

The *zntA* gene of *Escherichia coli* encodes a Zn(II)-translocating P-type ATPase

(zinc transport/zinc resistance/cadmium resistance)

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ABSTRACT The first Zn(II)-translocating P-type ATPase has been identified as the product of *o732*, a potential gene identified in the sequencing of the *Escherichia coli* genome. This gene, termed *zntA*, was disrupted by insertion of a kanamycin gene through homologous recombination. The mutant strain exhibited hypersensitivity to zinc and cadmium salts but not salts of other metals, suggesting a role in zinc homeostasis in *E. coli*. Everted membrane vesicles from a wild-type strain accumulated $^{65}\text{Zn(II)}$ and $^{109}\text{Cd(II)}$ by using ATP as an energy source. Transport was sensitive to vanadate, an inhibitor of P-type ATPases. Membrane vesicles from the *zntA::kan* strain did not accumulate these metal ions. Both the sensitive phenotype and transport defect of the mutant were complemented by expression of *zntA* on a plasmid.

All living organisms require zinc for continued growth and development. The numerous functions include both structural and catalytic roles in a number of enzymes (1–3). However, in excess zinc is toxic, so both eukaryotes and prokaryotes have developed mechanisms to prevent overaccumulation of zinc, for example induction of metallothioneins that sequester zinc (4, 5). Another mode of zinc regulation is likely to be at the level of transporters that facilitate zinc influx during deficiency and efflux during excess. Despite the obvious importance of zinc homeostasis, little is known about these transporters at the molecular level (6–10).

P-type ATPases form a large family of cation-transporting membrane proteins, with more than 50 members identified to date (11). A recently identified subfamily of putative soft metal P-type ATPases has been implicated in metal homeostasis (11–13). Representative members of this subfamily include bacterial enzymes such as CadA (14–16), CopA and CopB (17), CtaA (18), and eukaryotic Cu(I)-transporting ATPases such as the Menkes (19) and Wilson disease-associated proteins (20). Four ORFs potentially encoding soft metal-translocating ATPases were identified in the *Escherichia coli* chromosome: HRA1 (731 residues; accession no. U16658) (21), HRA2 (721 residues; accession no. U16659) (21), a probable copper-translocating ATPase (834 residues; accession no. U58330) (S. Das, E. Chuang, C. Vulpe, J. Goldman, and J. Gitschier, unpublished data), and ORF732 (732 residues; accession no. P37617) (22).

The most striking feature of soft metal P-type ATPases is the presence of 1–6 motifs, GXXCXXC or (M/H)XXM-DH(S/G)XM, at the N terminus of the molecule that are putative metal-binding domains (12, 13). Differences in the number of times this metal-binding motif is repeated provide the basis for the enormous variation in mass of these proteins. In addition, members of this group have either CPC or CPH

in a putative transmembrane helix that might be part of the cation channel. However, neither the cationic substrate nor the physiological function can be deduced from the primary structure. Only two members of this subgroup have been demonstrated to transport metals: CadA transports cadmium ions (15), and CopB transports copper and silver ions (23). Recently peptides derived from Menkes and Wilson proteins have been demonstrated to bind Cu(I) (24).

E. coli is intrinsically tolerant to high levels of zinc and cadmium. We predicted that ORF732, the closest homolog to CadA, would be responsible for resistance by catalyzing the active efflux of zinc or cadmium. In this report we disrupted the gene by insertion of a kanamycin gene. The resulting strain exhibited hypersensitivity to zinc and cadmium that could be complemented by *o732* (renamed *zntA*) on a plasmid. Everted membrane vesicles from the wild-type but not the disrupted strain exhibited ATP-dependent $^{65}\text{Zn(II)}$ and $^{109}\text{Cd(II)}$ accumulation by using ATP as an energy source. Because everted membrane vesicles have an orientation opposite to that of intact cells, accumulation into the vesicles is equivalent to extrusion from the cells. The Zn(II) transport defect of the mutant could similarly be restored by expression of *zntA* on a plasmid. Transport was inducible by zinc and inhibited by orthovanadate, an inhibitor of P-type ATPases. This is the first demonstration of Zn(II) transport by a P-type ATPase. Considering the importance of zinc homeostasis, we would predict the existence of a homolog of ZntA for zinc metabolism in humans, with inheritable metabolic diseases perhaps as severe as the Menkes and Wilson diseases.

