocistronic *PA2807* mRNA in addition to the polycistronic *ptrA-PA2807* mRNA.

CzcS/CzcR maintains Cu tolerance through PtrA during Zn excess

Owing that both PtrA and PA2807 proteins were annotated for Cu tolerance, we speculated that CzcS/CzcR might upregulate these two genes to elevate the tolerance of *P. aeruginosa* to Cu stress. To first test this speculation, we examined the growth of WT and Δ*czcR* strains in the absence and presence of 2 mM $CuSO_4$. As shown in Fig. 3A, there was no growth difference between the strains without Zn pretreatment when they were cultured in the both absence and presence of $CuSO_4$. However, the deletion of *czcR* caused an obvious growth defect when the mutant was pretreated with Zn and then grown in the presence of $CuSO_4$ (Fig. 3B), indicating the importance of CzcS/CzcR in maintaining Cu tolerance during Zn excess. To investigate if the reduced expression levels of *ptrA* and *PA2807* led to the susceptibility of Δ*czcR* to Cu, we overexpressed *ptrA*, *PA2807*, and the combination of *ptrA* and *PA2807*, respectively, in the Δ*czcR* mutant (Fig. 3C and D). It was shown that Cu tolerance of the Δ*czcR* mutant was enhanced to the WT level with the overexpression of *ptrA* or the simultaneous overexpression of *ptrA* and *PA2807* (Fig. 3D and S4). However, the Δ*czcR* mutant with overexpression of *PA2807* alone showed no growth difference as the mutant that contained the empty vector (EV) in the cell (Fig. 3D and S4), implying that CzcS/CzcR modulates Cu tolerance through PtrA during Zn excess.

It is interesting to notice that the promoter activities of both *ptrA* and *PA2807* were upregulated by Zn in the WT strain (Fig. 2D and E). However, the growth of the WT strain during Cu stress was almost unchanged with or without Zn pretreatment (Fig. 3C, D, and S4). These results suggested that the physiological function of CzcS/CzcR-upregulated *ptrA* is maintaining the capability of Cu tolerance which is impaired by excessive Zn through CzcS/CzcR-independent pathways.

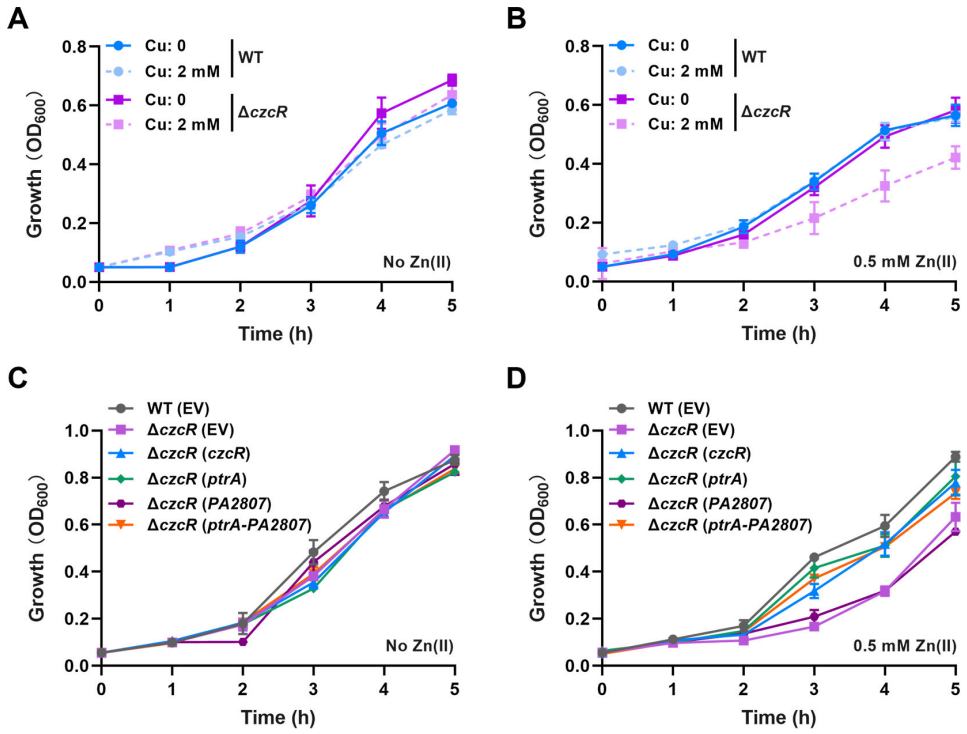


FIG 3 CzcS/CzcR positively regulates Cu tolerance through PtrA during Zn excess. (A) Growth of the WT strain and the Δ*czcR* mutant in the presence or absence of 2 mM $CuSO_4$ when the strains were not pretreated with Zn. (B) Growth of the WT strain and the Δ*czcR* mutant in the presence or absence of 2 mM $CuSO_4$ after the strains were pretreated with Zn. (C) Growth of the WT strain, Δ*czcR* mutant, and the Δ*czcR* mutant with the overexpression of *ptrA*, *PA2807,* or *ptrA-PA2807* in the presence of 2 mM $CuSO_4$ when the strains were not pretreated with Zn. (D) Growth of the WT strain, Δ*czcR* mutant, and the Δ*czcR* mutant with the overexpression of *ptrA*, *PA2807,* or *ptrA-PA2807* in the presence of 2 mM $CuSO_4$ after the strains were pretreated with Zn.

