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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 ^{65}Zn(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) homeostasis 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 ^{65}Zn(II) accumulation. Since no radioisotopes of Pb(II) are available, direct transport of Pb(II) was not assayed. However, Pb(II) inhibited ^{65}Zn(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 PROCUEDURES

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 digestions, 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)[1] 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 Qiagen 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 pYPK11 containing the pI258 *cad* operator/promoter was amplified by PCR. The fragment was engineered with an *Eco*RI at the 5′ end and a *Bam*HI site at the 3′ end. The fragment was ligated into plasmid pMLB1034 that had been digested with *Eco*RI and *Bam*HI, 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 pYPK11 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 pYSC1 were diluted 20-fold into a low phosphate minimal medium (14) containing 2% glucose plus the appropriate antibiotics. $Pb(OAc)_2$, $ZnSO_4$, $Cd(OAc)_2$, $HgCl_2$, $NaAsO_2$, $Bi(NO_3)_2$, $CuSO_4$, $NiCl_2$, or potassium anti-

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

TABLE I
Bacterial strains and plasmids used

Strains and plasmids	Genotype	Ref.
E. coli strains		
W3110	K12 F$^-$ IN(*rrnD-rrnE*)	29
RW3110	W3110 *zntA::km*	6
BL21(DE3)	*hsd*S *gal* (λc*Its857 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 *Xba*I fragment containing the *cadCA* operon in vector plasmid pT7–5 under control of the T7 promoter	11
pKJ3	2.6-kilobase pair *Xba*I fragment containing the 3′ end of *cadC* and the complete *cadA* gene from *S. aureus* in pET11a	K. J. Tsai
pYSC1	*CadC* gene under control of the T7 promoter in vector plasmid pACYC184 in which the Tc resistance was disrupted, Cm^r	This study
pYS2	121-basepair bp *Eco*RI/*Bam*HI fragment containing the *cad o/p* cloned into vector pMLB1034, Ap^r	This study

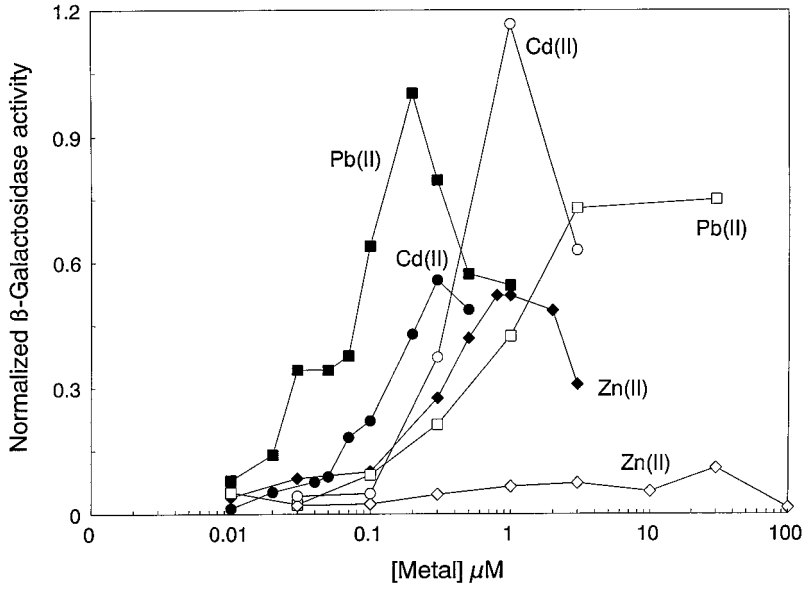

FIG. 1. ***In vivo* regulation by cadC.** Cells of *E. coli* strain BL21(DE3) (*open symbols*) or BL21(DE3) *zntA::km* (*closed symbols*) bearing both plasmid pYS2, in which *lacZ* was under control of the *cad* promoter, and pYSC1, in which *cadC* was under control of the T7 promoter, were assayed for 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: $Pb(OAc)_2$, ■, □; $Cd(OAc)_2$, ●, ○; $ZnSO_4$, ◆, ◇.

monyl tartrate were added at varying concentrations and the cells incubated at 37 °C for 5 h.

For assay of *lacZ* expression, portions (0.5 ml) of cultures were centrifuged at 13,000 × *g* and suspended in 0.5 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, pH 7) and permeabilized by addition of 30 μl of 0.1% SDS and 50 μl of chloroform, 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 μ1 of permeabilized cells, 0.1 ml 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 37 °C and expressed in Miller units.