MATERIALS AND METHODS

Growth of Cells. Cells of *E. coli* were grown in Luria-Bertani (LB) medium (25) at 37°C. Ampicillin (50 $\mu\text{g/ml}$), kanamycin (50 $\mu\text{g/ml}$), tetracycline (15 $\mu\text{g/ml}$), isopropyl β -D-thiogalactopyranoside (0.1 mM), and 5-bromo-4-chloro-3-indolyl- β -D-galactosidase (80 $\mu\text{g/ml}$) were added as required. The basal salts medium used for the determination of metal sensitivity was described by Poole *et al.* (26), except that zinc was omitted. For membrane preparation cells were grown overnight at 37°C in 20 ml of LB and diluted 50-fold in prewarmed medium. At an optical density of 0.8 at 600 nm the cultures were induced with 1 mM ZnSO_4 for 1 h. Controls were not induced.

Strain Construction and Plasmids. Plasmid DNA propagation, restriction enzyme treatment, ligation, and transformation were performed by minor modifications of published procedures (25). Plasmid pCGR2 was constructed by PCR amplification of the *zntA* gene, starting 97 bp upstream of the start codon and ending 78 bp downstream. The oligonucleotide

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Abbreviation: LB, Luria-Bertani.

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primers used for PCR were 5'-ATCGTCCGCTCGCTGTA-TCTCT-3' and 5'-CCGCCTTTTCCCCTCACCTAAC-3'. The PCR product was cloned into plasmid pGEM-T (Promega). Plasmid pCGR2 was then digested with *Sna*BI, which cuts at a unique site within *zntA*. The kanamycin resistance gene from plasmid pUC4K (Pharmacia) was excised as a *Sal*I fragment, and the ends were made blunt by using the Klenow fragment of *E. coli* DNA polymerase I. This gene was ligated with *Sna*BI-digested pCGR2, generating plasmid pCGR9. To disrupt the chromosomal *zntA* gene, pCGR9 was linearized with *Nco*I and *Sst*I, and the linear DNA was transformed into the *recD* strain JCB499 (27). Homologous recombination of the kanamycin resistance gene into *zntA* was confirmed by PCR with the above primers. The *zntA*-disrupted gene was transferred to strain W3110 by generalized transduction with P1 bacteriophage, with selection for kanamycin resistance.

Determination of Metal Sensitivity. Inhibition by metal salts was tested both in liquid and solid media. The minimal inhibitory concentration (MIC) is the concentration at which no growth was detected. Cells were grown overnight and

streaked to single colonies on LB plates containing varying concentrations of metal salts. Growth was monitored after 24 h at 37°C. In liquid culture, strains were grown overnight, diluted 1:100 in liquid medium containing the varying concentrations of metal salts, and incubated for 24 h at 37°C with shaking. Growth was monitored from the absorbance at 600 nm.

Transport Assays. Everted membrane vesicles were prepared essentially as described previously and stored at -70°C until use (28). Transport assays were performed at room temperature. Unless otherwise noted, the reaction mixture (1 ml) contained 20 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane, pH 6.0, 0.2 M KCl, 0.25 M sucrose, 1 mM 2-mercaptoethanol, 0.8–1 mg of membrane protein, either 5 mM Na₂ATP or ADP, and either 10 μM ⁶⁵ZnSO₄ or ¹⁰⁹Cd acetate (1.25 μCi/ml). The reaction was initiated by addition of 5 mM MgSO₄. At intervals, 0.1-ml samples were withdrawn, filtered through nitrocellulose filters (0.22-μm pore size, Whatman), and washed with 5 ml of the same buffer containing 10 mM MgSO₄ and either 20 mM ZnSO₄ or cadmium acetate. The filters were dried, and the radioactivity was

