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Functional analysis of the *Escherichia coli* zinc transporter ZitB

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Abstract

The membrane transporter ZitB responsible for Zn(II) efflux in *Escherichia coli* was studied by site-directed mutagenesis to elucidate the function of individual amino acid residues. Substitutions of several charged or polar residues, H53, H159, D163 and D186, located in predicted transmembrane domains resulted in loss of ZitB function. In contrast, neither the amino-terminal nor the carboxy-terminal regions, both histidine-rich, were required for function.

1. Introduction

Zinc is required for life in all organisms but is toxic in excess, which requires homeostatic mechanisms to control intracellular zinc levels. Efflux of zinc in *Escherichia coli* is accomplished by the P-type ATPase ZntA and the cation diffusion facilitator (CDF) ZitB [1]. The CDF family [2,3] of proteins has common structural characteristics, with (in most cases) six transmembrane helices and N- and C-terminal histidine-rich motifs predicted to extend into the cytosol. These membrane transporters are usually involved in zinc transport across cytoplasmic or organelle membranes [3–6]. Some prokaryotic CDF proteins also transport cobalt and cadmium [7–10]. Recently, Guffanti et al. [10] showed that CzcD from *Bacillus subtilis* utilizes an antiport mechanism. Antiporters are secondary transporters that couple electrochemical gradients of ions or organic solutes to drive transport reactions [11,12]. CzcD catalyzes active efflux of Zn^{2+} in exchange for K^+ and H^+ [10]. However, the amino acid residues that participate in catalysis are unknown. Secondary active transport proteins convert free energy stored in electrochemical ion gradients into work in the form of a concentration gradient. Comprehensively studied examples include the proton/substrate symporter lactose permease (LacY) and the Na^+/H^+ antiporters NhaA and NhaB [12,13]. Surprisingly, extensive use of site-directed mutagenesis demonstrated that only six amino acid residues in LacY are irreplaceable with respect to active lactose transport. Charge pairs have been identified that mediate substrate binding and H^+ translocation.

This report is the first attempt to elucidate the function of single amino acid residues in a prokaryotic CDF protein. Conserved residues in ZitB that could form a charge relay system for proton translocation were identified. Most amino acid residues examined and the histidine-rich terminus in the large, presumably cytosolic C-terminal domain did not appear to be essential for function. Other conserved amino acids located in transmembrane helices were not essential for function but contributed toward maximum efficiency.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

E. coli strains GG48 (Δ*zitB*::Cm *zntA*::Km) [1] and BL21 (Stratagene) were used for testing activity and overexpression of *E. coli* ZitB or each of the site-directed mutations cloned in plasmid pASK-IBA3 (IBA GmbH, Germany). ZitB with N- or C-terminal deletions were also cloned in pASK-IBA3. Plasmid pASK-IBA3 encodes a

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streptavidin tag that can be used for protein purification and detection using Western blot. The potassium transport-deficient *E. coli* TK2420 (Kdp$^-$ Kup$^-$ Trk$^-$) [14] was used for K^+ complementation experiments. Cells were grown at 37°C in Luria–Bertani (LB) broth supplemented with ampicillin (100 μg ml^{-1}) and anhydrotetracycline (200 μg l^{-1}) as inducer when needed. Strains were maintained on LB agar containing ampicillin (100 μg ml^{-1}) and were stored at −80°C in LB broth supplemented with 25% (v/v) glycerol.

Metal resistance of ZitB mutants was examined as described previously [1]. Complementation of *E. coli* TK2420 for growth on K^+ was measured in a defined medium containing Na^+ [14] and supplemented with different concentrations of Zn^{2+} or Cd^{2+}. The K^+ concentration used was 15 mM, which is much less than optimal for TK2420 [10].

2.2. Recombinant DNA techniques

Recombinant DNA methods including restriction endonuclease digestion, ligation, and transformation were performed according to standard protocols [15]. Plasmid DNA was purified using the Spin Miniprep kit (Qiagen) according to the manufacturer's instructions.