Zn and CzcS/CzcR reversely modulate pyochelin biosynthesis

In addition to Cu tolerance, the transcriptome result showed that the expression of multiple genes associated with the biosynthesis and transportation of pyochelin (PCH) and pyoverdine (PVD), two major siderophores playing important roles in Fe uptake (17), was different between WT and Δ*czcR* strains. Among them, most genes involved in PCH biosynthesis or transportation such as *pchA*, *pchB*, *pchC*, *pchD*, *pchE*, *pchF*, *pchG*, *pchR*, and *fptA* were induced at least by 2.51-fold in the Δ*czcR* mutant (Fig. 1D). In contrast, a small group of genes involved in PVD biosynthesis or transportation were slightly changed by the deletion of *czcR* with some of them such as *pvdA* and *pvdO* were upregulated by 1.49 and 2.27-fold, while some of them such as *pvdD*, *pvdL, pvdF,* and *pvdT* were downregulated by 2.28, 1.71, 1.85, and 1.69-fold, respectively (Table S1). These results suggested that CzcS/CzcR may also play a role in modulating Fe homeostasis.

To verify whether CzcS/CzcR regulates PCH and PVD productions, we selected some genes or operons (*pchE/pchF/pchG/PA4223/PA4222*, *pchD/pchC/pchB/pchA*, *pvdR/pvdT/opmQ*, Fig. 4A) involved in PCH biosynthesis and PVD transportation to construct promoter-*lux* transcription fusions and examine their transcriptional activities. It was shown that promoter activities of the PCH biosynthetic operons, i.e., P*pchD* and P*pchE*, were significantly elevated by the deletion of *czcR* in the presence but not absence of Zn (Fig. 4B and C). However, promoter activities of the PVD transporter gene operon P*pvdR* between WT and Δ*czcR* strains were not significantly different in both the presence and absence of Zn (Fig. 4B and C). These results suggested that CzcS/CzcR controls PCH biosynthesis. We next measured PCH and PVD productions in WT and Δ*czcR* strains. Consistent with the gene expression result, it was shown that PCH production was

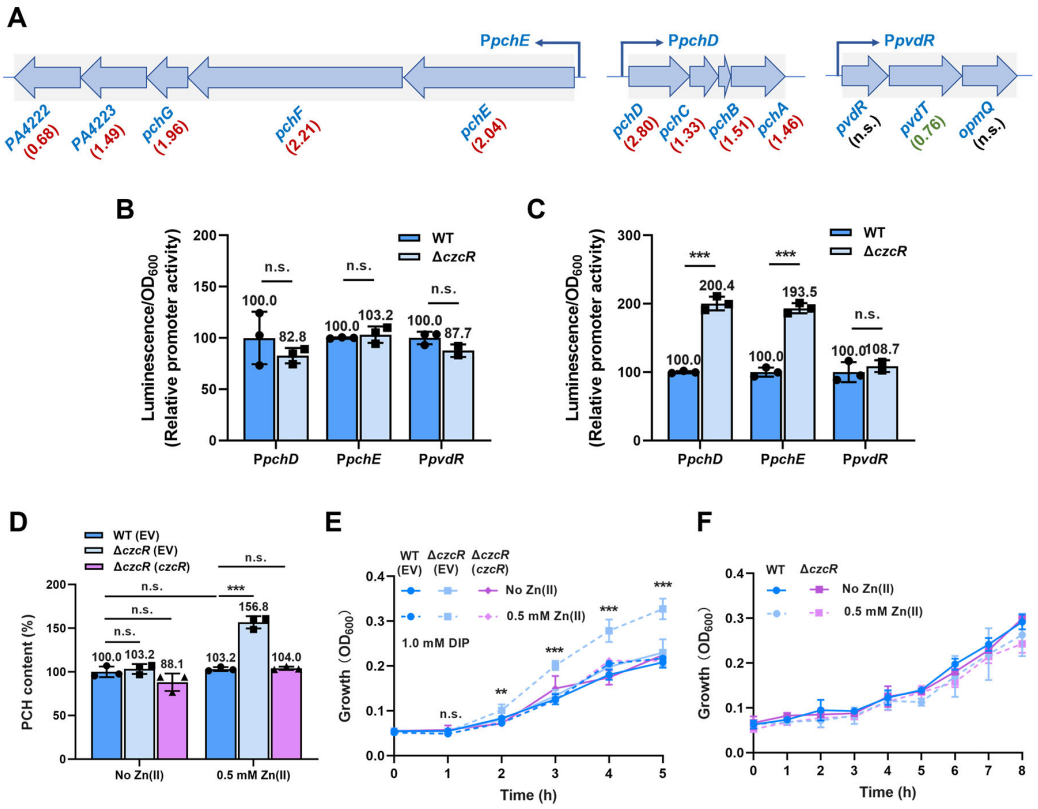


FIG 4 Loss of CzcS/CzcR signaling induces pyochelin production and promotes cell growth under Fe depletion and Zn excess conditions. (A) Three gene clusters involved in pyochelin (PCH) biosynthesis (*pchE/pchF/pchG/PA4223/PA4222* and *pchD/pchC/pchB/pchA*) and pyoverdine (PVD) transportation (*pvdR/pvdT/opmQ*) were shown. Values underneath gene names indicate Log_2Fold changes of gene expression in the Δ*czcR* mutant relative to that in the WT strain. Red and green colors indicated the upregulated and downregulated gene expression in the Δ*czcR* mutant, respectively. (B and C) Expression of P*pchD-lux*, P*pchE-lux*, and P*pvdR-lux* was measured in the WT and Δ*czcR* strains when they were cultured in the absence (B) or presence (C) of Zn. (D) Production of PCH was measured in WT and Δ*czcR* strains when they were cultured in the absence or presence of Zn. (E) Growth of the WT strain, Δ*czcR* mutant, and the Δ*czcR* mutant with the complementation of *czcR* in the presence of 1.0 mM 2,2′-dipyridyl (DIP) when the strains were pretreated or not pretreated with Zn. (F) Growth of WT and Δ*czcR* strains with or without Zn pretreatment in the minimal medium without Fe supplementation. Statistical significance was calculated compared to the WT group based on Student's *t*-test (B and C) and two-way ANOVA (D and E) (n.s., not significant; **, $P < 0.01$; ***, $P < 0.001$).

significantly higher in the Δ*czcR* mutant compared to the WT strain when they were cultured with Zn while there was no difference in PVD production between two strains (Fig. 4D and S5). Interestingly, it was also noticed that productions of PCH were not significantly different between the WT strains with or without Zn treatment (Fig. 4D), implying that Zn induces PCH biosynthesis independent of CzcS/CzcR while CzcS/CzcR simultaneously represses PCH biosynthesis.

