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The zinc ion-chelating agent TPEN reduces the CpxP-mediated negative regulation of the CpxAR two-component system in *Escherichia coli*

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CpxAR is a two-component signal transduction system found in Gram-negative bacteria that senses environmental and cellular stresses and regulates gene expression. The inner membrane bound protein CpxA senses cell envelope stress and transmits a signal to cytosolic CpxR via a phosphorylation cascade. CpxA is negatively regulated by the periplasmic protein, CpxP. The structure of CpxP has been solved and shown to adopt a dimeric alpha helical fold bound to zinc ions. We hypothesised that zinc plays a role in CpxP-mediated regulation of CpxAR activity. Using plasmid-encoded *lux* reporter genes, we show that in the presence of TPEN, a zinc (II) chelator, expression of genes regulated by the CpxAR system increases in wild type *Escherichia coli* DH5α, but not in CpxP-deficient mutants. CpxA and CpxR deficient mutants do not respond to changes in pH or the addition of TPEN. Our data suggest that zinc is required for CpxP-mediated inhibition of CpxAR activity.

To thrive in dynamic environments bacteria have evolved two-component signal transduction systems to sense changes in their surroundings and respond by altering gene expression. The CpxAR system is a two-component system that responds to envelope stress, protein misfolding, overexpression of secreted proteins, and pH stress (1, 2, 3), and is conserved among numerous Gram-negative bacteria (1). CpxA is a transmembrane sensor kinase and CpxR is a transcription factor that regulates the expression of stress response genes when phosphorylated by CpxA (2). The CpxAR system has been shown to regulate expression of hundreds of genes and plays a role in mediating attachment to cell surfaces and biofilm formation (3). Upon activation, CpxAR up-regulates genes such as *cpxAR, cpxP,* and the gene encoding spheroplast protein y *(spy)*, a protein suggested to be involved in zinc resistance and metabolism (4), and down-regulated genes such as *rpoE-rseABC,* a transcription factor subunit (5).

CpxP is a periplasmic protein that negatively regulates the CpxAR system by interacting with the periplasmic domain of CpxA, and inhibiting signal transduction (6). The mechanism by which CpxP and CpxA interact is not well understood (1). The structure of CpxP was solved by x-ray crystallography and shown to adopt the structure of a dimeric α-helical protein bound to zinc ions (1, 6). CpxP is structurally similar to several periplasmic metal ion-binding proteins that are known to sense and respond to changes in metal ion concentrations (1).

Zinc is an essential element involved in many biological processes including in the structure and function of proteins (7). Changes in zinc concentration have been shown to alter gene expression and affect basic cellular processes including growth, differentiation, and cell division (7). The presence of zinc in the CpxP structure has led to the suggestion that it may play a role in the CpxAR system, specifically through interactions with CpxP, however this has yet to be characterized (1, 2, 6). Other metal ions have also been shown to have a role in the regulation of CpxAR, including copper (8). For example, the *spy* gene was shown to be activated in both a zinc-dependent, through the BaeSR two-component system, and copper-dependent manner, through the CpxAR system (8). Thus, assaying *spy* expression could serve as a mechanism to integrate multiple environmental sensing two-component systems.

In this study, we investigated the role of zinc in CpxP mediated regulation of the CpxAR system using the zinc

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chelator N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamin (TPEN). TPEN is a divalent metal ion chelator that is cell permeable and has generally been accepted as a zinc-specific chelator (9, 10, 11, 12, 13), although some other divalent cations, such as copper, iron, cadmium, cobalt, and nickel, have also been shown to bind to TPEN (14). TPEN has been shown to alter the transcription of several genes in *E. coli*, including some that are regulated in response to zinc concentration (14). We hypothesized that zinc is involved in CpxP-mediated regulation of CpxA activity. We show that with treatment with TPEN, modelling low-zinc concentrations, the expression of CpxAR-regulated genes increases, dependent on the negative regulator CpxP. Thus, we provide evidence that CpxP-mediated inhibition of CpxAR system involves zinc.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JW3882-1 (Δ*cpxA*), JW3883-1 (Δ*cpxR*), and JW5558-1 (Δ*cpxP*) are *E. coli* BW25113 derivatives obtained from the Coli Genetic Stock Centre (CGSC) at Yale University. Bacterial strains used are summarized in Table 1. Plasmids were propagated in *E. coli* strain DH5α. The *lux* reporter plasmids, pJW1 (*cpxP*), pJW2 (*cpxAR*), pNLP15 (*spy*), and pNLP19 (*rpoE-rseABC*), were provided by Dr. Tracy Raivio at the University of Alberta (5). Plasmids used are summarized in Table 2.

TABLE 1 *E. coli* K12 derivative strain used and created.