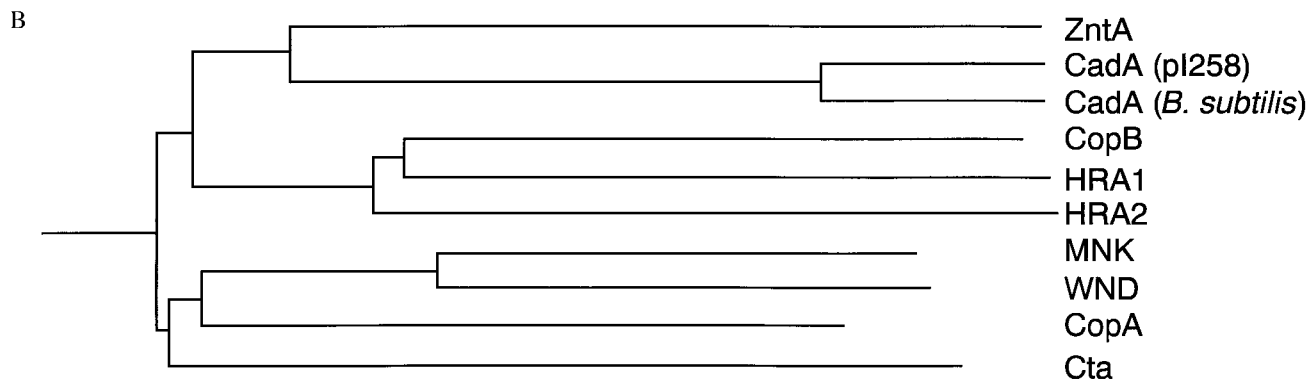
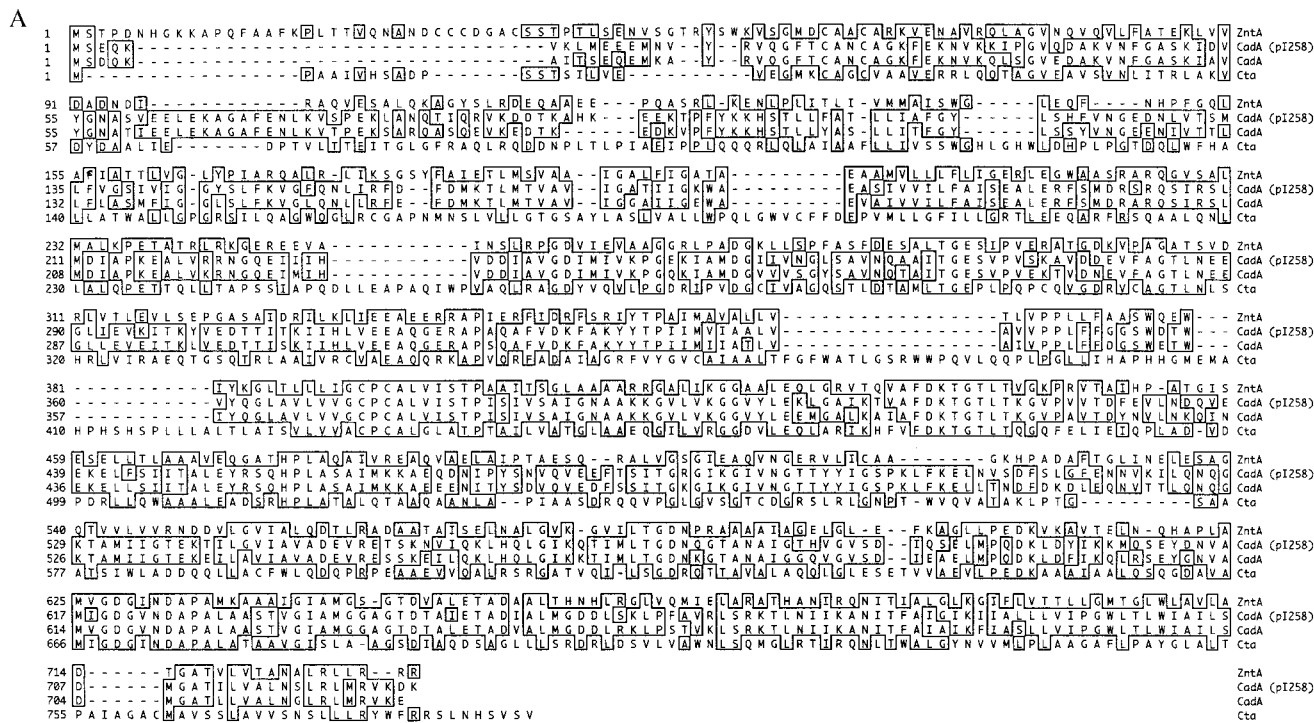


FIG. 1. Multiple alignment and phylogenetic relationship of the amino acid sequences of soft metal translocating P-type ATPases. (A) Alignment of the deduced amino acid sequence of ZntA (22); CadA (pI258) (14); CadA, *Bacillus firmus* (16); and CtaA, *Synechococcus* 7942 (18). Identical residues are boxed. (B) The dendrogram was made with MEGALIGN (DNastar). The branch lengths in the phylogenetic tree are proportional to the number of amino acid substitutions separating each pair. Proteins: ZntA (22), CadA (pI258) (14); CadA, *B. firmus* (16); CopB, *Enterococcus hirae* (17); HRA1, *E. coli* (21); HRA2, *E. coli* (21); Menkes protein (MNK) (19); Wilson protein (WND) (20); CopA, *Enterococcus hirae* (17); and CtaA, *Synechococcus* 7942 (18).