CzcS/CzcR inhibits cell growth during Fe depletion and Zn excess

Given that the function of PCH is to acquire Fe from the extracellular environment and Fe is indispensable for optimal bacterial growth, we then sought to explore whether the increased production level of PCH in Δ*czcR* will benefit its growth under Fe-depleted conditions. Growth of the WT and Δ*czcR* strains were monitored under Fe-depleted conditions which were generated by supplementing the Fe chelator 2,2′-dipyridyl (DIP) in the medium (18). When DIP was added in the medium at the concentration of 0.5 mM, growth of the WT and Δ*czcR* strains remained similar no matter whether the two strains were pretreated with Zn or not (Fig. S6). Noticeably, when DIP concentration

was increased to 1.0 mM, the Δ*czcR* mutant grew faster than the WT strain after Zn pretreatment despite the growth of both strains being inhibited by the high concentration of DIP (Fig. 4E and S6B). Consistent with the unchanged level of PCH production, there was no detectable difference in the growth of the WT strain with or without Zn pretreatment (Fig. 4E). Furthermore, when we replaced the culture medium with a minimal medium that did not contain Fe, the growth advantage of Δ*czcR* in the presence of Zn was fully abolished (Fig. 4F). These results showed that Zn promotes cell growth through competitive acquisition of extracellular Fe by PCH when the extracellular Fe concentration is depleted to a certain content, while this process could be counteractively inhibited by CzcS/CzcR. Under Fe-sufficient conditions, overproduction of PCH owing to the disruption of CzcS/CzcR did not cause any beneficial effects on cell growth.

CzcS/CzcR represses *pchR* to inhibit the expression of PCH biosynthetic genes

PCH biosynthesis in *P. aeruginosa* is activated by PchR which is a transcription factor belonging to the AraC-type family (19). The transcriptome result showed that the deletion of *czcR* not only caused the upregulation of PCH biosynthetic genes but also the induced expression of the gene encoding PchR (Fig. 1D). This led us to ask whether the upregulation of PchR activates PCH biosynthesis in Δ*czcR*. Promoter activity assay firstly confirmed the upregulation of *pchR* by the deletion of *czcR* when strains were cultured in the presence of Zn (Fig. 5A). In addition, the unchanged promoter activity of *pchR* in the WT strain after treatment of Zn was consistent with the unchanged production of PCH (Fig. 4D).

We then constructed the mutants Δ*pchR* and Δ*czcR* Δ*pchR* and measured the activity of promoters P*pchD* and P*pchE* in these strains when they were cultured in the presence of Zn. Deletion of *pchR* substantially inhibited the activity of both promoters (Fig. 5B and C), suggesting the key role of PchR in activating *pch* gene expression. Unlike the activation of both promoters by deleting *czcR* in the WT strain, deletion of *czcR* in the Δ*pchR* mutant did not induce the activity of the two promoters (Fig. 5B and C). Consistently, growth of the Zn-pretreated Δ*pchR* mutant was fully inhibited in the presence of 1.0 mM DIP and could not be promoted by further deleting *czcR* (Fig. 5D), indicating that the Zn-activated CzcS/CzcR inhibits cell growth under the Fe-depleted condition by repressing *pchR*.

Transcription of *pchR* is modulated by the ferric uptake regulator Fur as well as its product PchR. A Fur-binding region (Fur box) and a PchR-binding region (PchR box) were previously identified upstream of the *pchR* gene (Fig. 5E) (19). Since a 16-bp CzcR-binding motif was determined using the chromatin immunoprecipitation sequencing assay (20), we searched for potential CzcR-binding sites in the promoter of *pchR* by aligning the promoter sequence with the 16-bp CzcR-binding motif. Sequence alignment revealed a potential CzcR-binding site overlapping with the Fur box and the −10 box in the *pchR* promoter (Fig. 5E), suggesting that CzcR may regulate *pchR* expression by directly interacting with its promoter. In agreement with this analysis, EMSAs showed that CzcR can directly bind to the promoter of *pchR* (P*pchR*) but not the control promoter P*pvdR* (Fig. 5F). Taken together, these results demonstrated that CzcS/CzcR inhibits the expression of PCH biosynthetic genes by repressing *pchR* during Zn excess.

DISCUSSION

Zn is an important transition metal that is associated with numerous biological processes in all kingdoms of life. Although bacterial cells require sufficient Zn to support their growth, excessive Zn is also toxic which frequently competes with other metals or disrupts central carbon metabolism (21). Zn has great antimicrobial activity and the release of Zn from damaged or apoptotic cells and metallothionein is known to be an important line of defense during bacterial infection (22–25). *P. aeruginosa* can survive and thrive in different host environments, and the TCS CzcS/CzcR plays an important role in regulating genes involved in Zn detoxification. In addition to Zn efflux, accumulating evidence has indicated that the physiological functions of CzcS/CzcR in *P. aeruginosa*