Background Strain	Deletion	Plasmid	Resistance	Strain Name	Reference
DH5α	N/A	pJW1	Kanamycin	IKE14W-1P	This study
DH5α	N/A	pJW2	Kanamycin	IKE14W-1AR	This study
DH5α	N/A	pNLP15	Kanamycin	IKE14W-1SY	This study
DH5α	N/A	pNLP19	Kanamycin	IKE14W-1RE	This study
JW3882-1	Δ*cpxA*	N/A	None	IKE14W-2	This study
JW3882-1	Δ*cpxA*	pJW1	Kanamycin	IKE14W-2P	This study
JW3882-1	Δ*cpxA*	pJW2	Kanamycin	IKE14W-2AR	This study
JW3882-1	Δ*cpxA*	pNLP15	Kanamycin	IKE14W-2SY	This study
JW3882-1	Δ*cpxA*	pNLP19	Kanamycin	IKE14W-2RE	This study
JW3883-1	Δ*cpxR*	N/A	None	IKE14W-3	This study
JW3883-1	Δ*cpxR*	pJW1	Kanamycin	IKE14W-3P	This study
JW3883-1	Δ*cpxR*	pJW2	Kanamycin	IKE14W-3AR	This study
JW3883-1	Δ*cpxR*	pNLP15	Kanamycin	IKE14W-3SY	This study
JW3883-1	Δ*cpxR*	pNLP19	Kanamycin	IKE14W-3RE	This study
JW5558-1	Δ*cpxP*	N/A	None	IKE14W-4	This study
JW5558-1	Δ*cpxP*	pJW1	Kanamycin	IKE14W-4P	This study
JW5558-1	Δ*cpxP*	pJW2	Kanamycin	IKE14W-4AR	This study
JW5558-1	Δ*cpxP*	pNLP15	Kanamycin	IKE14W-4SY	This study
JW5558-1	Δ*cpxP*	pNLP19	Kanamycin	IKE14W-4RE	This study

TABLE 2 Names and descriptions of bacterial plasmids used in this study.

Plasmid	Resistance	Description	Reference
pCP20	Ampicillin	Heat-curable containing a heat-inducible FLP recombinase	15
pJW1	Kanamycin	*cpxP* promoter *lux* reporter (*cpxP::lux)*	5
pJW2	Kanamycin	*cpxAR* promoter *lux* reporter (*cpxAR::lux)*	5
pNLP15	Kanamycin	*spy* promoter *lux* reporter (*spy::lux*)	5
pNLP19	Kanamycin	*rpoE-rseABC* promoter *lux* reporter (*rpoE-rseABC::lux*)	5

Media and stock solutions. Lysogeny broth (LB) (1% tryptone, 0.5% yeast extract, 1% NaCl) was autoclaved with 2% agar for solid media. LB was adjusted to pH 6, pH 7, or pH 8 using a 0.2 M sodium phosphate buffer. Transformation buffer (10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$, pH 6.7) used to prepare competent cells was stored at 4°C. N,N,N',N'-tetrakis(2-pyridylmethyl)ethane-1,2-diamin (TPEN) (Sigma-Aldrich®) was dissolved in DMSO as a 350 µM stock solution and stored short-term at 25°C to be used as a divalent zinc ion chelator. $ZnCl_2$ (Sigma-Aldrich®) was dissolved in dH_2O as a 50 mM stock solution and stored at 25°C. Ampicillin sodium salt (Sigma-Aldrich®) and kanamycin monosulfate (Gibco®) were dissolved in dH_2O as 50 mg/ml stock solutions and stored at -20°C. Reagents and media components that were not autoclaved were filter sterilized with a 0.22 µm nitrocellulose filter (EMD MilliporeTM).

Bacterial culture and induction of the CpxAR system. Overnight culture in LB was performed by inoculating a loopful of bacteria in 5 ml of sterile LB and incubating at 37°C, shaking at 100 rpm for 16-20 hours. The CpxAR system was induced or repressed by inoculating a 10^2 dilution of overnight culture in LB adjusted to pH 6, pH 7, or pH 8. The role of zinc ions in induction was assessed by inoculating a 10^2 dilution of overnight culture in LB adjusted to pH 7 or pH 8. $ZnCl_2$ was added to a concentration of 1 mM or chelated by adding TPEN to a concentration of 10 µM.

Preparation of competent cells and plasmid transformation. Plasmids were isolated using a PureLink HiPure Plasmid Miniprep Kit (Life Technologies). Plasmid transformations were performed as previously described (15). Overnight culture was inoculated in 30 ml of LB at a 1/100 dilution and grown with shaking at 37°C until an OD_{600} of 0.4 was achieved. A 15 ml aliquot of culture was spun down in each 50 ml centrifuge tube at 2500 rcf for 10 minutes at 4°C and the pellet was resuspended in 5 ml transformation buffer, incubated on ice for 10 minutes and centrifuged as before. Pellets were resuspended in 1.25 ml transformation buffer. Competent cell aliquots of 100 µl were mixed with 10 ng of plasmid DNA and incubated on ice for 30 minutes. Cells were heat-shocked in a 42°C water bath for 30 seconds and incubated on ice for 2 minutes. Cells were allowed to recover in LB for 1 hour at 37°C with shaking. Cells were spread on solid LB plates with the appropriate antibiotic (50 µg/ml ampicillin for pCP20, and 50 µg/ml kanamycin for the *lux* reporter plasmids) and grown at 30°C for pCP20 and 37°C for *lux* reporter plasmids.