Table 1. Minimal inhibitory concentrations of metal salts

Metal salt	Apparent MIC, mM		
	W3110	RW3110	RW3110 (pCGR2)
ZnSO ₄	2	0.5	2
Cd(OAc) ₂	1.5	0.03	0.8
CuCl ₂	4	4	4
NiCl ₂	4	4	4
CoCl ₂	1.6	1.6	1.6
HgCl ₂	0.03	0.03	0.03
AgNO ₂	0.6	0.6	0.6
NaAsO ₂	5	5	5
Potassium antimonyl tartrate	1.2	1.2	1.2

Cells were grown overnight and streaked onto LB plates containing varying concentrations of metal salts. Growth was monitored after 24 hr at 37°C. The apparent minimal inhibitory concentration (MIC) is the concentration at which no colonies were observed.

quantified in a liquid scintillation counter. A blank value, obtained by filtering 0.1 ml of assay mixture without membrane vesicles, was subtracted from all points. To determine the initial rates, 1- and 2-min time points were used.

Other Methods. Protein sequence alignment and construction of a phylogenetic tree were executed with MEGALIGN from DNASTAR (Madison, WI). Protein content was estimated by a modified procedure of Lowry *et al.* (29).

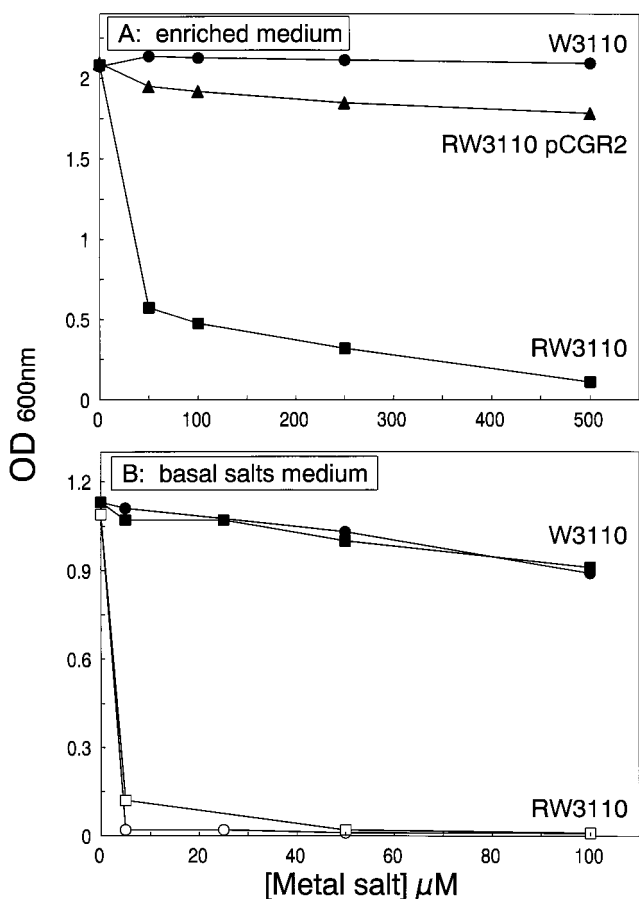


FIG. 2. Cadmium and zinc ion resistance. Metal ion resistance was assayed in cells of *E. coli* grown either in LB medium (A) or a basal salts medium (B) with the indicated concentrations of metal ion salts for 24 hr at 37°C with shaking, following which turbidity at 600 nm was measured. (A) Cadmium sensitivity in strain W3110 (wild type) (●), RW3110 (*zntA::kan*) (■), or RW3110 pCGR2 (*zntA*) (▲). (B) ■, Zinc sensitivity in W3110; □, zinc sensitivity in RW3110; ●, cadmium sensitivity in W3110; ○, cadmium sensitivity in RW3110.

RESULTS

The *zntA* Gene Confers Zinc and Cadmium Tolerance.

CadA, a Cd(II)-translocating P-type ATPase, is encoded by the *cadA* gene of plasmid pI258 from *Staphylococcus aureus* (14). The closest homolog to CadA is the putative product of *o732* (Fig. 1A), one of several potential soft metal-translocating P-type ATPases identified in the *E. coli* genome (21, 22). These enzymes form a subfamily of the cation-translocating P-type ATPases that transport soft metals, including Cu(I), Ag(I), and Cd(II) (Fig. 1B) (11–13). Even though the *o732* gene product is closely related to CadA, the two proteins have only 35% identical residues. In particular, the *o732* gene product has an N-terminal extension containing an additional cysteine triplet that may be of possible functional significance. Thus it would not be unexpected for the chromosomally encoded *o732* and the plasmid-encoded *cadA* gene products to have different physiological activities.