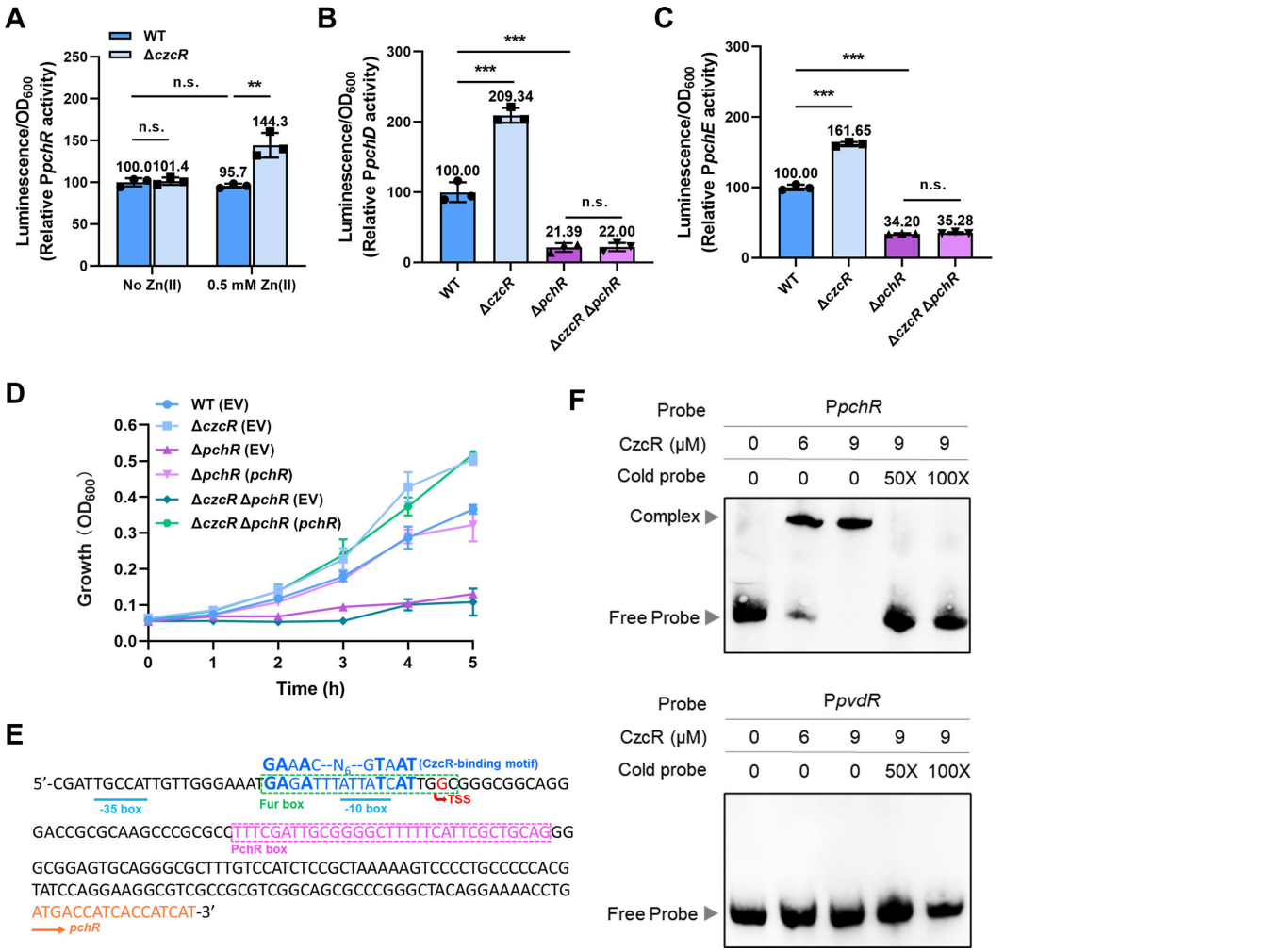


FIG 5 CzcS/CzcR represses PCH biosynthetic gene expression by inhibiting the expression of *pchR*. (A) Expression of P*pchR-lux* was measured in the WT and Δ*czcR* strains when they were cultured in the absence or presence of Zn. (B and C) Expression of P*pchD-lux* (B) and P*pchE-lux* (C) was measured in the WT, Δ*czcR,* Δ*pchR,* and Δ*czcR* Δ*pchR* strains when they were cultured in the presence of Zn. (D) Growth of the WT(EV), Δ*czcR*(EV), Δ*pchR*(EV), Δ*pchR*(*pchR*), Δ*czcR* Δ*pchR*(EV), and Δ*czcR* Δ*pchR*(*pchR*) strains in the presence of 1.0 mM DIP when the strains were pretreated with Zn. (E) Prediction of the CzcR-binding site in the promoter of *pchR*. (F) EMSAs showed the binding ability of CzcR at the promoter of *pchR* (P*pchR*) but not the promoter of *pvdRT-opmQ* (P*pvdR*). Statistical significance was calculated compared to the indicated groups based on two-way ANOVA (A) or one-way ANOVA (B and C) (n.s., not significant; **, $P < 0.01$; ***, $P < 0.001$).

are still not fully understood. In the present study, we performed comparative transcriptome analysis to comprehensively investigate the regulatory function of CzcS/CzcR and found its global regulatory effects on 2,653 genes. Focusing on various metal-associated cellular processes, we revealed the molecular details about the upregulation of the Cu tolerance gene *ptrA* and downregulation of the pyochelin biosynthesis regulatory gene *pchR* under the control of CzcS/CzcR during Zn excess. More interestingly, we further demonstrated that *ptrA* is upregulated to rescue the impaired capacity of Cu tolerance, and *pchR* is downregulated to prevent pyochelin overproduction during Zn excess (Fig. 6).

