Cd(II), Pb(II) and Zn(II) ions regulate expression of the metal-transporting P-type ATPase ZntA in *Escherichia coli*

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Abstract ZntA is a cation-translocating ATPase which exports from *Escherichia coli* Cd(II) and Pb(II), as well as Zn(II). The metal-dependent ATP hydrolysis activity of purified ZntA was recently characterised and showed a specificity for Cd(II), Pb(II) and Zn(II). *zntA* expression has been reported to be up-regulated primarily by Zn(II), mediated by the regulatory protein ZntR, belonging to the MerR transcriptional regulator family. In contrast to previous claims, we now show, using a Φ(*zntA-lacZ*) monolysogen, that Cd(II) is the most effective inducer of *zntA*, which is also induced significantly by Pb(II). The Cd(II)- and Pb(II)-dependent transcriptional up-regulation of *zntA* is also mediated by ZntR.

Key words: Zn(II); Cd(II); Pb(II); P-type ATPase ZntA; Transcriptional regulator ZntR; *Escherichia coli*

1. Introduction

Zn(II) is a key structural component of a large number of proteins in all living systems and plays an important catalytic role in numerous enzymes [1,2]. However, above critical concentrations, Zn(II) is toxic [3] and so its concentration must be carefully controlled by excluding excess Zn(II) from the cell or by Zn(II) sequestration. In prokaryotes, homeostasis is maintained mainly by regulating the uptake and efflux of Zn(II).

In *Escherichia coli*, an ABC-type transporter, ZnuABC, is responsible for high affinity Zn(II) uptake when external Zn(II) concentrations are low. Under conditions of Zn(II) sufficiency, expression of the pump is repressed by Zur, a Fur homologue [4]. Zn(II) was also demonstrated to be transported with a low affinity by PitA, an inorganic phosphate transport system [5]. When internal Zn(II) concentrations are high, a Zn(II) export system ZntA is switched on. ZntA is a cation-translocating ATPase which exports Cd(II) and Pb(II) as well as Zn(II) [6–8]. The metal-dependent ATP hydrolysis activity of purified ZntA was recently characterised and showed a specific activity for Pb(II), Cd(II) and Zn(II) [9].

zntA expression is regulated by ZntR, a transcriptional regulator belonging to the MerR family, which activates *zntA* expression in the presence of Zn(II) [10]. Surprisingly, despite the established role of ZntA in transporting Pb(II) and Cd(II), these metal ions gave only slight induction of a plasmid-borne *zntA* fusion when added to growing broth cultures [10]. A very recent paper demonstrated that ZntR activates the transcription of *zntA* by a DNA distortion mechanism similar to MerR regulation of Hg(II) resistance genes [11]. In general, expression of metal transporters is induced by the metal transported itself; one of many examples is the activation of Hg(II) resistance by Hg(II) [12].

The purpose of this work was to re-examine the regulation of *zntA* using a monolysogen, thereby avoiding complications of gene dosage effects associated with multicopy vectors. In this paper, we demonstrate that *zntA* is maximally induced by Cd(II), not Zn(II), as well as by Pb(II), and that the Cd(II)- and Pb(II)-dependent transcriptional regulation of *zntA* is also mediated by ZntR. Thus, Cd(II) and Pb(II), metals transported by ZntA, are indeed inducers of this export system.

2. Materials and methods

2.1. Strains, plasmids, phage and growth conditions

E. coli K-12 strains, plasmids and λ specialised transducing bacteriophage used in this study are described in Table 1. Methods for genetic crosses, P1-mediated transduction, restriction endonuclease digestion and ligation of DNA, plasmid DNA isolation and transformation of bacteria were described by Poole et al. [15]. Cells were grown in LB broth or MOPS-buffered defined medium, initial pH 7.0 [15]. Kanamycin, chloramphenicol and ampicillin were used in media at final concentrations of 50, 30 and 150 μg/ml, respectively.