2.3. Site-directed mutagenesis

Site-directed mutations were PCR-generated by the overlap extension method [16]. Two PCR reactions are performed. First round PCR products were digested with *Dpn*I endonuclease to prevent the amplification of wild-type *zitB*. PCR reactions were performed using DNA polymerase mix of the Expand long template PCR system (Roche). PCR-generated mutants were cloned into pASK-IBA3 as *Eco*RI/*Pst*I fragments. Primers used are listed at http://ag.arizona.edu/SWES/people/CV%27s/rensing.htm and were from Sigma Genosys.

2.4. Nucleotide sequencing

Plasmid DNA was sequenced by the dideoxy chain termination method [17] at the University of Arizona sequencing core facility.

2.5. Western blotting

Total membrane protein of *E. coli* BL21 transformed with pZITB, pASK-IBA3, or site-directed mutants of *zitB* was isolated from 100 ml of LB broth cultures induced with 200 μg l^{-1} anhydrotetracycline. Cultures of each strain were grown until the OD_{600nm} reached 1.5, harvested by centrifugation (5000 rpm, 15 min, 4°C) and resuspended in 15 ml of 100 mM Tris–HCl at pH 8.0. Cells were ruptured by sonication and the lysate was centrifuged (5000 rpm, 15 min, 4°C). The cleared lysate was centrifuged (29 000 rpm, 90 min, 4°C). The resulting membrane pellet was resuspended in Tris buffer. Aliquots of each sample were separated by denaturing SDS–PAGE electrophoresis and transferred onto an immunoblot PVDF (polyvinylidene difluoride) membrane (Bio-Rad, Hercules, CA, USA) using a trans-blot apparatus (Bio-Rad). Strep-tactin conjugated to horseradish peroxidase (dilution 1:1000, IBA, Germany) that specifically binds to Strep-tagged proteins was added as described by the supplier. ZitB with bound conjugate was detected using its peroxidase activity in a color reaction with 4-chloronaphthol as chromogen.

3. Results

3.1. Conservation of amino acid residues among ZitB and other CDF proteins

Topological mapping of the integral inner membrane CDF protein family suggests six transmembrane helices for most members with both the N- and C-termini located in the cytoplasm (Fig. 1). Alignment of 12 members of the CDF family identified conserved residues including conserved charged residues in transmembrane helices (alignment at http://ag.arizona.edu/SWES/people/CV%27s/rensing.htm). In addition, there are residues that are conserved only in CDF members that were shown to function in zinc transport such as CzcD and ZitB but not in other CDF proteins such as YiiP where the function is yet unknown. These residues could be involved in conferring metal specificity.

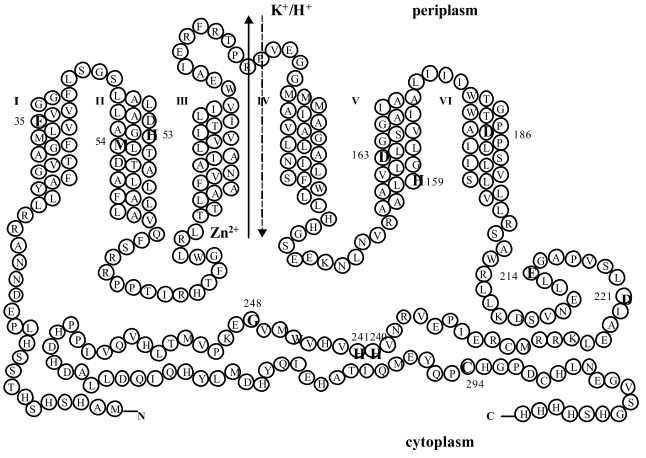

Fig. 1. Topological model of ZitB. The amino acid sequence of ZitB and its proposed topology are shown. Roman numbers I–VI denominate segments of ZitB predicted to span the cytoplasmic membrane. Bold letters indicate amino acid residues residues chosen for site-directed mutagenesis. Numbers represent the amino acid residue position.