Crosstalk of Zn and Fe homeostatic systems has been reported in some bacterial species (26, 27), but connections between these two metal homeostatic systems in *P. aeruginosa* are rarely known. In this study, we found that the production of PCH involved in Fe uptake is not influenced by Zn excess in the PAO1 WT strain, while interestingly, its production can be induced by Zn in the mutant lacking CzcS/CzcR. This finding displays

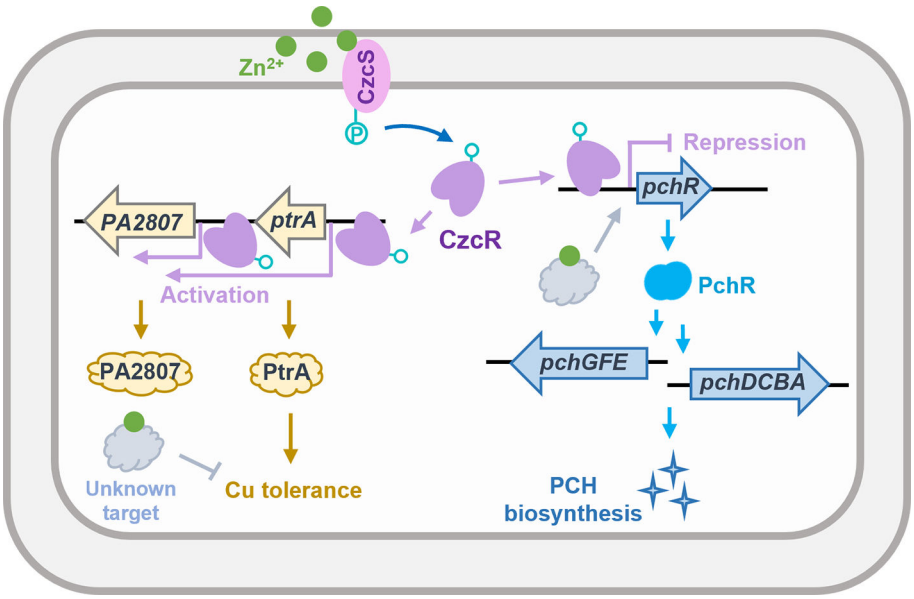


FIG 6 A schematic diagram showing the regulation of Cu tolerance and PCH biosynthesis by CzcS/CzcR during Zn excess in *P. aeruginosa*. Zn excess disrupts *P. aeruginosa* tolerance to Cu, while simultaneously, CzcS/CzcR is activated which induces the expression of *ptrA* to remedy the capability of *P. aeruginosa* to tolerate Cu stress. Zn is also shown to induce the expression of *pchR*, which leads to the overproduction of PCH and enhanced growth under Fe-depleted conditions. Concurrently, CzcS/CzcR antagonizes the induction of *pchR* during Zn excess by the direct binding of CzcR to the promoter of *pchR* to inhibit its expression.

the importance of CzcS/CzcR in maintaining Fe homeostasis in *P. aeruginosa* when the pathogen encounters Zn excess. Induced PCH production in the Δ*czcR* mutant is ascribed to the de-repression of *pchR*, a gene encoding the PCH biosynthesis regulatory protein PchR. Transcriptional expression of *pchR* is positively regulated by Fur and PchR. Sequence analysis showed that the CzcR-binding motif is overlapped with the Fur box at the −10 box which is located upstream of the PchR box in the promoter region, it is possible that the expression of *pchR* and PCH biosynthetic genes are reversely regulated by CzcR and Fur during Zn excess and consequently the production of PCH in the PAO1 WT strain is not affected during Zn excess.

Since Fe is the most abundant metal ion that is indispensable for many essential cellular processes such as DNA replication and oxygen transport (28), the ability to acquire Fe from the environment is critical for the survival of pathogens, especially at the host-pathogen interface. We demonstrated that the increased production of PCH was indeed beneficial for cell growth under Fe-depleted conditions. It is curious why *P. aeruginosa* activates CzcS/CzcR to inhibit the overproduction of PCH caused by Zn. We speculated that the Zn-activated CzcS/CzcR inhibits PCH biosynthesis to prevent excessive PCH production and Fe acquisition when Fe is sufficient. Because PCH and Fe accumulation could be harmful to cells by inducing the production of intracellular reactive oxygen species and causing oxidative damage through the Fenton reaction (29, 30), this is supported by the result that most oxidative tolerance genes including *katA*, *katB*, *ankB*, *ahpC*, *ahpF*, and *ahpB* in the Δ*czcR* mutant were upregulated (Fig. 1D). We thus expected that the lack of CzcS/CzcR would increase the cellular tolerance to oxidative stress during Zn excess. However, we noticed that the Δ*czcR* mutant exhibited lower tolerance to H_2O_2-induced oxidative stress than the WT strain, and the altered susceptibility to H_2O_2 does not seem to be ascribed to PCH or Fe accumulation because the Δ*czcR* Δ*pchR* mutant still exhibited the same growth pattern as the Δ*czcR* mutant (Fig. S7).

Association between Zn excess and Cu tolerance in *P. aeruginosa* has been previously linked to PtrA and PA2807, which not only suggested the importance of PtrA and PA2807 in activating Cu tolerance during Zn excess but also showed the co-transcription of *ptrA* and *PA2807* from the same promoter P*ptrA* (8). Here, we further showed that *PA2807* is also transcribed from its own promoter which can be directly targeted by CzcR. Although both *ptrA* and *PA2807* are annotated for Cu tolerance, we demonstrated that only *ptrA* contributes to Cu tolerance in the Δ*czcR* mutant during Zn excess. Interestingly, *ptrA* is upregulated by Zn in the WT strain but enhanced Cu tolerance was not observed as we expected. Instead, we found that Cu tolerance of the Δ*czcR* mutant was compromised in the presence of Zn, meaning that the CzcS/CzcR-dependent upregulation of *ptrA* in *P. aeruginosa* represents a parallel pathway to rescue the capability of Cu tolerance destructed by Zn through unclear targets rather than to further elevate the level of Cu tolerance.

In summary, this study not only expanded our understanding of the large CzcS/CzcR regulon but also highlighted the importance of CzcS/CzcR in maintaining the balance of some cellular processes such as Cu tolerance and PCH biosynthesis in *P. aeruginosa* during Zn excess. Findings in this study might provide a new target of CzcS/CzcR for the development of anti-infective strategies by administrating concentrations of diverse metal ions.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and growth conditions

Bacterial strains, plasmids, and primers are listed in Table S2. Luria-Bertani broth (Invitrogen) was used to culture bacterial cells. The medium was supplemented with 0.5 mM $ZnSO_4$ for Zn treatment. Antibiotics were added for plasmid construction and propagation when required: tetracycline, 15 µg/mL; gentamycin, 50 µg/mL; kanamycin, 50 µg/mL for *Escherichia coli* DH5α, tetracycline, 15 µg/mL; kanamycin, 500 µg/mL for *E. coli* SM10, and tetracycline, 50 µg/mL; gentamycin, 50 µg/mL; kanamycin, 500 µg/mL for *P. aeruginosa* PAO1.

