Pb(II)-translocating P-type ATPases*

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The cad operon of Staphylococcus aureus plasmid pI258, which confers cadmium resistance, encodes a transcriptional regulator, CadC, and CadA, an ATP-coupled Cd(II) pump that is a member of the superfamily of cation-translocating P-type ATPases. The Escherichia coli homologue of CadA, termed ZntA, is a Zn(II)/Cd(II) pump. The results described in this paper support the hypothesis that ZntA and CadA are Pb(II) pumps. First, CadC is a metal-responsive repressor that responds to soft metals in the order Pb > Cd > Zn. Second, both CadA and ZntA confer resistance to Pb(II). Third, transport of 65Zn(II) in everted membrane vesicles of E. coli catalyzed by either of these two P-type ATPase superfamily members is inhibited by Pb(II).

Exposure to environmental sources of lead is a serious public health concern. In humans chronic lead exposure produces neurotoxicity, anemia, and kidney damage, and acute lead toxicity can be fatal. Neither the specific lead transporters nor the regulatory elements that control the expression of the transporter genes have been identified. As models for human metal toxicity, we have been characterizing transporters for toxic metals and their genetic regulation (1, 2) and report here the identification of two P-type ATPases that are responsible for Pb(II) extrusion and resistance in bacteria.

Bacterial metal ion resistance probably arose early in evolution due to widespread geological occurrence of metals. Bacterial cells have chromosomally and plasmid-encoded mechanisms for extrusion of antimicrobial substances, including toxic soft metals (3). While the ionic forms of some of these metals such as zinc and copper are essential for all organisms, all of these ions are toxic in excess. ZntA from Escherichia coli and CadA from plasmid pI258 of Staphylococcus aureus are both members of the superfamily of P-type cation-translocating ATPases but belong to a subgroup of soft metal transporters that includes CopA, a Cu(I) pump from Enterococcus hirae, and eukaryotic Cu(I) homoeostasis proteins such as the Menkes and Wilson disease-associated proteins (1, 4, 5). ZntA has been shown to catalyze ATP-dependent transport of Zn(II) and Cd(II) (6), and CadA has been shown to transport Cd(II) (7). Both have been shown to confer resistance to cadmium and zinc ions (8–10). The pI258 cadCA operon is regulated at the transcriptional level by the product of the cadC gene, which encodes the 122-residue CadC repressor (11–13).

In this report, we show that CadC repression of the cad promoter is relieved upon addition of soft metals, with the order of effectiveness Pb(II) > Cd(II) > Zn(II). In E. coli Zn(II) responsiveness could be observed only in a zntA-disrupted strain. The zntA-disrupted strain of E. coli exhibited hypersensitivity to Pb(II) that was complemented by cadA, indicating that both soft metal-translocating P-type ATPases are essential for Pb(II) resistance in bacteria. Everted membrane vesicles from cells expressing either zntA or cadA exhibited ATP-dependent 65Zn(II) accumulation. Since no radioisotopes of Pb(II) are available, direct transport of Pb(II) was not assayed. However, Pb(II) inhibited 65Zn(II) transport, indicating that Pb(II) is a substrate of the two P-type ATPases. These results support the concept that ZntA and CadA are Pb(II) pumps with physiological functions that include to provide resistance to environmental lead.

EXPERIMENTAL PROCEDURES

Growth of Cells—The bacterial strains and plasmids used in this study are listed in Table I. Cells were grown in LB medium at 37 °C. Ampicillin (50 μg/ml), kanamycin (50 μg/ml), chloramphenicol (80 μg/ml), isopropyl-β-D-thiogalactopyranoside (0.1 mM) and 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (80 μg/ml) were added as required. For determination of sensitivity to metal ions, a basal salts medium was used (14) with the omission of zinc salts. The pH of the medium was adjusted to 5.5 to prevent precipitation of lead salts. Cells were grown overnight, diluted 50-fold in the same medium containing metal ion salts, and incubated for 24 h at 37 °C with shaking. Growth was monitored from the absorbance at 600 nm.