Zinc minimum inhibitory concentration assay. Overnight culture of *E. coli* DH5α was diluted 10^2 in 200 µl of LB in a 96-well plate and grown in the presence of 10 µM TPEN, each well containing concentrations of zinc chloride increasing by 150 µM, performed in triplicate. Concentrations of 0 µM to 5 mM $ZnCl_2$

were used. After incubation at 37°C for 8 hours, OD_{600} was measured with a Bio-Rad Microplate Reader Model 3550.

Generation of deletion mutants. Single colonies of kanamycin-resistant bacterial mutant strains obtained from the CGSC containing pCP20 were inoculated in 5 ml LB and grown at 42°C overnight as previously described (15). Kanamycin cassettes containing flanking homologous regions (*frt* sites) were recombined and removed through the use of a heat-inducible flippase (FLP) encoded on the heat-curable pCP20 plasmid. Culture was diluted 10^{-6} and 50 µl was plated on LB and grown at 30°C to obtain single colonies. Single colonies were streaked on LB supplemented with 50 µg/ml ampicillin, or 50 µg/ml kanamycin. The absence of growth in the presence of both antibiotics indicated successful removal of both pCP20 and resistance cassette.

Analysis of CpxAR activity through *lux* reporter assays. Single colonies from *E. coli* DH5α and Δ*cpxP*, Δ*cpxA*, and Δ*cpxR* strains containing *lux* reporter plasmids were incubated overnight and diluted 100-fold in 200 µl of LB in a 96-well plate as described in (5), in the conditions used to induce or repress the CpxAR system, and grown at 37°C for 8 hours. OD_{600} and luminescence was measured with an Infinite® 200 Pro series Tecan microplate reader to determine *lux* reporter activity. A media control was subtracted from measured luminescence (counts per second) and culture density (OD_{600}), and luminescence was normalized by culture density.

Statistical analysis. Significant differences were determined as significant using unpaired, non-parametric Mann-Whitney U tests, with an $\alpha = 0.1$.

RESULTS

Alkaline pH induces expression of *cpxAR*, *cpxP*, and *spy* but not *rpoE-rseABC* in *E. coli* DH5α. In order to ensure that the CpxAR system was responding to known inducers, expression of *cpxAR*, *cpxP*, *spy*, and *rpoE-rseABC* was assessed at varying pH. Using *lux* reporters fused to promoters of CpxAR-regulated genes encoded on plasmids, transcription was assayed by measuring photon emission normalized against culture density of each *E. coli* clone. As expected, each reporter strain showed significantly higher levels of photon emission compared to negative control *E. coli* strain DH5α that did not contain a *lux* reporter plasmid (data not shown). As previously shown (5), expression of *cpxAR::lux* (Fig 1A), *cpxP::lux* (Fig 1B), and *spy::lux* (Fig 1C) in *E. coli* strain DH5α increases as culture pH increases. However, expression of *rpoE-rseABC::lux,* which is not positively regulated by the CpxAR system, did not change in response to increasing pH (Fig 1D). Notably, photon emission of *cpxP::lux* was approximately tenfold higher than the other CpxAR regulated genes (Fig 1), as seen previously (5). These data indicate that alkaline pH induces the expression of genes positively regulated by the CpxAR system in *E. coli* strain DH5α, which is consistent with previous studies using *E. coli* strain MC4100 (5).

Chelation of zinc with TPEN increases expression of *cpxAR*, *cpxP*, and *spy*, but not *rpoE-rseABC* at pH 7 and pH 8 in *E. coli* strain DH5α. In order to understand the effect of changing the concentration of extracellular zinc on the CpxAR system, the expression of the *lux* reporter constructs were assayed in *E. coli* strain DH5α grown in the presence of different concentrations of zinc. We attempted to deplete the concentration of free zinc by adding 10 µM TPEN, a zinc chelator, the concentration of TPEN used when determining the minimum inhibitory concentration of zinc chloride as 2 mM (Fig S1). Zinc concentrations were increased by adding exogenous zinc chloride to a final concentration of 1 mM, which was determined to be one half of the minimum inhibitory concentration in *E. coli* strain DH5α.