Assay of $^{65}Zn(II)$ 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 $ZnSO_4$ for 1 h and then with 0.5 mM $ZnSO_4$ for 2 h. For induction of *cadA* 0.1 mM $Cd(OAc)_2$ was used in place of $ZnSO_4$. The cells were harvested and washed twice in 75 mM HEPES-KOH, pH 7.5, 0.2 M KCl, 2.5 mM $MgSO_4$. The cells were suspended 5 ml/g wet cells in a buffer consisting of 10 mM HEPES-KOH, pH 7.5, containing 0.25 M sucrose, 0.2 M KCl, and 0.5 mM EDTA and lysed by a single passage through a French pressure cell at 6,000 p.s.i. The protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (200 μM) was immediately added to the lysate. After addition of 2 mM $MgSO_4$ and DNase I (20 μg/ml), cells were incubated on ice for 30 min. Cell debris was pelleted by centrifugation at 10,000 × *g*, the supernatant was centrifuged at 100,000 × *g* for 60 min to isolate the membranes. The pelleted membrane vesicles were washed once with the same buffer without EDTA and stored at −70 °C until use.

Transport assays were performed at room temperature as described previously (6). The reaction mixture consisted of 20 mM BisTris-propane, 0.2 M KCl, 0.25 M sucrose, 10 μM $^{65}ZnSO_4$ (1.25 μCi), 0.8–1 mg of membrane protein, with or without 5 mM Na_2ATP 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 $MgSO_4$. At intervals 0.1-ml samples were withdrawn, filtered through nitrocellulose filters (0.2 μm pore size, Whatman), and washed with the same buffer containing 10 mM $MgSO_4$ and 20 mM $ZnSO_4$. The filters were dried and the radioactivity quantified in a liquid scintillation counter. The amount of binding in the absence of ATP was subtracted from all values. Membrane vesicles were incubated with inhibitors for 1 min prior to addition of $^{65}ZnSO_4$. Protein content was estimated by a modified procedure of Lowry *et al*. (20)

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*[2] 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

[2] S. G. Chikramane and D. T. Dubin, unpublished GenBank™ accession number L10909.

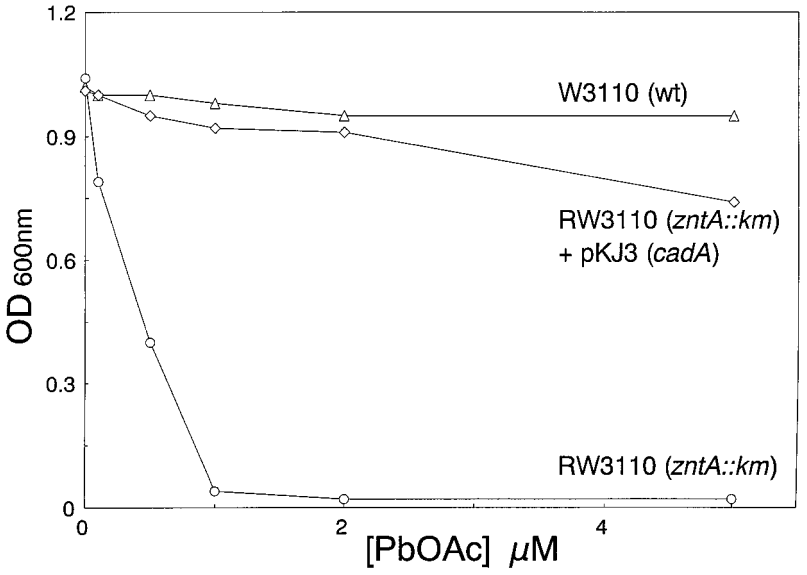

FIG. 2. **ZntA and CadA confer resistance to Pb(II).** Metal ion resistance was assayed in cells of *E. coli* grown in a basal salts medium with the indicated concentrations of $Pb(OAc)_2$ for 24 h at 37 °C with shaking, following which turbidity at 600 nm was measured. Strains: W3110 (wild type), △; RW3110 (*zntA*::*km*), ○; RW3110 pKJ3 (*cadA*), ◇.

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 $ZnSO_4$. 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 μM $ZnSO_4$ (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 nM $Pb(OAc)_2$ and complete response by 200 nM. In cells of the wild type, 3 μM $Pb(OAc)_2$ 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 pKPY11, which carries the entire *cadCA* operon (data not shown). Thus *cadA* also confers Pb(II) resistance.

CadA Catalyzes ATP-dependent Transport of $^{65}Zn(II)$—Expression of *cadA* in either *B. subtilis* or *E. coli* has been shown to produce cells and everted membrane vesicles capable of transporting $^{109}Cd(II)$ (7). We have shown previously that *cadA* can complement the Zn(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 $^{65}Zn(II)$ was observed in everted membrane vesicles prepared from cells of the *zntA*-disrupted *E. coli* strain RW3110 bearing plasmid pKPY11, which has the pI258 *cadCA* operon (Fig. 3*A*). In that experiment the cells were induced with 0.2 mM $Cd(OAc)_2$. Ex-