DNA Manipulation—Preparation of plasmid DNA was performed using a Wizard DNA purification kit (Promega). Endonuclease digests, electrophoretic separations and isolations, ligations, transformations, and Klenow fragment fill in were performed according to standard procedures (15) unless otherwise noted. The conditions for polymerase chain reaction (PCR)† were as described previously (16). Restriction endonucleases, T4 DNA ligase, Klenow fragment of DNA polymerase I, and Taq polymerase were from Life Technologies, Inc. For DNA sequencing, double-stranded DNA was isolated with a plasmid minikit from Qagen and then sequenced by the method of Sanger et al. (17) using an ALFexpress system and a Cy5-labeled sequence kit (Pharmacia Biotech Inc.).

In Vivo Measurement of Inducer Specificity of cadC—The lacZ reporter gene plasmid pYS2 was constructed to monitor the regulatory properties of the cadC gene product. A 121-base pair fragment from plasmid pYPK1 containing the pI258 cad operator/promoter was amplified by PCR. The fragment was engineered with an EcoRI at the 5′ end and a BamHI site at the 3′ end. The fragment was ligated into plasmid pMLB1034 that had been digested with EcoRI and BamHI, generating plasmid pYS2, in which a lacZ gene is controlled by the cad operator/promoter. In several steps the pI258 cadC gene was amplified by PCR from plasmid pYPK1 and cloned as a 0.5-kilobase pair fragment into plasmid PACYC184 under control of the T7 promoter.

Overnight LB + 2% glucose cultures of E. coli strains BL21(DE3) or BL21(DE3) zntA::km harboring compatible plasmids pYS2 and pYS1 were diluted 20-fold into a low phosphate minimal medium (14) containing 2% glucose plus the appropriate antibiotics. Pb(OAc)2, ZnSO4, Cd(OAc)2, HgCl2, NaAsO2, Bi(NO3)3, CuSO4, NiCl2, or potassium anti-

† The abbreviations used are: PCR, polymerase chain reaction; Bis-Tris, 2-[bis(2-hydroxyethyl)amin]-2-(hydroxyethyl)-propane-1,3-diol.

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mixture contained 50 mM HEPES-KOH, pH 7.5, 0.2 M KCl, 2.5 mM MgSO4.

Assayed for under control of the T7 promoter, were cadC promoter, and pYSC1, in which lacZ was under control of the cadC promoter, and pYS2, in which constitutively express lacZ. Cells were induced at 37 °C for 5 h.

Cells of E. coli strain BL21(DE3) (open symbols) or BL201(DE3) (closed symbols) bearing both plasmid pYS2, in which lacZ was under control of the cad promoter, and pYSC1, in which cadC was under control of the T7 promoter, were assayed for β-galactosidase activity as described under “Experimental Procedures.” Values were normalized to the activity of cells with only plasmid pYS2, which constitutively express lacZ. Cells were induced with the indicated concentrations of metals: Pb(OAc)2, Cd(OAc)2, CdCl2, PbCl2, ZnSO4, or ZnCl2.

**Assay of 65Zn(II) Accumulation—**Everted membrane vesicles were prepared as described (19). Cells were grown overnight at 37 °C in 20 ml of LB and diluted 50-fold in prewarmed medium. At an optical density of 0.5 at 600 nm the cultures were induced with 0.1 mM ZnSO4 for 1 h with or without 5 mM Na2ATP in a final volume of 1 ml. The pH of the reaction mixture was adjusted to 6.0, and no precipitation of either Pb(II) or Zn(II) salts was observed under the conditions of this assay. The reaction was initiated by addition of 5 mM Pb(II) or Zn(II) and the complete cadA gene from S. aureus in pET11a and the complete cadA gene from S. aureus in pET11a was under control of the cad promoter in vector plasmid pACYC184 in which the Tc resistance was disrupted, Cm

**RESULTS**

*CadC Responds to Pb(II), Cd(II), and Zn(II) in Vivo—**In cells of *S. aureus* CadC represses transcription from the pI258 cad operator/promoter in the absence of metals (21). The response to metal ions has been examined in both *S. aureus* and *E. coli* (12). However, those results are complicated by the presence of chromosomally encoded efflux pumps in *S. aureus* and *E. coli* (6, 9) that limit the ability of the cells to accumulate intracellular metal ions.