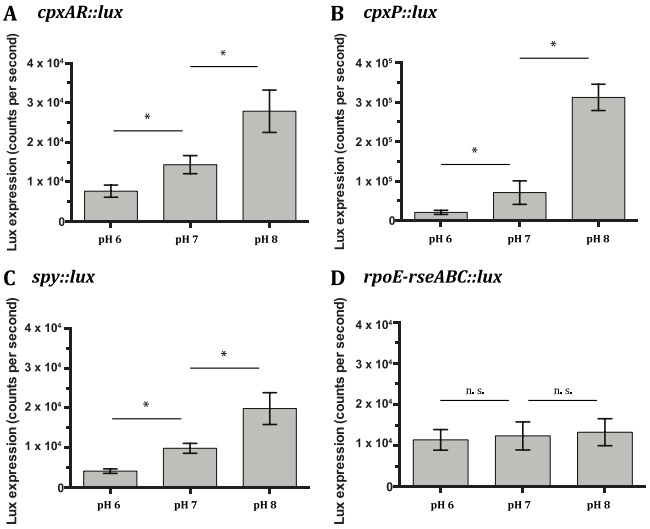

FIG 1 CpxAR positively regulated genes were up-regulated in response to increasing pH, using promoter-fused plasmid *lux* reporter strains in *E. coli* DH5α. Photon emission was normalized to culture density. Data is shown as the mean of 3 replicates and error bars are expressed as standard deviation of the mean. Asterisks denote significant differences as determined by Mann-Whitney U tests ($p<0.1$). (A) Expression of *cpxAR::lux,* in *E. coli* DH5α. (B) Expression of *cpxP::lux* in *E. coli* DH5α. (C) Expression of *spy::lux* in *E. coli* DH5α. (D) Expression of *rpoE-rseABC::lux* in *E. coli* DH5α.

The addition of TPEN resulted in increased expression of genes positively regulated by the CpxAR system. Expression of *cpxAR::lux* increased two-fold in the presence of TPEN in comparison to both the untreated culture and a culture to which 1 mM of exogenous zinc chloride was added (Fig 2A). When 1 mM of zinc chloride was added to the culture, differences in expression were not observed compared to the untreated culture (Fig 2A). Increased expression of the *cpxP::lux* reporter was observed in culture treated with TPEN compared to the untreated control (Fig 2B). Expression of *spy::lux* increased in the presence of TPEN, but an increase in zinc concentration showed no effect (Fig 2C). A significant change in the expression of *rpoE::rseABC* was not seen when the concentration of zinc was altered (Fig 2D). Taken together, these data suggest that the transcription of

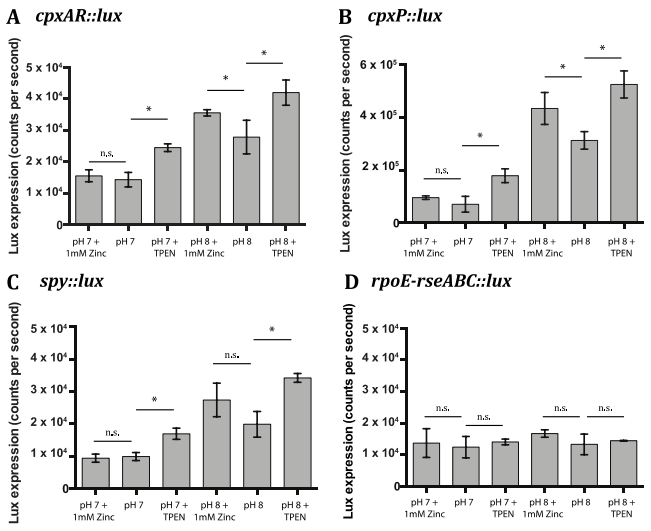

FIG 2 Genes positively regulated by CpxAR were up-regulated in the presence of a zinc chelator, TPEN (10 μM), as assessed using promoter-fused plasmid *lux* reporter strains in *E. coli* DH5α in response to altering concentration of zinc chloride at pH 7 and pH 8. Photon emission was normalized to culture density. Data is shown as the mean of 3 replicates and error bars are expressed as standard deviation of the mean. Asterisks denote significant differences as determined by Mann-Whitney U tests ($p<0.1$). **(A)** Expression of *cpxAR::lux* in *E. coli* DH5α. **(B)** Expression of *cpxP::lux* in *E. coli* DH5α. **(C)** Expression of *spy::lux* in *E. coli* DH5α. **(D)** Expression of *rpoE-rseABC::lux* in *E. coli* DH5α.

CpxAR positively regulated genes is upregulated under growth conditions where free zinc is likely limited, as modeled by the addition of TPEN (Fig 2).