Construction of gene deletion mutants

Gene deletion was conducted using the chromosomally integrated type I-F CRISPR-Cas system (31). Briefly, an editing plasmid was first constructed which contains a mini-CRISPR to express a crRNA targeting a 32-bp protospacer in the gene and a donor fragment consisting of ~500-bp upstream and ~500-bp downstream sequences of the target gene. The editing plasmid was then introduced into $PAO1^{IF\lambda}$ through conjugation from *E. coli* SM10. The desired gene deletion was confirmed by PCR and Sanger sequencing. After gene deletion, the integrated type I-F CRISPR-Cas machinery was removed by an additional round of editing using the editing plasmid pAY7401.

RNA sequencing

PAO1 WT and Δ*czcR* strains were cultured in the presence or absence of Zn for 8 h until OD_{600} reached 2.0. Subsequently, 0.5 mL cell culture was then collected, and total RNA was extracted using the Eastep Super Total RNA Extraction Kit (Promega). Library construction and high-throughput sequencing were conducted using Illumina Novaseq 6000 at Novogene (Beijing, China). Quality control of raw reads was conducted using Trimmomatic v0.32 (32). Mapping of qualified reads was performed against the reference genome of PAO1 (NC_002516.2) using BWA v0.7.17-r1188 (33), SAMtools v1.15 (34), and bamkeepgoodreads of Stampy v1.0.32 (35). FeatureCounts of Subread v2.0.3 (36) was used to generate a count matrix which was submitted to DEseq2 (37) for identifying differentially expressed genes. KEGG pathway enrichment analysis was performed using clusterProfile v4.0 (38).

Reverse transcription-quantitative PCR

One milliliter bacterial culture was harvested by centrifugation after incubation in the presence or absence of Zn for 8 h. Total RNA was extracted, cDNA was reverse transcribed, and qPCR was performed using the Eastep Super Total RNA Extraction Kit (Promega), TransScript OneStep gDNA Removal and cDNA Synthesis SuperMix (TransGen), and the ChamQ Universal SYBR qPCR Master Mix (Vazyme), respectively. The housekeeping *recA* gene was used as the internal reference control. All experiments were conducted with three independent biological repeats.

Growth curve measurement

Overnight *P. aeruginosa* culture was 1:50 diluted into fresh LB broth with or without the supplementation of Zn and grown for 6 h. After adjusting the cell number to approximately 1×10^7, $CuSO_4$ was added into the medium to a final concentration of 2 mM, DIP was added to the concentrations of 0.5 mM or 1.0 mM, and H_2O_2 was added to the concentration of 0.75 mM. Cell growth was monitored by measuring the OD_{600} value every 1 h. All experiments were conducted with three independent biological repeats.

Promoter activity assay

Promoter sequences were amplified by PCR using the genomic DNA of PAO1 as a template and then inserted between the HindIII and BamHI sites in the mini-CTX-*lux* plasmid. The promoter-*lux* fusions were integrated into the genomes of *P. aeruginosa* strains at the *attB* site by conjugation from the *E. coli* SM10 strain. Overnight cultures of *P. aeruginosa* strains containing promoter-*lux* fusions were 1:50 diluted into fresh LB broth with or without the presence of Zn. Luminescence and OD_{600} were monitored in a microplate reader (BioTek) after cells were grown for 6 h. All experiments were conducted with at least three independent biological repeats.

Electrophoretic mobility shift assay

Expression of the His_6-tagged CzcR in *E. coli* BL21(DE3) was induced by 0.5 mM isopropyl-β-d-thiogalactoside. His_6-tagged CzcR protein was purified with a Ni^{2+}-affinity column (Smart-Lifesciences). Promoter fragments were first obtained by PCR using the genomic DNA of PAO1 as a template and then biotin-labeled by Biotin 3′ End DNA Labeling Kit (ThermoFisher Scientific). Each biotin-labeled promoter fragment with or without unlabeled promoter fragment (cold probe) was incubated with CzcR and then subjected to polyacrylamide gel electrophoresis. The LightShift Chemiluminescent EMSA Kit (ThermoFisher Scientific) was used to examine potential interactions between CzcR and target DNA fragments. Uncropped gel images of the EMSA results are shown in Fig. S8.

Measurement of PCH and PVD

For PCH measurement, *P. aeruginosa* strains was cultured with or without the supplementation of Zn for 8 h. PCH was extracted twice from the supernatant of the cell culture using an equal volume of acidified ethyl acetate (0.1% acetic acid). The organic phase was collected and dried with a centrifugal vacuum evaporator. The dried compounds were dissolved in 1 mL methanol (HPLC grade) for liquid chromatography–mass spectrometry analysis using a Q Exactive Focus Hybrid Quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific). For PVD, *P. aeruginosa* strains was cultured in minimal medium (absence of sucrose) with or without the supplementation of Zn. After adjusting the cell number to approximately 1×10^9, PVD was quantified by measuring the absorbance of culture supernatants after the supernatants were 1:10 diluted in 100 mM Tris-HCl (pH 8.0) at 405 nm using a spectrophotometer. All experiments were conducted with at least three independent biological repeats.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No. 32100020 and 32370188), Guangdong Basic and Applied Basic Research Foundation (No. 2023A1515012775 and 2022 A1515010194), Guangzhou Basic and Applied Basic Research Foundation (No. 202201010613), and the Health and Medical Research Fund (No. 19201901).