We have developed a method to probe the ability of metals to release CadC from the operator/promoter in vivo. The method relies on the construction of a strain of *E. coli* in which zntA has been disrupted (6), with introduction of a two-plasmid reporter

![Graph](http://www.jbc.org/)

**TABLE I**

| Strains and plasmids | Genotype | Ref. |
|----------------------|----------|-----|
| **E. coli strains**  |          |     |
| W3110                | K12 F' IN(rrnD-rrnE) | 29  |
| RW3110               | zntA::km  | 6   |
| BL21(DE3)            | hsdS gal (λctts857 ind1 Sam7 nin5 lacUV5-T7 gene1) | Novagen |
| BL21(DE3) zntA::km   | P1 transduction of zntA::km from RW3110 to BL21(DE3) | This study |
| Plasmids             |          |     |
| pCGR2                | zntA gene from *E. coli* in pGEM-T (Promega) | 6   |
| pYPK11               | 3.0-kilobase pair XbaI fragment containing the cadCA operon | 11  |
| pKJ3                 | 2.6-kilobase pair XbaI fragment containing the T7 promoter | K. J. Tsai |
| pYSC1                | CadC gene under control of the T7 promoter in vector plasmid pACYC184 | This study |
| pYS2                 | 121-basepair bp EcoRI/BamHI fragment containing the cad o/p cloned into vector pMLB1034, Ap' | This study |

![Graph](http://www.jbc.org/)

**Fig. 1. In vivo regulation by cadC.** Cells of *E. coli* strain BL21(DE3) (open symbols) or BL201(DE3) (closed symbols) bearing both plasmid pYS2, in which lacZ was under control of the cad promoter, and pYSC1, in which cadC was under control of the T7 promoter, were assayed for β-galactosidase activity as described under “Experimental Procedures.” Values were normalized to the activity of cells with only plasmid pYS2, which constitutively express lacZ. Cells were induced at 37 °C for 10 min. The β-galactosidase assay reaction mixture contained 50 μM of permeabilized cells, 0.1 μl of 8 mg/ml o-nitrophenyl-β-D-galactopyranoside and 0.85 ml of Z buffer.

**Assay of 65Zn(II) Accumulation—**Everted membrane vesicles were prepared as described (19). Cells were grown overnight at 37 °C in 20 ml of LB and diluted 50-fold in prewarmed medium. At an optical density of 0.5 at 600 nm the cultures were induced with 0.1 mM ZnSO4 for 1 h and then with 0.5 mM ZnSO4 for 2 h. For induction of cadA 0.1 mM Cd(OAc)2 was used in place of ZnSO4. The cells were harvested and washed twice in 10 mM HEPES-KOH, pH 7.5, 0.2 M KCl, 2.5 mM MgSO4, followed by vigorous mixing by vortexing for 1 min (18). The cells were incubated at 37 °C for 10 min. The β-galactosidase assay reaction mixture contained 50 μl of permeabilized cells, 0.1 μl of 8 mg/ml o-nitrophenyl-β-D-galactopyranoside and 0.85 ml of Z buffer. β-Galactosidase activity was estimated from the release of nitrophenol at 420 nm at 600 nm the cultures were induced with 0.1 mM ZnSO4 for 1 h. Protein content was estimated by a modified procedure of Lowry et al. (20).
gene system. The cadC gene was cloned behind the T7 promoter in one plasmid, termed pYSC1, to allow expression in a strain harboring the gene for the T7 polymerase. A second plasmid, pYS2, was constructed that had the lacZ gene cloned behind the cad-operator/promoter. Expression of cadC in E. coli strain BL21(DE3) resulted in almost complete transcriptional repression of the lacZ gene (Fig. 1). Since this strain has a chromosomal zntA gene, expression was insensitive to 0.1 mM ZnSO4. In contrast, when a zntA disruption was transduced into E. coli strain BL21(DE3), which was subsequently transformed with plasmids pYSC1 and pYS2, repression could be relieved by low concentrations of Zn(II), with maximal induction at 0.8 mM ZnSO4 (Fig. 1), an increase in metal responsiveness of about 3 orders of magnitude. This result demonstrates that the inability to observe derepression in wild type cells of E. coli is the result of ZntA activity.

Repression by cadC in the zntA disrupted strain was relieved most effectively by Pb(II), with significant derepression at 25 mM Pb(OAc)2 and complete response by 200 mM. In cells of the wild type, 3 mM Pb(OAc)2 was required to give the same response, a 15-fold increase. Derepression by Cd(II) was intermediate, with maximal induction at 300 nM. Interestingly, the wild type response to Cd(II) was only shifted by a factor of two relative to the zntA disrupted strain. It is not clear why there is so little difference between wild type and mutant with this metal ion. Other metal ions, including Hg(II), Cu(II), Ni(II), As(III), Sb(III), and Bi(III), showed little or no induction (data not shown).