3.2. Effect of deletions and substitutions of conserved amino acids in ZitB on zinc resistance and accumulation

In order to identify residues required for function, single amino acid mutations of conserved residues in ZitB were generated by site-directed mutagenesis [16]. All mutants were sequenced to ensure that there were no secondary mutations. The consequence of distinct amino acid changes in ZitB or deletions of its N- or C-termini was assessed by expressing mutated *zitB* in *E. coli* GG48 and monitoring zinc resistance (Fig. 2).

Zinc resistance was abolished in five of the 12 mutants. The H159R and D163E substitutions in transmembrane helix 5 and D186A in transmembrane helix 6 resulted in complete loss of resistance (Fig. 2A). The H53R mutation in transmembrane helix 2 resulted in a mutated ZitB transporter unable to confer zinc resistance in *E. coli* GG48 indicating that this amino acid residue may also contribute to activity (Fig. 2A). Glu214 located in the carboxy-terminal domain is only conserved in bacterial zinc transporters and appears to be involved in ZitB function. E214A was not capable of complementing zinc sensitivity in *E. coli* GG48 (Fig. 2A).

Other charged residues in transmembrane domains with a possible function in cation and/or proton translocation were also examined. Amino acid residue E35 in the transmembrane helix 1 is conserved in all known zinc-transporting CDF proteins. E35A exhibited a slight reduction in zinc resistance. A similar result was obtained with an E35D substitution. Therefore, Glu53 is not essential for function (Fig. 2B). Amino acid residue M54 in transmembrane helix 2 is also conserved in all zinc-translocating CDF proteins and M54L caused a zinc-sensitive phenotype but not a complete loss of function. These results indicate that M54 is also not essential for function. G248T resulted in a slight reduction in ZitB-mediated zinc resistance (Fig. 2B). ^{65}Zn(II) accumulation assays were performed with mutations in ZitB that conferred an intermediate zinc resistance phenotype. All of these ZitB mutants accumulated significantly less zinc than the control strain *E. coli* GG48 (pASK-IBA3) (Fig. 3) confirming the results of zinc resistance phenotype.

In addition to conserved residues in predicted transmembrane helices other charged residues in the large cytoplasmic carboxy-terminal domain are conserved in some CDF proteins. However, D221A, H240R and H241D had no effect on the function of ZitB (Fig. 2C).

An interesting feature of many CDF proteins including ZitB is the presence of numerous histidine residues at the amino-terminus and/or at the carboxy-terminus. Deletion of the N-terminal His-rich region from Met1 to His11 or the C-terminal His-rich region from His308 to His313 had no effect on the function of ZitB. Only a double deletion with both the N-terminus from Met1 to His11 and the C-terminus from Cys294 to His313 deleted lost the ability to confer zinc resistance (data not shown). Intracellular cysteine residues can be part of metal binding sites. There are only three cysteines in ZitB, C230, C294 and C299. The presence of Cys294 at the C-terminus was not required for function since neither a C294S mutation nor the deletion from Cys294 to His313 (including C299) affected zinc resistance (data not shown).

3.3. Expression of ZitB mutants

To show that reduction of zinc resistance of ZitB containing site-directed mutations was not the result of synthesis of a truncated protein, failure of the protein to be inserted in the membrane, or improper insertion or misfolding of the protein that might render it more susceptible to proteolysis, membrane preparations of *E. coli* transformed with pASK-IBA3, pZITB, or each of the site-di-