The function of *o732* was investigated by disruption of the gene. The gene was cloned into plasmid pGEM-T by PCR, creating plasmid pCGR2. A kanamycin cassette was inserted into *o732*, and the chromosomal *o732* of the *recD* strain JCB499 was replaced with the disrupted gene by homologous recombination. The disruption was transduced into strain W3110, producing strain RW3110. The growth of the two strains was compared on solid LB medium containing various metal ion salts (Table 1). The two strains showed the same growth in the presence of copper, nickel, cobalt, mercury,

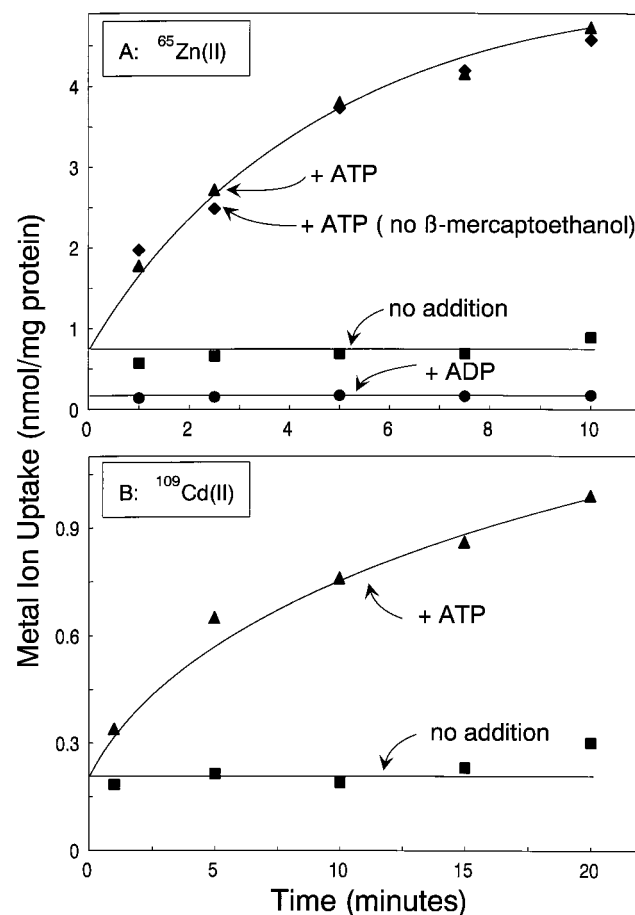


FIG. 3. ATP-dependent uptake of (A) ⁶⁵Zn(II) and (B) ¹⁰⁹Cd(II) in everted membrane vesicles of *E. coli*. Vesicles were prepared from cells of strain W3110 induced with 1 mM ZnSO₄. Transport was assayed with 10 μM ⁶⁵ZnSO₄ or ¹⁰⁹Cd acetate. Additions: (■), no energy source; (▲), 5 mM MgATP; (●), 5 mM MgADP; (◆), 5 mM MgATP but 2-mercaptoethanol was omitted from the assay buffer.

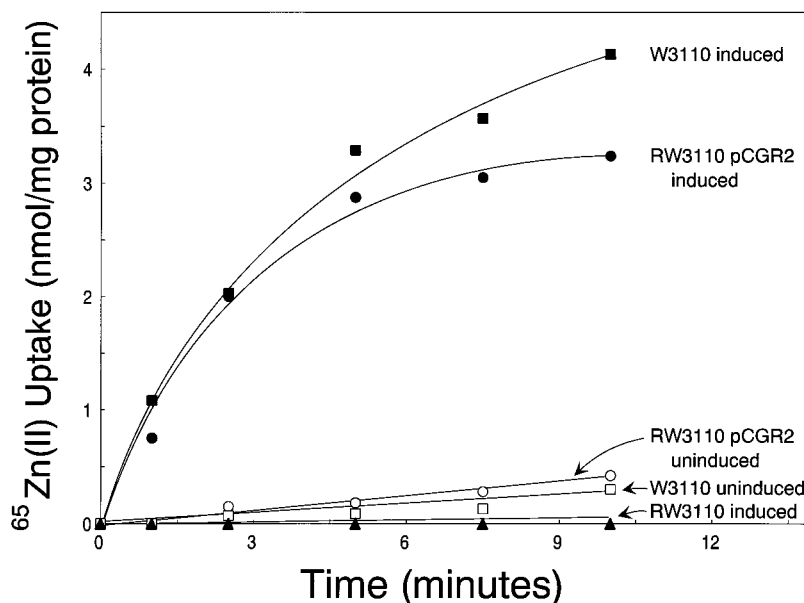


FIG. 4. Inducibility of ATP-dependent ⁶⁵Zn(II) transport in everted membrane vesicles and complementation by *zntA*. Vesicles were prepared from zinc-induced (filled symbols) or uninduced (open symbols) cells of *E. coli* W3110 (squares) or RW3110 (triangles) or RW3110 pCGR2 (circles). The values at each time were corrected for nonspecific binding by subtraction of the nonenergy-dependent values.