The zntA and cadA Genes Confer Pb(II) Resistance—Disruption of zntA rendered the cells hypersensitive to Pb(II) (Fig. 2). The wild type could grow in concentrations as high as 200 μM (data not shown). In contrast, E. coli strain RW3110 (znt::km) showed growth inhibition even at 100 nM, and no growth was observed at a concentration of 1 μM. Thus zntA confers Pb(II) resistance. Pb(II) sensitivity was complemented by plasmid pKJ3, which carries a pI258 cadA gene (Fig. 2). Complementation was also observed with plasmid pCGR2 (zntA) (B and C). Transport was assayed with 10 μM 65ZnSO4 with 5 mM MgATP as an energy source. In A and B transport was measured in the absence (closed symbols) or presence (open symbols) of 10 μM Pb(OAc)2. In C the accumulation of 65Zn(II) was measured 2 min after addition of MgSO4 in the presence of the indicated concentrations of Pb(OAc)2 (B) or Cd(OAc)2 (C).

CadA Catalyzes ATP-dependent Transport of 65Zn(II)—Expression of cadA in either B. subtilis or E. coli has been shown to produce cells and everted membrane vesicles capable of transporting 109Cd(II) (7). We have shown previously that cadA can complement the Znt(II)-sensitive phenotype of the zntA-disrupted E. coli strain (10). However, the ability of CadA, which is only 30% identical to ZntA, to catalyze transport of Zn(II) has not been examined. Accumulation of 65Zn(II) was observed in everted membrane vesicles prepared from cells of the zntA-disrupted E. coli strain RW3110 bearing plasmid pPKY11, which has the pI258 cadCA operon (Fig. 3A). In that experiment the cells were induced with 0.2 mM Cd(OAc)2. Ex-
expression of CadA-catalyzed Zn(II) transport could also be induced with Zn(II) (data not shown). In the absence of a source of energy, no time-dependent uptake of $^{65}$Zn(II) was observed. Addition of MgATP produced time-dependent accumulation of $^{65}$Zn(II) in the strain expressing cadA. Thus CadA, like ZntA, is a Zn(II) pump.

$^{65}$Zn Transport by ZntA and CadA Is Inhibited by Pb(II)—The ability of ZntA and CadA to confer resistance to Pb(II) implies that those two cation-transporting P-type ATPases pump Pb(II). Since there are no available lead isotopes, direct measurement of transport was not possible, so the effect of Pb(II) on $^{65}$Zn(II) accumulation in inverted membrane vesicles was examined. At 10 mM $^{65}$ZnSO$_4$, a concentration near the $K_m$ value, 10 mM Pb(OAc)$_2$ inhibited the initial rate of ATP-dependent accumulation of $^{65}$Zn (II) catalyzed by either CadA (Fig. 3A) or ZntA (Fig. 3B). The concentration dependence of inhibition of ZntA transport by Pb(II) and Cd(II) was determined (Fig. 3C). With both cations, half-maximal inhibition was observed in a range of 2–4 μM, indicating $K_i$ values for both Pb(II) and Zn(II) within the same range as the $K_m$ for Zn(II). Since ZntA has been shown to transport both Zn(II) and Cd(II), these results suggest that it also transports Pb(II) and that the affinity of the pump is within the same order of magnitude for each of the three cations.

**DISCUSSION**

In humans chronic exposure to low levels of lead may cause neurological, reproductive, and developmental problems. Lead exposure is especially harmful to children, and nearly one million American children below the age of 5 years have blood lead levels that exceed those considered as elevated by the Centers for Disease Control and Prevention (22). Even though lead affects virtually every organ and tissue in the body, little is known about the routes of lead ion uptake and extrusion. Even less is known about Pb(II)-regulated gene transcription, and there are no genetic markers for lead exposure. We have undertaken a study of Pb(II)-responsive genes and transporters. The CadC repressor and CadAZntA pumps represent the first proteins demonstrated to have a physiological function by orthovanadate, a classical inhibitor of P-type ATPases (5).

Bacterial Pb(II) Pumps

Copper pumps are widely distributed in nature, and genetic diseases such as Menkes and Wilson’s result from mutations in the genes for these pumps. Elucidation of these bacterial model systems may also lead to the development of biomarkers for lead exposure and susceptibility in humans.

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