Genes positively regulated by CpxAR do not respond to TPEN in the absence of CpxR or CpxA. In order to test whether zinc-mediated regulation of the CpxAR-regulated genes functions through the CpxAR system, the effect of altering the concentration of free zinc on the transcription of CpxAR-regulated genes in the absence of CpxA or CpxR was assayed in *E. coli* strains with deletions of *cpxA* or *cpxR*. As shown, in the absence of CpxA or CpxR, all CpxAR-regulated genes assayed were not up-regulated or down-regulated in response to the addition of TPEN or exogenous zinc chloride (Fig 3), or pH (data not shown). In the *cpxAR::lux*, *cpxP::lux*, and *spy::lux* reporter strains, a decrease in expression was observed in both Δ*cpxA* and Δ*cpxR* in comparison to *E. coli* DH5α for all growth conditions (Fig 3). The negatively CpxAR-regulated *rpoE-rseABC::lux* reporter did not show a significant change in expression in Δ*cpxR* in comparison to the control *E. coli* DH5α strain under all growth conditions (Fig 3D). Interestingly, in Δ*cpxA* there was a significant decrease in expression of *rpoE-rseABC::lux* in Δ*cpxA* in comparison to *E. coli* strain DH5α at pH 7 treated with TPEN and at pH 8 in all zinc concentrations (Fig 3D). Taken together, these data show that *cpxA* and *cpxR* are required for zinc-mediated upregulation of *cpxAR*, *cpxP*, and *spy*, and thus likely the CpxAR system.

Zinc-mediated regulation of *cpxAR, cpxP, spy,* and *rpoE-rseABC* involves CpxP. In order to test if zinc-mediated regulation of the CpxAR system involves CpxP, *lux* reporters of CpxAR-regulated genes were analyzed in *E. coli* bearing a deletion of *cpxP*. As shown in Figure 4, the expression of *cpxAR::lux* did not change in response to the addition of TPEN in the media (Fig 4A). Similar results were obtained for *cpxP::lux* (Fig 4B) and *spy::lux* (Fig 4C). However, the CpxAR system was still induced when pH was increased (Fig 4). The expression of *rpoE-rseABC::lux* did not change in response to the addition of TPEN or exogenous zinc in the media (Fig 4D). These data suggest that *cpxP* is required for zinc-mediated regulation of CpxAR.

DISCUSSION

The objective of this study was to determine whether or not zinc ions play a role in CpxP-mediated inhibition of the CpxAR system. Based on the observation of zinc ions bound to CpxP in the crystal structure reported by Thede *et al.* (6), we hypothesized that zinc may play a role in mediating interactions between CpxP and CpxA, which in turn may modulate the activity of the CpxAR system. The data presented herein suggest that CpxP-mediated negative regulation of the CpxAR system may involve zinc.

Expression of *cpxP*, *cpxAR*, *spy* and *rpoE-rseABC* using promoters fused to *luxABCDE* reporters on plasmids were measured to determine the induction of the CpxAR system in *E. coli* strain DH5α. With increasing pH, we observed an increase in gene expression of known CpxAR up-regulated genes (*cpxAR, cpxP*, and *spy*), and a lack of up-regulation of *rpoE-rseABC*, known to be down-regulated by the CpxAR system (5) (Fig 1). Differences in the *E. coli* background strain may account for variability regulation patterns of genes assayed; previous studies were conducted using *E. coli* strain MC4100 (5). CpxP up-regulation upon activation of the CpxAR two-component system can be explained by the dual function of CpxP as a negative regulator and a chaperone protein (1). This could allow CpxP to assist in alleviating envelope stress while simultaneously ensuring that the CpxAR system remains regulated (1).

We showed that varying zinc concentration in growth media affects the expression of genes regulated by the CpxAR system. Interestingly, increasing available zinc ions through addition of exogenous zinc chloride produced no significant changes in gene expression, with the exception of a small decrease in expression of *cpxAR, cpxP,* and *spy* expression at pH 8. It has been suggested that *cpxP* and *spy* expression are up regulated