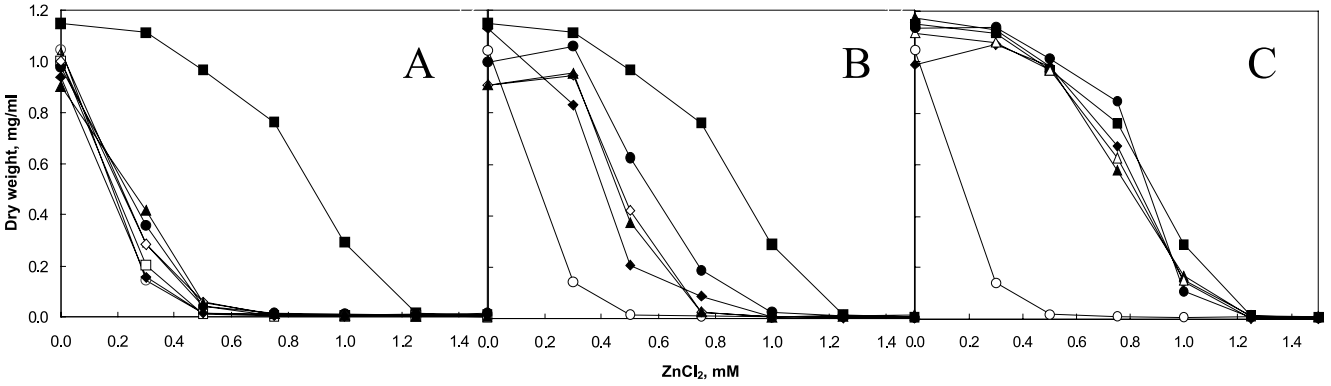

Fig. 2. Zinc resistance of *E. coli* strain GG48 (Δ*zitB*::Cm *zntA*::Km), expressing different mutated *zitB* derivatives. Growth with different $ZnCl_2$ concentrations is shown. Overnight cultures were diluted 1:500 into fresh LB broth with indicated concentrations of $ZnCl_2$ and cell growth after 16 h incubation at 37°C with shaking monitored as OD_{600n} and converted to dry weight (mg ml^{-1}). A: *E. coli* strain GG48 (Δ*zitB*::Cm *zntA*::Km/hypersensitive control) (○), *E. coli* GG48 (pZITB wild-type) (■), GG48 (pZITB, H53R) (□), GG48 (pZITB, H159R) (△), GG48 (pZITB, D163A) (●), GG48 (pZITB, D163E) (◇), GG48 (pZITB, D186A) (◆), GG48 (pZITB, E214A) (▲). B: *E. coli* strain GG48 (hypersensitive control) (○), GG48 (pZITB wild-type) (■), GG48 (pZITB, E35A) (◇), GG48 (pZITB, E35D) (▲), GG48 (pZITB, M54L) (◆), GG48 (pZITB, G248T) (●). C: *E. coli* strain GG48 (○), GG48 (pZITB wild-type) (■), *E. coli* GG48 (pZITB, D221A) (◆), *E. coli* GG48 (pZITB, H240R) (▲), *E. coli* GG48 (pZITB, H241D) (△), *E. coli* GG48 (pZITB, C294S) (●).