AUTHOR AFFILIATIONS

[1]Guangdong Province Key Laboratory of Microbial Signals and Disease Control, Integrative Microbiology Research Centre, South China Agricultural University, Guangzhou, China
[2]Department of Microbiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China

AUTHOR ORCIDs

Zeling Xu http://orcid.org/0000-0003-4662-8111

FUNDING

Funder	Grant(s)	Author(s)
MOST | National Natural Science Foundation of China (NSFC)	32100020, 32370188	Zeling Xu
Guangdong Basic and Applied Basic Research Foundation	2023A1515012775, 2022A1515010194	Zeling Xu
Guangzhou Basic and Applied Basic Research Foundation	202201010613	Zeling Xu
Health and Medical Research Fund (HMRF)	19201901	Huiluo Cao

AUTHOR CONTRIBUTIONS

Ting Li, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft | Huiluo Cao, Data curation, Formal analysis, Funding acquisition, Investigation, Visualization, Writing – review and editing | Cheng Duan, Investigation | Shuzhen Chen, Investigation | Zeling Xu, Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – review and editing

DATA AVAILABILITY

The transcriptome data were deposited in the NCBI under the project accession number PRJNA896733.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Fig S1-S8, Table S2 (AEM02327-23-s0001.pdf). Supplemental figures and table.
Table S1 (AEM02327-23-s0002.xlsx). List of differentially expressed genes (P<0.05) in the Δ*czcR* mutant compared to the wild-type strain as measured during Zn excess.

REFERENCES

1. Palmer LD, Skaar EP. 2016. Transition metals and virulence in bacteria. Annu Rev Genet 50:67–91. https://doi.org/10.1146/annurev-genet-120215-035146
2. Murdoch CC, Skaar EP. 2022. Nutritional immunity: the battle for nutrient metals at the host-pathogen interface. Nat Rev Microbiol 20:657–670. https://doi.org/10.1038/s41579-022-00745-6