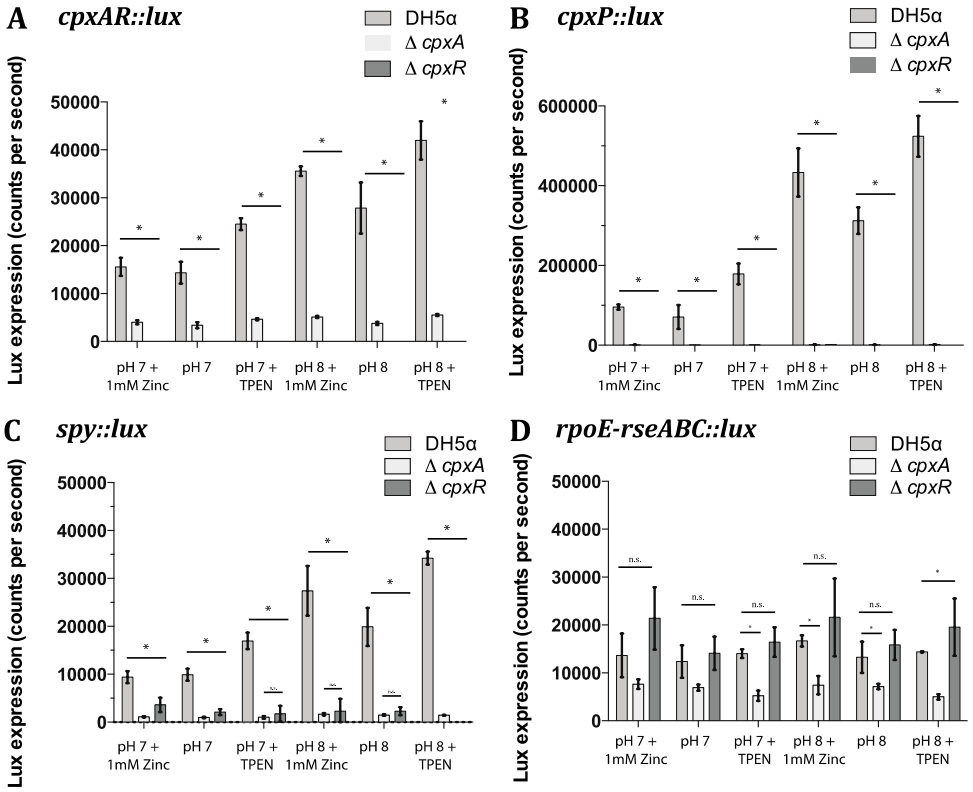

FIG 3 Genes positively regulated by CpxAR are not up-regulated in *ΔcpxA* and *ΔcpxR* in response to TPEN; however, negatively regulated genes are expressed, independent of zinc ions, as determined through a comparison of the effects of zinc chloride between *E. coli* DH5α, *ΔcpxA*, and *ΔcpxR* strains on gene expression of CpxAR-regulated genes using promoter-fused plasmid *lux* reporter strains at pH 7 and pH 8. Photon emission was normalized to culture density. Data is shown as the mean of 3 replicates and error bars are expressed as standard deviation of the mean. Significant differences between DH5α and *ΔcpxA,* and DH5α and *ΔcpxR* are denoted by asterisks as determined by Mann-Whitney U tests (p<0.1). **(A)** Expression of *cpxAR::lux.* **(B)** Expression of *cpxP::lux.* **(C)** Expression of *spy::lux.* **(D)** Expression of *rpoE-rseABC::lux*.

in the presence of zinc (17). As there are approximately 30 different two-component systems in *E. coli*, it is not surprising that zinc may induce or repress the expression of some genes independent of the CpxAR system (16, 18). For example, the BaeSR system can induce the expression of *spy* in a copper-dependent manner (8, 19). The CpxAR negatively-regulated gene, *rpoE-rseABC*, was not up-regulated in response to the addition of TPEN, which is consistent with the hypothesis that zinc is a positive regulator of the CpxAR system. All experiments involving zinc performed at pH 8 demonstrated significantly higher expression than those at pH 7 (Fig 2-4), consistent with results obtained when inducing of the CpxAR system at pH 8 (Fig 1).

To assess whether or not the sensor kinase, CpxA, and the response regulator, CpxR, are important for zinc-mediated up-regulation of the CpxAR system in *E. coli*, gene expression in Δ*cpxA* and Δ*cpxR* was measured through *lux* reporter analysis in pH-adjusted media (data not shown) and in different concentrations of zinc (Fig 3). Consistent with previous results, Δ*cpxA* and Δ*cpxR* showed decreased expression of the CpxAR up-regulated genes, *cpxAR*, *cpxP*, and *spy*, compared to our wild-type control *E. coli* strain DH5α (5). Expression of *rpoE-rseABC* showed no significant change between *ΔcpxR* and DH5α in all conditions used, but loss of CpxA showed decreased gene expression of *rpoE-rseABC* in conditions of CpxAR induction compared to wild type DH5α. It is possible that a deficiency in CpxA may not be as severe as a deficiency CpxR in altering the expression of CpxAR-regulated genes. This is not surprising as the large number of two-component systems encoded in the *E. coli* genome may lead to redundancy in signal transduction systems (18). It is possible that there is an alternate method for the activation of CpxR in the absence of CpxA.

To determine whether or not the TPEN-mediated increase in gene expression involves CpxP, a loss of function experiment was performed in an *E. coli cpxP* deletion mutant. We expected that mutant strains deficient in CpxP would demonstrate no significant difference in gene expression when growth media zinc concentration is altered. Consistent with our hypothesis, in the absence of CpxP, gene expression of *cpxAR, cpxP, spy* and *rpoE-rseABC* did not change when the concentration of free zinc was altered in the growth media (Fig 4). Taken together, these data support the conclusion that metal ion-mediated repression of the CpxAR system requires CpxP.