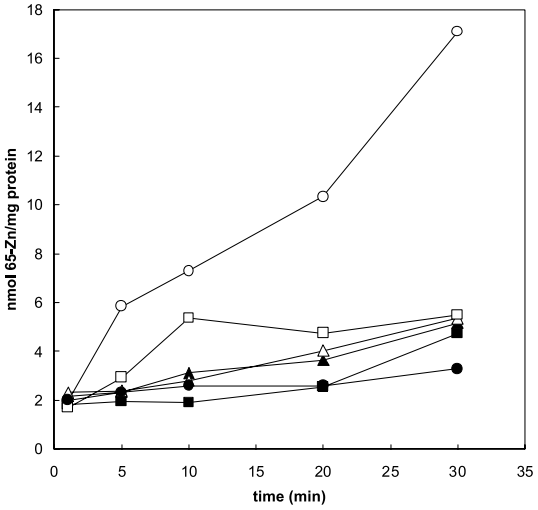

Fig. 3. $^{65}Zn(II)$ uptake by cells of *E. coli* strain GG48 expressing mutant *zitB*. Cells were grown overnight in LB medium and diluted 100-fold into fresh LB medium and grown to an optical density of 0.8 OD_{600nm} and induced with 200 μg l^{-1} anhydrotetracycline. After growth for 2.5 h, the cells were washed with buffer A (10 mM Tris–HCl, pH 7.0, 2 g l^{-1} glucose, 10 mM Na_2HPO_4) and concentrated four-fold in the same buffer. $^{65}ZnSO_4$ was added to a final concentration of 5 μM, cells incubated at 37°C, 0.1-ml aliquots were filtered through nitrocellulose membranes (0.45 μm) at various times and washed with 10 ml of buffer B (10 mM Tris–HCl, pH 7.0, 10 mM $MgCl_2$). The membranes were dried, and radioactivity measured using a liquid scintillation counter. The protein concentration was determined using the BCA kit (Sigma), and the amount of Zn(II) per mg protein was calculated. *E. coli* strain GG48 (○), *E. coli* GG48 (pZITB wild-type) (■), *E. coli* GG48 (pZITB, E35A) (▲), *E. coli* GG48 (pZITB, E35D) (△), *E. coli* GG48 (pZITB, M54L) (□), *E. coli* GG48 (pZITB, G248T) (●).

rected mutations were assayed by Western blot with a Strep-tag specific peroxidase conjugate (Fig. 4). All ZitB site-directed mutants produced a band of the appropriate size, with approximately equal amounts. Only the double

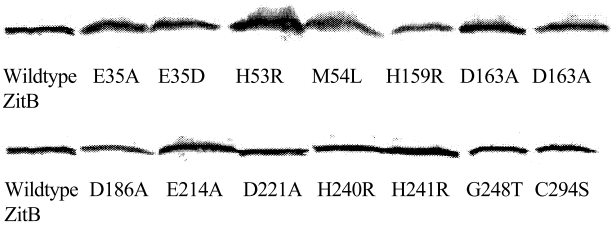

Fig. 4. Western blot analysis of mutant ZitBs. Mutant *zitB* genes were overexpressed in *E. coli* strain BL21 under control of the *tet* promoter on vector pASK-IBA3. Total membrane proteins were prepared, separated by SDS–PAGE, and transferred onto a PVDF membrane. ZitB proteins were detected using a Strep-tag specific peroxidase conjugate and bands visualized in a color reaction.

deletion from Met1 to His11 (N-terminus) and from Cys294 to His313 (C-terminus) did not produce a cross-reacting protein (data not shown).

3.4. *ZitB complements the K^+ uptake defect of E. coli TK2420 in the presence of zinc*

E. coli TK2420 was transformed with pZITB. Expression of *zitB* was induced by addition of anhydrotetracycline. The addition of zinc enhanced the growth of TK2420 on a limiting concentration of 15 mM K^+ in the presence of ZitB (Fig. 5A). Growth enhancement was not observed in the presence of Cd^{2+} and only to a slight degree with Co^{2+} (data not shown). At a concentration of 80 mM K^+ all strains were able to grow.

4. Discussion

In this report the effect of substitutions of conserved amino acids of the CDF transporter ZitB was examined.