silver, arsenic, and antimony. In contrast, RW3110 exhibited a 50-fold increase in sensitivity to cadmium and a 4-fold increase in zinc sensitivity. The cadmium sensitivity of the mutant in liquid LB medium could be complemented by pCGR2, demonstrating that the disruption was responsible for the phenotype (Fig. 2A). The response to both zinc and cadmium was more dramatic in a basal salts medium (Fig. 2B). The reason for the greater sensitivity to zinc in a basal medium compared with an enriched medium is not known, but complexation of zinc with components of LB medium is a possibility. Zinc resistance could also be restored by pCGR2 (data not shown). Whereas this work was in progress Poole and coworkers (30) reported the isolation of a zinc-sensitive strain by random transposon mutagenesis, designating the disrupted gene *zntA*.

They showed the sequence of *zntA* to be identical to *o732*. The gene name *zntA* is used hereafter.

ATP-Dependent Accumulation of ⁶⁵Zn(II) and ¹⁰⁹Cd(II) in Everted Membrane Vesicles of *E. coli*. Everted membrane vesicles were prepared from cells of *E. coli* strain W3110. In the absence of a source of energy or in the presence of MgADP no time-dependent uptake of ⁶⁵Zn(II) (Fig. 3A) or ¹⁰⁹Cd(II) (Fig. 3B) was observed. Addition of MgATP produced time-dependent accumulation of both metals in the wild type. In subsequent experiments transport was corrected for nonenergy-dependent binding. Although 2-mercaptoethanol was included in most assays, it apparently was not required (Fig. 3A).

Properties of ZntA-Catalyzed Zinc Transport in *E. coli*. To demonstrate that transport requires expression of the *zntA*

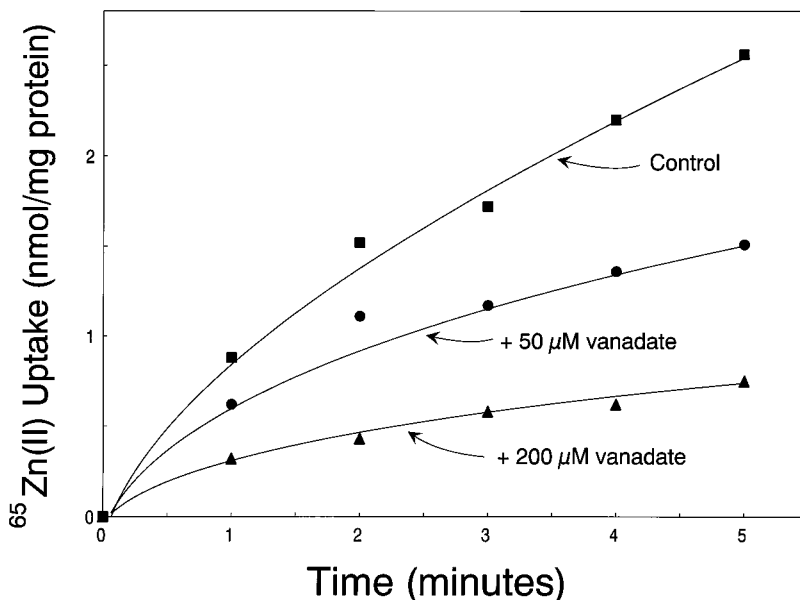


FIG. 5. Vanadate inhibition of ⁶⁵Zn(II) transport. Transport was assayed in vesicles prepared from zinc-induced cells of *E. coli* W3110 in the presence of the following concentrations of sodium orthovanadate: ■, none; ●, 50 μM; ▲, 200 μM. 5 mM MgATP was present as an energy source. The values at each time were corrected for nonspecific binding by subtraction of the non-energy-dependent values.

gene, accumulation of $^{65}\text{Zn(II)}$ was compared in everted membrane vesicles from cells of the wild-type strain W3110, the mutant *zntA::kan* strain RW3110, and the mutant complemented with a *zntA* gene on plasmid pCGR2 (Fig. 4). In uninduced cells only a basal level of activity was observed. Induction with 1 mM ZnSO_4 for 1 hr resulted in a dramatic increase in transport activity. Vesicles of the mutant exhibited no accumulation of $^{65}\text{Zn(II)}$, but the transport defect was complemented by the *zntA* gene on a plasmid. Zinc transport exhibited saturability, with an apparent K_m for Zn(II) of 9 μM (data not shown). From its sequence, ZntA would be predicted to be a P-type ATPase. This class of enzyme is sensitive to inhibition by vanadate. Consistent with this assumption, transport of $^{65}\text{Zn(II)}$ was inhibited by vanadate, with approximately 50% inhibition with 50 μM sodium orthovanadate (Fig. 5).