Despite widespread acknowledgement of TPEN as a zinc (II)-specific chelator (9, 10, 11, 12, 13), other reports have shown that TPEN may have an important effect on

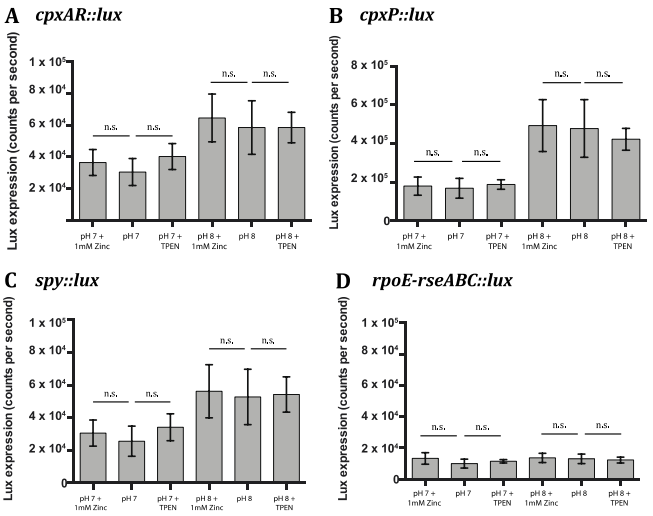

FIG 4 Gene expression of CpxAR-regulated genes was not altered when zinc ions were chelated using 10 µM TPEN, as determined using promoter-fused plasmid *lux* reporter strains in *ΔcpxP* in response to altering concentration of zinc chloride at pH 7 and pH 8. Photon emission was normalized to culture density. Data is shown as the mean of 3 replicates and error bars are expressed as standard deviation of the mean. Asterisks denote significant differences as determined by Mann-Whitney U test ($p<0.1$). **(A)** Expression of *cpxAR::lux* in *E. coli ΔcpxP*. **(B)** Expression of *cpxP::lux* in *E. coli ΔcpxP*. **(C)** Expression of *spy::lux* in *E. coli ΔcpxP*. **(D)** Expression of *rpoE-rseABC::lux* in *E. coli ΔcpxP*.

other metal ions (14). It is known to form tight bounds with cadmium (II), cobalt (II), nickel (II) and copper (II) as well as forming stable complexes with iron (II) (14). Thus, it is possible that other divalent metal cations are influencing the interaction between CpxP and CpxA. Our data strongly suggest that zinc plays an important role in the CpxP-mediated negative regulation of the CpxAR system, however, we cannot conclusively rule out the possibility that other divalent metal cations with similar chemical properties to zinc (such as copper) may also play a role in CpxP-mediated regulation of the CpxAR system. Also, it is possible that DMSO used to dissolve TPEN may have some effects on transcription, however TPEN is well known to act on the transcription of many genes (14), and thus likely the major contributor to changes in gene expression. Notably, the crystal structure of CpxP was solved in the presence of high concentrations of zinc acetate (6). Therefore, the structure of CpxP and the presence of zinc binding domains should be regarded with some caution. It is also possible that TPEN may chelate divalent metal ions from periplasmic proteins, causing misfolding, which may indirectly induce the envelope stress response, due to the nature of CpxP as a chaperone protein (20). This alternate model may propose an indirect role for the induction of the CpxAR system by TPEN, in a mechanism that does not directly involve zinc. The exact mechanism, by which TPEN induces transcription of *cpxP*, *cpxAR*, and *spy*, remains to be elucidated.

Gram-negative bacteria commonly use two-component systems to sense their environmental conditions and respond appropriately; a mechanism for which *E. coli* can adapt to low metal ion concentrations would increase fitness in dynamic environments. Our results suggest that zinc may inhibit the induction of the CpxAR envelope stress response system through interactions with CpxP. We propose a model to explain how our results fit into the CpxAR system (Fig 5). Alkaline pH induces the CpxAR system resulting in phosphorylation of CpxA, which causes CpxR in turn to be phosphorylated, which can promote or repress the transcription of specific genes (Fig 5) (1). We suggest that CpxP mediates repression of CpxA in the presence of zinc ions (Fig 5). Insights into the CpxP structure have suggested that an arginine and an aspartate residue, R60 and D61 respectively, may be involved in interactions with CpxA (6). Divalent cations such as zinc may play a direct role in mediating the interaction between CpxP and CpxA at these residues, or rather an indirect role, causing a conformational change in CpxP, increasing the affinity between these two proteins. *In vitro*