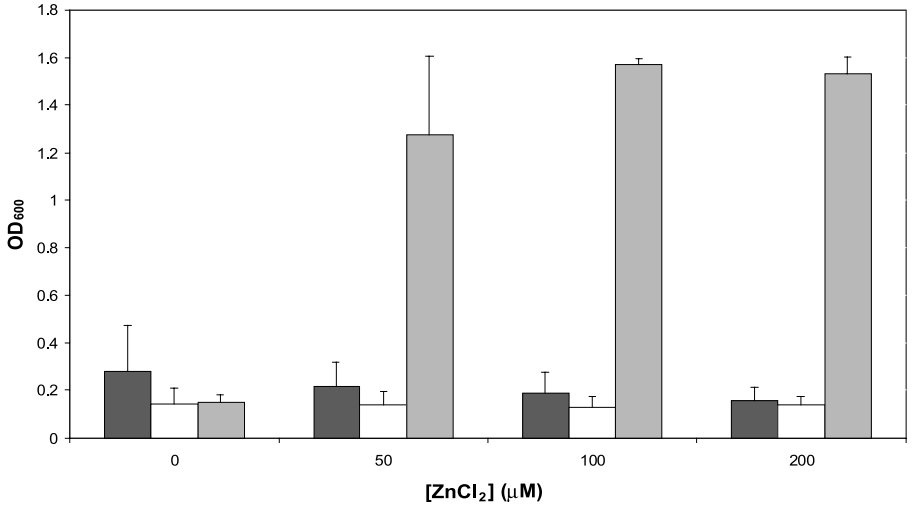

Fig. 5. ZitB-dependent complementation of the K^+ uptake defect of *E. coli* TK2420. The growth of *E. coli* TK2420 transformed with pASK-IBA3 (control), pZITB or without plasmid in minimal salts medium [14] was monitored by reading the optical density at 600 nm (OD_{600}). Medium containing 15 mM KCl was supplemented with increasing concentrations of $ZnCl_2$. These experiments were done in triplicate, shown are the means with the error bars representing standard deviations. *E. coli* TK2420 (black bars), TK2420 pASK-IBA3 (white bars) and TK2420 pZITB (gray bars).

According to the topology model (Fig. 1), it is evident that most of the essential residues examined in this report are located in transmembrane helices and clustered in or near potential motifs (Fig. 1).

There are several charged or polar amino acid residues in ZitB that are located in predicted transmembrane domains that could potentially be involved in cation and potassium/proton transport. Substitutions of three conserved amino acids in transmembrane helices resulted in loss of zinc efflux and resistance. These residues are conserved in all CDF proteins and could form a charge relay system [3]. In addition, a H53R substitution also resulted in loss of resistance. His53 is present in all known zinc transporters but not in all CDF proteins. ZitB (H53M54) and CzcD both have a HM sequence in this region but in Znt2 from *Rattus norvegicus* it is HL and in YiiP from *E. coli* it is DS. Possibly, this residue is needed for zinc transport but not transport of other metals.

It cannot be assumed that each of these amino acids directly interacts with the zinc or potassium/proton. They could participate in stabilization of the overall protein structure or be involved in conformational changes between substrate binding and release. The presence of uncompensated charged amino acid residues in transmembrane helices of membrane proteins would be energetically very unfavorable. Thus, it is possible that many charged residues in hydrophobic regions may be neutralized by a neighboring residue of opposite charge.

In this report, we also identified residues and motifs that are not required for function. The histidine-rich N- and C-termini of ZitB are not essential but might increase zinc transport when there are only very few ZitB transporters present in the cell. Another possibility is that these motifs have a regulatory role. In other families of metal transporters potential metal binding motifs were also not required for function. A deletion of the His-rich carboxy-terminus in NreB, a nickel-transporting member of the major facilitator superfamily, did not result in a loss of the ability to confer nickel resistance [18]. In members of metal-translocating P-type ATPases the amino-terminal CXXC motif(s) does not appear to be required for function [19–21].

The CDF transporter ZitB is an antiporter exchanging Zn^{2+} for K^+ since expression of ZitB could complement the potassium deficiency of strain *E. coli* TK2420 in the presence of zinc. ZitB might be more specific for zinc than CzcD, which appears to have a broader substrate specificity. However, both transporters utilize antiport to exchange cytoplasmic zinc for extracellular potassium. In transport experiments Guffanti et al. [10] were able to show that protons can also serve as a coupling ion both in concert with K^+ and alone. This probably is also true for ZitB. A better understanding about the role of amino acids involved in the transport process can only come from direct transport measurement and the determination of kinetic parameters.

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References

[1] Grass, G., Fan, B., Rosen, B.P., Franke, S., Nies, D.H. and Rensing, C. (2001) ZitB (YbgR), a member of the cation diffusion facilitator family, is an additional zinc transporter in *Escherichia coli*. J. Bacteriol. 183, 4664–4667.