DISCUSSION

In this work we demonstrate that *o732*, a potential gene in the *E. coli* genome, is responsible for specific resistance to zinc and cadmium in *E. coli*. While this work was in progress Poole and coworkers (30) isolated by transposon mutagenesis a Zn(II)/Cd(II) -sensitive mutant of *E. coli*, designating the mutated gene *zntA*. The sequence of *zntA* was identical to that of *o732*, and we have adopted that gene name. *zntA* was predicted to code for a soft metal-translocating P-type ATPase (13). Our data clearly establish that ZntA catalyzes ATP-dependent zinc transport in everted membrane vesicles of *E. coli*. Because everted membranes have an orientation opposite to that of the inner membrane of intact cells, accumulation in vesicles is the equivalent of extrusion from cells (28).

Although the closest homolog of ZntA is CadA, the two proteins share only 35% identity. The physiological role of the plasmid-encoded CadA is clearly cadmium resistance. On the other hand, we would predict that ZntA has a dual role physiologically. Whereas it would confer cadmium resistance in the rare event that cells of *E. coli* would be exposed to cadmium, a more routine function would be in zinc homeostasis. Consistent with that idea, the pump appears to transport $^{65}\text{Zn(II)}$ approximately 4-fold more effectively than $^{109}\text{Cd(II)}$ (Fig. 3).

Total zinc in *E. coli* has been estimated as a nominal concentration of 0.6 mM if it were all free (31). In mammalian cells this value has been estimated at 0.2 mM and free intracellular Zn(II) at 20 μM (6). However, free intracellular zinc concentrations are difficult to measure. ZntA-mediated zinc transport in everted membrane vesicles exhibits a K_m in the range of 10^{-5} M zinc. *A priori*, it is likely that a K_m in the range of the free intracellular concentration would provide the greatest ability to respond to changes in cytosolic zinc. Although wild-type *E. coli* can tolerate zinc concentrations in the millimolar range, a strain with a disruption in *zntA* is sensitive to micromolar concentrations. These results suggest that free intracellular zinc may be in the range of 10^{-5} M.

Zinc transport was induced by growth in zinc. The *cadA* gene is regulated by the CadC repressor (32), a member of the ArsR family of metalloregulatory proteins (33). However, no genes for homologs of CadC or ArsR are located near *zntA*, and there are no nearby unknown reading frames encoding potential DNA-binding proteins (22). Expression of *zntA* from its own promoter on a plasmid remained inducible, even though the cloned sequence contained no additional reading frames. However, expression from that high copy number plasmid did not produce an increase in either resistance or transport. These results imply a requirement for a chromosomally encoded regulatory protein. Because expression from a high copy number plasmid would be likely to titrate out a small number of repressor proteins, positive regulation of *zntA* expression is a possibility. The sequence of the putative *zntA* promoter has

similarities with the positively regulated promoter of the mercury resistance (*mer*) operon. Thus, whereas the mechanism of zinc regulation remains obscure, we would predict the existence of an activator.

Zinc is an essential metal required by all organisms. Zinc ions play both structural and catalytic roles in zinc-dependent proteins (1–3). In addition, transcription factors recently have been implied to control developmental changes by a *zinc-occupancy* switch that is dependent on the availability of Zn(II) inside cells (34). In spite of the many physiological roles of Zn(II) , in high concentrations it is toxic. In *E. coli* ZntA can be considered as the outward half of a zinc homeostatic mechanism. The other half would be comprised of one or more zinc uptake systems. Other organisms have developed a variety of mechanisms to maintain intracellular zinc homeostasis. In both eukaryotes and prokaryotes Zn(II) hyperaccumulation is prevented by binding of metal to metallothionein (4, 5). Secondary carriers confer zinc resistance by movement of zinc out of cells or into intracellular compartments. For example, the Czc antiporter of *Alcaligenes eutrophus* CH34 produces resistance to Co(II) , Zn(II) , and Cd(II) by extrusion in exchange for protons (9, 10). The mammalian transporters Znt-1 and Znt-2 mediate resistance to Zn(II) by translocation of Zn(II) across various membranes (6, 7). However, zinc resistance produced by a primary ATP-coupled pump has not been demonstrated to date in eukaryotes or other prokaryotes. Considering the inborn errors of copper homeostasis produced by mutations in the Wilson and Menkes genes, energy-dependent active efflux of zinc is likely to be important in humans, predictably by ZntA homologs.