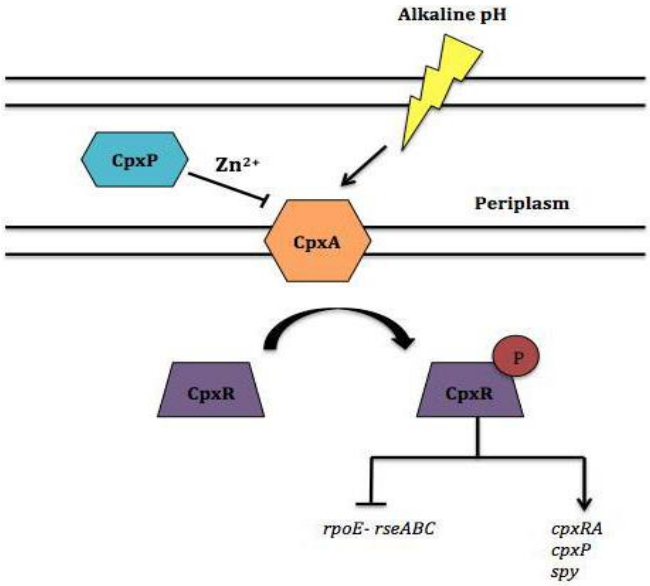

FIG 5 Proposed model for zinc mediating the CpxP-mediated negative regulation of the CpxAR system. Alkaline pH is a known inducer of the CpxAR system that causes autophosphorylation of CpxA, allowing the transfer of the phosphate group to CpxR, activating its transcriptional activity and acting on downstream genes. CpxP is a known negative regulator of CpxA and thus we propose that zinc ions mediate the interaction between CpxP and CpxA and are necessary for the negative regulation of the CpxAR system.

biochemical assessment of the role of zinc on the activity of CpxP will allow us to understand whether this interaction is direct or indirect. The structure of CpxP suggests that there may be several zinc-binding sites (6). However, the mechanism by which zinc may mediate the inhibition of CpxA remains unclear, rendering it an important area for future investigation.

CpxP is a known negative regulator of the CpxAR two-component envelope system, however, the mechanism by which this interaction is regulated has yet to be characterized. Here we provide evidence that supports zinc ion concentration as a regulator for this system. Our results demonstrate that TPEN, a zinc chelator, in the *E. coli* strain DH5α causes an increase in expression of CpxAR positively regulated genes, demonstrating that zinc ion concentration may regulate the CpxAR two-component system. Moreover, TPEN does not affect expression of CpxAR positively regulated genes when CpxP is removed from the system, suggesting that the negative regulation of the CpxAR system mediated by metal cofactors, specifically zinc, requires CpxP. We further showed that Δ*cpxA* and Δ*cpxR* failed to respond to changes in environmental conditions, when adding TPEN or adjusting pH, suggesting these proteins play a direct role in regulation of the CpxAR system. We present a model proposing that CpxP requires zinc ions to inhibit the CpxAR system. Our results support the existence of a role for environmental metal ions in the regulation of two-component systems.

FUTURE DIRECTIONS

This study provided preliminary evidence showing that zinc may play an important role in the inhibition of the CpxAR system through CpxP. In this study, zinc chelation by TPEN was not confirmed. To do this, a fluorometric zinc ion quantification kit must be used to confirm that zinc is in fact being depleted from the media. Furthermore, complementation of zinc concentration by supplementing exogenous zinc in the presence of TPEN would be sufficient to determine zinc specificity. A DMSO vehicle control would also be useful to ensure there was no undesirable transcriptional activity of DMSO on the CpxAR-regulated genes. Additionally, this study was performed using *E. coli* strain DH5α as the wild type control. These experiments should be repeated using the *E. coli* wild type strain BW25113, as the deletion mutants Δ*cpxP*, Δ*cpxA*, and Δ*cpxR* were constructed from this background. As both DH5α and BW25113 are *E. coli* K12 derivate strains that have a functional CpxAR system, we expect the results to be comparable. It may also be insightful to perform both the *lux* reporter gene expression quantification at varying concentrations of zinc ions for Δ*cpxP*, Δ*cpxR*, Δ*cpxA* and *E. coli* BW25113 at pH 6, where the system is not being induced, to assess whether zinc allows negative regulation of the CpxAR system, or zinc ions induce expression of the CpxAR system. Based on our model, the CpxAR system should not respond to zinc at pH 6, since the CpxAR system is not induced and consequently there should be no CpxP-mediated repression (21).

As CpxP shares structural similarities with other periplasmic metal ion binding proteins (1), such as those that bind nickel, iron, or cobalt, it would be of interest to determine whether or not these metal ions have similar effects on the CpxAR system. Repeating experimental conditions using the *lux* reporter strains in DH5α and Δ*cpxP* with differential concentrations of other metal cations using chelators specific to each ion would be a feasible. This experiment would give insight into the extent at which CpxP is involved in metal ion sensing, whether it is limited to zinc or whether it can respond to a broad range of metal ions. Based on our results, we would expect that zinc would be the major metal ion to have a role in the CpxP-mediated negative regulation of CpxAR. If more than one metal ion was capable of interacting with CpxP to the same extent as zinc, we would likely not have seen as significant of a reduction in CpxAR negative regulation in the presence of TPEN, due to redundant function of other metal ions. Lastly, it will be important to assess the role of zinc directly on the activity of CpxP *in vitro* using biochemical approaches. This will help elucidate whether zinc has a direct or indirect role on the repression of the CpxAR system in order to support or refute our model.

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