2.2. Culture conditions

Cultures (10 ml) in LB in the presence of different metals were inoculated with 2% of their volume with overnight precultures and shaken at 200 rpm at 37°C in 250 ml conical flasks with matched glass tubes of Klett dimensions as a side arm. Turbidity was measured with a Klett colourimeter (Manostat Corporation) fitted with a red filter. After growth to the mid-exponential phase (about 60–70 Klett units or equivalent OD_{600}), cells were harvested at $5000 \times g$ for 10 min at 4°C. The following high purity metal salts were used: $ZnSO_4$ (Sigma, 101% pure), $CdCl_2$ (Fluka, 98%), $Pb(CH_3COO)_2$ (Sigma, 99%), $HgCl_2$ (Sigma ACS reagent, 100%), $NiSO_4$ (BDH AnalaR, 99%), $CuSO_4$ (BDH AnalaR, 99.5%), $CoCl_2$ (BDH AnalaR, 99%).

2.3. β-Galactosidase assay

Assays were carried out at room temperature as described before [15]. Cell pellets were suspended in 5 ml of buffer and stored on ice. β-Galactosidase activity was measured in $CHCl_3$- and sodium dodecyl sulfate-permeabilised cells by monitoring the hydrolysis of *o*-nitroparaphenyl-β-D-galactopyranoside. Activities are expressed in terms of the OD_{600} of cell suspensions using the formula of Miller [15]. Each culture was assayed in triplicate; results were confirmed in at least two independent experiments.

2.4. Construction of a zntA operon fusion

Fusions of *zntA* to *lacZ* were constructed on plasmids and then transferred to λ phage by recombination in vivo using the method of Simons et al. [14]. A PCR using RP107 (5′-GCTGTTACTGGCCTGGCAAGCGCCG-3′) and RP108 (5′-GTCCATGCCGCTGACTTTCCAG-3′) as primers with *Eco*RI/*Bam*HI sites was performed. The derivative of pRS415 carrying the *zntA* promoter was

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created by digesting the PCR product and ligating with pRS415 cut with *Eco*RI/*Bam*HI.

The required recombinant plasmid was isolated by transformation of strain RK4353 (Δ*lac*) [15]. The fusion was recombined onto λRS45 to give λMB1. Several single-copy fusions to the chromosome of VJS676 (Δ*lac*) were isolated and verified using β-galactosidase assays and Ter tests as described before [15]. One such fusion strain (RKP2910) was used. The mutant allele *zntR* was transduced from TG1 *zntR::kan* [10] to give strain RKP2997 and the mutant allele *zntA* was transduced from SJB101 [6] to give strain RKP2926.

3. Results

3.1. Zn(II), Cd(II), Pb(II) and other metal ions up-regulate zntA expression

A chromosomal operon fusion Φ(*zntA-lacZ*) was used to monitor expression of the *zntA* promoter in response to a range of metal ions. Strain RKP2910 carries a single-copy operon fusion comprising 200 bp of the coding region of *zntA* and the promoter region 558 bp upstream of the translational start site. Expression of the fusion in LB medium was 13-fold higher with 0.5 mM Zn(II) than without Zn(II) (Fig. 1).

ZntA confers Cd(II) resistance and exports Cd(II); we therefore tested whether Cd(II) increases *zntA* expression. The minimal inhibitory concentration of Cd(II) in LB medium for this strain is 0.5–0.75 mM (results not shown). At these concentrations, the growth was very slow and thus the concentration of Cd(II) chosen for expression studies was 0.1 mM. Even at this concentration, a 44-fold induction of the fusion was observed, considerably exceeding the up-regulation by 0.5 mM Zn(II) (Fig. 1). When the mutation *zntA* was introduced into the fusion Φ(*zntA-lacZ*), the basal level of induction increased dramatically by 7-fold (compared to the fusion in a wild-type background) in the absence of added metals in the culture. In the presence of 0.5 mM Zn(II) or 0.1 mM Cd(II), the induction of the fusion in the *zntA* mutant strain was similar to that in the wild-type (Fig. 1).

To determine the effect of medium composition on *zntA* induction by metals, MOPS minimal medium was used. In such defined media, the metal concentrations tolerated were much lower than in LB medium. However, the same pattern of induction was observed: 3-fold induction of the fusion was obtained with 10 μM Zn(II) and 13-fold induction of the fusion was obtained with 10 μM Cd(II).

Other metal ions were also tested on the fusion, namely Pb(II), Co(II), Cu(II) and Ni(II), each at 0.5 mM, and Hg(II) at 5 μM; greatest activation of the promoter could be seen with Hg(II) at 5 μM giving 18.7-fold induction (Table 2). Ni(II) increased expression (up to 4-fold induction) but only at higher concentrations (2 mM, not shown).