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- Hughes, M. N. & Poole, R. K. (1989) *Metals and Micro-organisms* (Chapman & Hall, London), pp. 245–248.
- Vallee, B. L. & Auld, D. S. (1995) *Experientia* **73**, 259–277.
- O'Halloran, T. (1993) *Science* **261**, 715–725.
- Palmiter, R. D. (1987) *Experientia* **52**, 63–80.
- Huckle, J. W., Morby, A. P., Turner, J. S. & Robinson, N. (1993) *Mol. Microbiol.* **7**, 177–187.
- Palmiter, R. D. & Findley, S. D. (1995) *EMBO J.* **14**, 639–649.
- Palmiter, R. D., Cole, T. B. & Findley, S. D. (1996) *EMBO J.* **15**, 1784–1791.
- Palmiter, R. D., Cole, T. B., Quaife, C. J. & Findley, S. D. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 14934–14939.
- Nies, D. H. (1995) *J. Bacteriol.* **177**, 2707–2712.
- Rensing, C., Pribyl, T. & Nies, D. H. (1997) *J. Bacteriol.* **179**, 6871–6879.
- Lutsenko, S. & Kaplan, J. H. (1995) *Biochemistry* **34**, 15607–15613.
- Soliz, M. & Vulpe, C. (1996) *Trends Biochem. Sci.* **21**, 237–241.
- Silver, S. (1996) in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, eds. Neidhardt, F. C., Curtiss, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M. & Umberger, H. E. (Am. Soc. Microbiol., Washington, DC), 2nd Ed., pp. 1091–1102.
- Nucifora, G., Chu, L., Misra, T. K. & Silver, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3544–3548.
- Tsai, K. J., Yoon, K.-P. & Lynn, A. R. (1992) *J. Bacteriol.* **174**, 116–121.
- Ivey, D. M., Guffanti, A. A., Shen, Z., Kudyan, N. & Krulwich, T. A. (1992) *J. Bacteriol.* **174**, 4878–4884.
- Odermatt, A., Suter, H., Krapf, R. & Soliz, M. (1993) *J. Biol. Chem.* **268**, 12775–12779.
- Phung, L. T., Ajlani, G. & Haselkorn, R. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 9651–9654.

19. Vulpe, C., Levinson, B., Whitney, S., Packman, S. & Gitschier, J. (1993) *Nat. Genet.* **3**, 7–13.
20. Bull, P. C., Thomas, G. R., Rommens, J. M., Forbes, J. R. & Cox, D. W. (1993) *Nat. Genet.* **5**, 327–337.
21. Trenor, C. C., Lin, W. & Andrews, N. C. (1994) *Biochem. Biophys. Res. Commun.* **205**, 1644–1650.
22. Sofia, H. J., Burland, V., Daniels, D. L., Plunkett, G., III, & Blattner, F. R. (1994) *Nucleic Acids Res.* **22**, 2576–2586.
23. Solioz, M. & Odermatt, A. (1995) *J. Biol. Chem.* **270**, 9217–9221.
24. Lutsenko, S., Petrukhin, K., Cooper, M. J., Gilliam, C. T. & Kaplan, J. H. (1987) *J. Biol. Chem.* **272**, 18939–18944.
25. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
26. Poole, R. K., Williams, H. D., Downie, J. A. & Gibson, F. (1989) *J. Gen. Microbiol.* **135**, 1865–1874.
27. Bardwell, J. C., McGovern, K. & Beckwith, J. (1991) *Cell* **67**, 581–589.
28. Kuroda, M., Dey, S., Sanders, O. I. & Rosen, B. P. (1997) *J. Biol. Chem.* **272**, 326–331.
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
30. Beard, S. J., Hashim, R., Membrillo-Hernandez, J., Hughes, M. N. & Poole, R. K. (1997) *Mol. Microbiol.* **25**, 883–892.
31. Kung, F. C., Raymond, J. & Glaser, D. A. (1976) *J. Bacteriol.* **126**, 1089–1095.
32. Endo, G. & Silver, S. (1995) *J. Bacteriol.* **177**, 4437–4441.
33. Shi, W. P., Wu, J. H. & Rosen, B. P. (1994) *J. Biol. Chem.* **269**, 19826–19829.
34. Suhy D. A. & O'Halloran, T. V. (1996) *Met. Ions Biol. Syst.* **32**, 557–578.