3. Andreini C, Banci L, Bertini I, Rosato A. 2006. Zinc through the three domains of life. J Proteome Res 5:3173–3178. https://doi.org/10.1021/pr0603699
4. Qin S, Xiao W, Zhou C, Pu Q, Deng X, Lan L, Liang H, Song X, Wu M. 2022. *Pseudomonas aeruginosa*: pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. Signal Transduct Target Ther 7:199. https://doi.org/10.1038/s41392-022-01056-1
5. Winstanley C, O'Brien S, Brockhurst MA. 2016. *Pseudomonas aeruginosa* evolutionary adaptation and diversification in cystic fibrosis chronic lung infections. Trends Microbiol. 24:327–337. https://doi.org/10.1016/j.tim.2016.01.008
6. Gonzalez MR, Ducret V, Leoni S, Perron K. 2019. *Pseudomonas aeruginosa* zinc homeostasis: key issues for an opportunistic pathogen. Biochim Biophys Acta Gene Regul Mech 1862:722–733. https://doi.org/10.1016/j.bbagrm.2018.01.018
7. Perron K, Caille O, Rossier C, Van Delden C, Dumas J-L, Köhler T. 2004. CzcR-CzcS, a two-component system involved in heavy metal and carbapenem resistance in *Pseudomonas aeruginosa*. J Biol Chem 279:8761–8768. https://doi.org/10.1074/jbc.M312080200
8. Ducret V, Abdou M, Goncalves Milho C, Leoni S, Martin-Pelaud O, Sandoz A, Segovia Campos I, Tercier-Waeber M-L, Valentini M, Perron K. 2021. Global analysis of the zinc homeostasis network in *Pseudomonas aeruginosa* and its gene expression dynamics. Front Microbiol 12:739988. https://doi.org/10.3389/fmicb.2021.739988
9. Wang D, Chen W, Huang S, He Y, Liu X, Hu Q, Wei T, Sang H, Gan J, Chen H. 2017. Structural basis of Zn(II) induced metal detoxification and antibiotic resistance by histidine kinase CzcS in *Pseudomonas aeruginosa*. PLoS Pathog 13:e1006533. https://doi.org/10.1371/journal.ppat.1006533
10. Dieppois G, Ducret V, Caille O, Perron K. 2012. The transcriptional regulator CzcR modulates antibiotic resistance and quorum sensing in *Pseudomonas aeruginosa*. PLoS One 7:e38148. https://doi.org/10.1371/journal.pone.0038148
11. Lee J-H, Kim Y-G, Cho MH, Lee J. 2014. ZnO nanoparticles inhibit *Pseudomonas aeruginosa* biofilm formation and virulence factor production. Microbiol Res 169:888–896. https://doi.org/10.1016/j.micres.2014.05.005
12. Ducret V, Gonzalez MR, Scrignari T, Perron K. 2016. OprD repression upon metal treatment requires the RNA chaperone Hfq in *Pseudomonas aeruginosa*. Genes (Basel) 7:82. https://doi.org/10.3390/genes7100082
13. Chen S, Cao H, Xu Z, Huang J, Liu Z, Li T, Duan C, Wu W, Wen Y, Zhang LH, Xu Z. 2023. A type I-F CRISPRi system unveils the novel role of CzcR in modulating multidrug resistance of *Pseudomonas aeruginosa*. Microbiol Spectr 11:e01123-23. https://doi.org/10.1128/spectrum.01123-23
14. Liu Z, Xu Z, Chen S, Huang J, Li T, Duan C, Zhang LH, Xu Z. 2022. CzcR is essential for swimming motility in *Pseudomonas aeruginosa* during zinc stress. Microbiol Spectr 10:e0284622. https://doi.org/10.1128/spectrum.02846-22
15. Elsen S, Ragno M, Attree I. 2011. PtrA is a periplasmic protein involved in cu tolerance in *Pseudomonas aeruginosa*. J Bacteriol 193:3376–3378. https://doi.org/10.1128/JB.00159-11
16. Quintana J, Novoa-Aponte L, Argüello JM. 2017. Copper homeostasis networks in the bacterium *Pseudomonas aeruginosa*. J Biol Chem 292:15691–15704. https://doi.org/10.1074/jbc.M117.804492
17. Cornelis P, Dingemans J. 2013. *Pseudomonas aeruginosa* adapts its iron uptake strategies in function of the type of infections. Front Cell Infect Microbiol 3:75. https://doi.org/10.3389/fcimb.2013.00075
18. McHugh JP, Rodríguez-Quinoñes F, Abdul-Tehrani H, Svistunenko DA, Poole RK, Cooper CE, Andrews SC. 2003. Global iron-dependent gene regulation in *Escherichia coli*: a new mechanism for iron homeostasis. J Biol Chem 278:29478–29486. https://doi.org/10.1074/jbc.M303381200
19. Michel L, González N, Jagdeep S, Nguyen-Ngoc T, Reimmann C. 2005. PchR-box recognition by the AraC-type regulator PchR of *Pseudomonas aeruginosa* requires the siderophore pyochelin as an effector. Mol Microbiol 58:495–509. https://doi.org/10.1111/j.1365-2958.2005.04837.x
20. Fan K, Cao Q, Lan L. 2021. Genome-wide mapping reveals complex regulatory activities of BfmR in *Pseudomonas aeruginosa*. Microorganisms 9:485. https://doi.org/10.3390/microorganisms9030485
21. Ong CY, Walker MJ, McEwan AG. 2015. Zinc disrupts central carbon metabolism and capsule biosynthesis in *Streptococcus pyogenes*. Sci Rep 5:10799. https://doi.org/10.1038/srep10799
22. Botella H, Peyron P, Levillain F, Poincloux R, Poquet Y, Brandli I, Wang C, Tailleux L, Tilleul S, Charrière GM, Waddell SJ, Foti M, Lugo-Villarino G, Gao Q, Maridonneau-Parini I, Butcher PD, Castagnoli PR, Gicquel B, de Chastellier C, Neyrolles O. 2011. Mycobacterial P_1-type ATPases mediate resistance to zinc poisoning in human macrophages. Cell Host Microbe 10:248–259. https://doi.org/10.1016/j.chom.2011.08.006
23. Ong CY, Gillen CM, Barnett TC, Walker MJ, McEwan AG. 2014. An antimicrobial role for zinc in innate immune defense against group A *Streptococcus*. J Infect Dis 209:1500–1508. https://doi.org/10.1093/infdis/jiu053
24. Na-Phatthalung P, Min J, Wang F. 2021. Macrophage-mediated defensive mechanisms involving zinc homeostasis in bacterial infection. Infect Microbes Dis 3:175–182. https://doi.org/10.1097/IM9.-0000000000000058
25. Eijkelkamp BA, Morey JR, Neville SL, Tan A, Pederick VG, Cole N, Singh PP, Ong C-LY, Gonzalez de Vega R, Clases D, Cunningham BA, Hughes CE, Comerford I, Brazel EB, Whittall JJ, Plumptre CD, McColl SR, Paton JC, McEwan AG, Doble PA, McDevitt CA. 2019. Dietary zinc and the control of *Streptococcus pneumoniae* infection. PLoS Pathog 15:e1007957. https://doi.org/10.1371/journal.ppat.1007957
26. Xu Z, Wang P, Wang H, Yu ZH, Au-Yeung HY, Hirayama T, Sun H, Yan A. 2019. Zinc excess increases cellular demand for iron and decreases tolerance to copper in *Escherichia coli*. J Biol Chem 294:16978–16991. https://doi.org/10.1074/jbc.RA119.010023
27. Xu FF, Imlay JA. 2012. Silver(I), mercury(II), cadmium(II), and zinc(II) target exposed enzymic iron-sulfur clusters when they toxify *Escherichia coli*. Appl Environ Microbiol 78:3614–3621. https://doi.org/10.1128/AEM.07368-11
28. Skaar EP. 2010. The battle for iron between bacterial pathogens and their vertebrate hosts. PLoS Pathog 6:e1000949. https://doi.org/10.1371/journal.ppat.1000949
29. Cornelis P, Wei Q, Andrews SC, Vinckx T. 2011. Iron homeostasis and management of oxidative stress response in bacteria. Metallomics 3:540–549. https://doi.org/10.1039/c1mt00022e
30. Ong KS, Cheow YL, Lee SM. 2017. The role of reactive oxygen species in the antimicrobial activity of pyochelin. J Adv Res 8:393–398. https://doi.org/10.1016/j.jare.2017.05.007
31. Xu Z, Li Y, Cao H, Si M, Zhang G, Woo PCY, Yan A. 2021. A transferrable and integrative type I-F Cascade for heterologous genome editing and transcription modulation. Nucleic Acids Res 49:e94. https://doi.org/10.1093/nar/gkab521
32. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30:2114–2120. https://doi.org/10.1093/bioinformatics/btu170
33. Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics 25:1754–1760. https://doi.org/10.1093/bioinformatics/btp324
34. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Genome Project Data Processing S. 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25:2078–2079. https://doi.org/10.1093/bioinformatics/btp352
35. Lunter G, Goodson M. 2011. Stampy: a statistical algorithm for sensitive and fast mapping of Illumina sequence reads. Genome Res 21:936–939. https://doi.org/10.1101/gr.111120.110
36. Liao Y, Smyth GK, Shi W. 2014. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30:923–930. https://doi.org/10.1093/bioinformatics/btt656
37. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550. https://doi.org/10.1186/s13059-014-0550-8
38. Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, Feng T, Zhou L, Tang W, Zhan L, Fu X, Liu S, Bo X, Yu G. 2021. clusterProfiler 4.0: a universal enrichment tool for interpreting omics data. Innovation (Camb) 2:100141. https://doi.org/10.1016/j.xinn.2021.100141