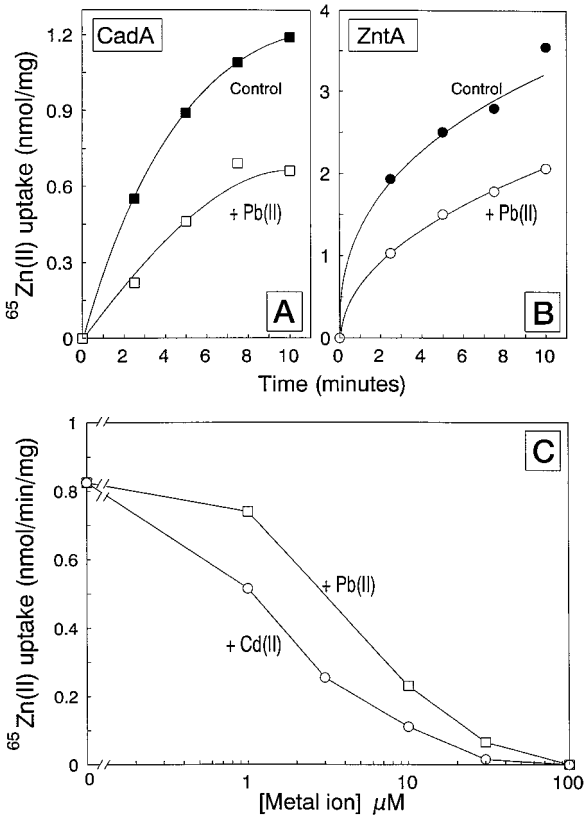

FIG. 3. **Inhibition of ATP-dependent uptake of $^{65}Zn(II)$ in everted membrane vesicles of *E. coli* by lead and cadmium ion.** Vesicles were prepared from cells of strain RW3110 (*zntA*::*km*) bearing plasmid pYPK11 (*cadCA*) (*A*) or plasmid pCGR2 (*zntA*) (*B* and *C*). Transport was assayed with 10 μM $^{65}ZnSO_4$ 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 $^{65}Zn(II)$ was measured 2 min after addition of $MgSO_4$ in the presence of the indicated concentrations of $Pb(OAc)_2$ (□) or $Cd(OAc)_2$ ((○).

pression 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-translocating 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 everted membrane vesicles was examined. At 10 μM $^{65}ZnSO_4$, a concentration near the K_m value, 10 μM $Pb(OAc)_2$ inhibited the initial rate of ATP-dependent accumulation of ^{65}Zn (II) catalyzed by either CadA (Fig. 3*A*) or ZntA (Fig. 3*B*). The concentration dependence of inhibition of ZntA transport by Pb(II) and Cd(II) was determined (Fig. 3*C*). 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 CadA/ZntA pumps represent the first proteins demonstrated to have a physiological function that includes providing the host organism with a protective response to environmental lead stress.

CadA and ZntA are members of the superfamily of P-type cation-translocating ATPases, but belong to a group of soft metal transporters that includes bacterial enzymes such as the CopA Cu(I) pump (23), the yeast CCC2 Cu(I) pump (24), and the human Cu(I)-transporting ATPases such as MNK (25) and WND (26). The soft metal pumps can be further subdivided into the Cu(I)/Ag(I)-translocating ATPases and the Zn(II)/Cd(II)/Pb(II) ATPases (1). While none of these proteins has yet been demonstrated to catalyze ATP hydrolysis, several have been shown to have properties consistent with being cation-translocating ATPases. First, transport requires ATP and is inhibited by orthovanadate, a classical inhibitor of P-type ATPases (5). Second, P-type ATPases form a β-acylphosphate intermediate, and several soft metal pumps have been shown to form these intermediates (27, 28).

CadA had been shown to catalyze ATP-coupled, vanadate-sensitive Cd(II) transport (7) and to form a phosphoenzyme intermediate (27). ZntA has been shown to transport both Zn(II) and Cd(II) in an ATP-requiring, vanadate-sensitive reaction (6). In this paper we report that CadA also transports Zn(II). Despite the unavailability of lead isotopes for direct transport assays, the results described here are consistent with Pb(II) transport catalyzed by members of this subgroup of P-type ATPases. First, Pb(II) induces expression of CadA (Fig. 1). Second, both ZntA and CadA confer Pb(II) resistance (Fig. 2). Third, Pb(II) inhibits ^{65}Zn(II) transport by both ZntA and CadA (Fig. 3, *A* and *B*). Fourth, inhibition of ZntA activity by Pb(II) was essentially identical to inhibition by Cd(II), a known pump substrate (Fig. 3*C*).

Copper pumps are widely distributed in nature, and genetic diseases such as Menkes and Wilsons result from mutations in the genes for these pumps (25, 26). We predict that Zn(II)/Cd(II)/Pb(II) P-type ATPases exist in humans, and it is not unreasonable to expect that there are diseases related to defects 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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