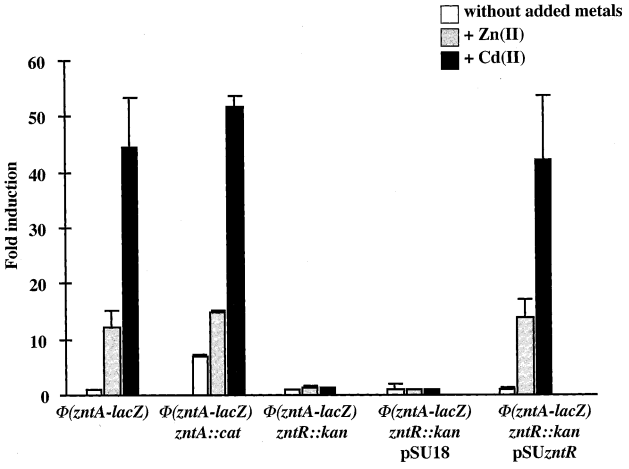

Fig. 1. Effects of Zn(II) and Cd(II) and *zntA* and *zntR* mutations on Φ(*zntA-lacZ*) expression. Zn(II) and Cd(II) were used at 0.5 mM and 0.1 mM, respectively. The values are the mean (column) and S.E.M. (vertical bars) of at least triplicate data sets. Induction of the fusion, measured as Miller units of β-galactosidase activity, is expressed relative to activity of the fusion in the wild-type background without metals.

Pb(II) gave a 3.8-fold induction of the promoter at 0.5 mM. We focused more on the Pb(II) induction rather than Hg(II) induction as Pb(II), but not Hg(II), is transported by ZntA.

3.2. Regulation of zntA by Zn(II), Cd(II) and Pb(II) is mediated by ZntR

Zn(II) activation of the *zntA* promoter has already been shown to be mediated by ZntR [10]. In a *zntR* mutant, the Zn(II) induction of the *zntA* promoter was not observed, but the Zn(II) induction was restored by complementation with $zntR^+$ in *trans*. ZntR has been demonstrated to bind DNA specifically at the *zntA* promoter region [10] and activate transcription by a DNA distortion mechanism [11].

To determine whether ZntR is also a Cd(II)- and Pb(II)-responsive regulator of *zntA* expression, we introduced the mutation *zntR::kan* into the strain carrying the operon fusion Φ(*zntA-lacZ*) and looked at the induction of β-galactosidase activity in the presence of Cd(II) and Pb(II) (Figs. 1 and 2).

Table 1
Strains, plasmids and phage used

Strains, plasmids, phage	Relevant genotype/properties	Reference or source
E. coli strains		
RK4353	*araD139 Δ(argF-lac)U169 gyr A219 non-9 rpsL150*	[13]
RKP2910	Same as VJS676 but Φ(*zntA-lacZ)1*	This work
RKP2926	Same as RKP2910 but *zntA::cat*	This work
RKP2997	Same as RKP2910 but *zntR::kan*	This work
SJB101	*zntA::cat*	[6]
VJS676	*Δ(argF-lac)U169*	V.J. Stewart
Plasmids		
pRS415	Ap^R *bla-Tl4-lacZ′* operon fusion vector	[14]
pSJB07	Ap^R, $zntA^+$	[6]
pSU*zntR*	Cm^R, $zntR^+$	[10]
Phage		
λMB1	Φ(*zntA-lacZ)1* (gene fusion)	This work
λRS45	*bla′-lacZsc* att^+ int^+ imm^{21}	[14]

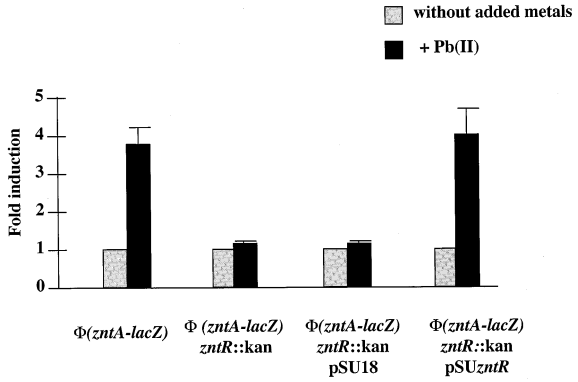

Fig. 2. Effect of Pb(II) and a *zntR* mutation on Φ(*zntA-lacZ*) expression. Pb(II) was used at 0.1 mM. Other details are as in Fig. 1.