[2] Nies, D.H. and Silver, S. (1995) Ion efflux systems involved in bacterial metal resistances. J. Ind. Microbiol. 14, 186–199.

[3] Paulsen, I.T. and Saier, M.J. (1997) A novel family of ubiquitous heavy metal ion transport proteins. J. Membr. Biol. 156, 99–103.

[4] Eide, D.J. (1998) The molecular biology of metal ion transport in *Saccharomyces cerevisiae*. Annu. Rev. Nutr. 18, 441–469.

[5] Palmiter, R.D. and Findley, S.D. (1995) Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. EMBO J. 14, 639–649.

[6] Miyabe, S., Izawa, S. and Inoue, Y. (2000) Expression of *ZRC1* coding for suppressor of zinc toxicity is induced by zinc-starvation stress in Zap1-dependent fashion in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Comm. 276, 879–884.

[7] Xiong, A. and Jayaswal, R.K. (1998) Molecular characterization of a chromosomal determinant conferring resistance to zinc and cobalt ions in *Staphylococcus aureus*. J. Bacteriol. 180, 4024–4029.

[8] Kuroda, M., Hayashi, H. and Ohta, T. (1999) Chromosome-determined zinc-responsible operon *czr* in *Staphylococcus aureus* strain 912. Microbiol. Immunol. 43, 115–125.

[9] Anton, A., Grosse, C., Reissmann, J., Pribyl, T. and Nies, D.H. (1999) CzcD is a heavy metal ion transporter involved in regulation of heavy metal resistance in *Ralstonia* sp. strain CH34. J. Bacteriol. 181, 6876–6881.

[10] Guffanti, A.A., Wei, Y., Rood, S.V. and Krulwich, T.A. (2002) An antiport mechanism for a member of the cation diffusion facilitator family: divalent cations efflux in exchange for K^+ and H^+. Mol. Microbiol. 45, 145–153.

[11] Rosen, B.P. and Kashket, E.R. (1978) Energetics of active transport. In: Bacterial Transport (Rosen, B., Ed.), pp. 559–620. Marcel Dekker, New York.

[12] Padan, E. and Schuldiner, S. (1994) Molecular physiology of the Na^+/H^+ antiporter in *Escherichia coli*. J. Exp. Biol. 196, 443–456.

[13] Kaback, H.R., Sahin-Toth, M. and Weinglass, A.B. (2001) The kamikaze approach to membrane transport. Nat. Rev. Mol. Cell. Biol. 2, 610–620.

[14] Epstein, W. and Kim, B.S. (1971) Potassium transport loci in *Escherichia coli* K-12. J. Bacteriol. 108, 639–644.

[15] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

[16] Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R. (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77, 51–59.

[17] Sanger, F., Micklen, S. and Coulson, A.R. (1977) DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 551–559.

[18] Grass, G., Fan, B., Rosen, B.P., Lemke, K., Schlegel, H.G. and Rensing, C. (2001) NreB from *Achromobacter xylosoxidans* 31A is a nickel-induced transporter conferring nickel resistance. J. Bacteriol. 183, 2803–2807.

[19] Fan, B., Grass, G., Rensing, C. and Rosen, B.P. (2001) *Escherichia coli* CopA N-terminal $Cys(X)_2Cys$ motifs are not required for either copper resistance or transport. Biochem. Biophys. Res. Commun. 286, 414–418.

[20] Mitra, B. and Sharma, R. (2001) The cysteine-rich amino-terminal domain of ZntA, a Pb(II)/Zn(II)/Cd(II)-translocating ATPase from *Escherichia coli*, is not essential for function. Biochemistry 40, 7694–7699.

[21] Voskoboinik, I., Strausak, D., Greenough, M., Brooks, H., Petris, M., Smith, S., Mercer, J.F. and Camakaris, J. (1999) Functional analysis of the N-terminal CXXC metal-binding motifs in the human Menkes copper-transporting P-type ATPase expressed in cultured mammalian cells. J. Biol. Chem. 274, 22008–22012.