As expected, the mutant showed an increased sensitivity to Zn(II), Cd(II) and Pb(II) in liquid cultures (results not shown); thus the metal concentrations were adjusted to allow good growth in the presence of these metals. The concentrations used were 0.5 mM Zn(II), 0.1 mM Cd(II) and 0.1 mM Pb(II).

As shown in Figs. 1 and 2, no transcriptional activation of the *zntA* promoter could be observed in the presence of Zn(II), Cd(II) or Pb(II) in the *zntR* mutant strain carrying the Φ(*zntA-lacZ*) fusion. When the plasmid vector pSU18 was introduced in this strain as a control, no transcriptional activation could be seen, whereas the introduction of pSU*zntR* (carrying *zntR*$^+$) re-established the Zn(II)-, Cd(II)- and Pb(II)-dependent induction of the *zntA* promoter.

4. Discussion

We have demonstrated that Cd(II) and Pb(II), substrates of the *zntA*-encoded exporter, are also inducers of the expression of this system. Indeed, we showed that Cd(II) was a much more effective inducer of *zntA* expression than was Zn(II), previously considered as the major inducer of the system. Induction by both Cd(II) and Pb(II) is mediated by ZntR, as for Zn(II), and thus the metal selectivity of ZntR is not restricted to Zn(II).

Evidence that ZntA is functional in metal ion export even in media that have not been supplemented with extra metal comes from the observation that a *zntA* mutant exhibits a higher basal level of *zntA* expression than does a wild-type strain. One explanation of this result is that, in the absence of the ZntA transporter, accumulated metal (probably Zn(II)) is sufficient to up-regulate *zntA* expression, though not to the extent elicited by added 0.5 mM Zn(II) (Fig. 1).

Table 2
Expression of Φ(*zntA-lacZ*) in the presence of different metals

Metal added	Concentration (mM)	β-Galactosidase specific activity (Miller units) (fold induction ± S.D.)
–	–	1
Hg(II)	0.005	18.7 ± 2.0
Pb(II)	0.5	3.80 ± 0.41
Ni(II)	0.5	1.12 ± 0.04
Co(II)	0.5	1.40 ± 0.03
Cu(II)	0.5	0.57 ± 0.04

The striking difference in results obtained with Cd(II) in the present work compared with an earlier study [10] is probably due to the nature of the fusion construct. Brocklehurst et al. [10] used a pUC-based reporter plasmid carrying the *zntA* promoter, whereas in the present work, the *zntA-lacZ* fusion was present on the chromosome as a single-copy lysogen. Multiple copies of the promoter may titrate transcriptional regulators, rendering the fusion relatively insensitive to changes in the population of active regulator. Alternatively, variability in plasmid copy number and other factors may also pose difficulties [13]. Both studies used rich broth media in which metal chelation by medium components [16] is likely to be similar, and changing to MOPS minimal media still revealed Cd(II) to be the more effective inducer. It should be pointed out that reporter enzyme assays in the present work were performed on cultures grown throughout with the inducing metal, whereas Brocklehurst et al. [10] challenged growing cultures with metal ions and measured reporter luminescence after 2 h treatment.

The interaction of Zn(II)-ZntR with the *zntA* promoter region was characterised recently [11] but the interaction of ZntR with Pb(II) and Cd(II) remains to be explored.

There are three cysteine residues in ZntR at positions corresponding to those responsible for binding Hg(II) in MerR [10] and which may be responsible for the cross-induction of the *zntA* promoter by Hg(II). MerR is specific for Hg(II) and does not bind Zn(II) or Cd(II) [17]. MerR specificity could be extended to Cd(II) by single mutations [18], but none of these mutations occurred at the three cysteines comprising the Hg(II) metal binding sites of MerR. These Cd(II)-responsive MerR mutants did not respond at all to Zn(II). Thus, in MerR, the sites of metal recognition lie not only immediately adjacent to the known metal ligand thiols but throughout the core metal binding domain and the putative coupling region between it and the DNA binding domain [18].

The metal binding sites in ZntR are yet to be discovered. We can hypothesise that cysteine and/or histidine residues may be responsible for binding Zn(II), Cd(II) and Pb(II) and that other residues confer specificity for these metals. Identification of the metal binding sites on ZntR may lead to understanding the basis of metal recognition and signal transduction and perhaps engineering of the protein to modify metal specificity.

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