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Membrane Topology of the pl258 CadA Cd(II)/Pb(II)/Zn(II)-Translocating P-Type ATPase

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Plasmid pl258 carries the *cadA* gene that confers resistance to cadmium, lead, and zinc. CadA catalyzes ATP-dependent cadmium efflux from cells of *Staphylococcus aureus*. It is a member of the superfamily of P-type ATPases and belongs to the subfamily of soft metal ion pumps. In this study the membrane topology of this P-type ATPase was determined by constructing fusions with the topological reporter genes *phoA* or *lacZ*. A series of 44 C-terminal truncated CadAs were fused with one or the other reporter gene, and the activity of each chimeric protein was determined. In addition, the location of the first transmembrane segment was determined by immunoblot analysis. The results are consistent with the pl258 CadA ATPase having eight transmembrane segments. The first 109 residues is a cytosolic domain that includes the $Cys(X)_2Cys$ motif that distinguishes soft metal ion-translocating P-type ATPases from their hard metal ion-translocating homologues. Another feature of soft metal ion P-type ATPases is the CysProCys motif, which is found in the sixth transmembrane segment of CadA. The phosphorylation site and ATP binding domain conserved in all P-type ATPases are situated within the large cytoplasmic loop between the sixth and seventh transmembrane segments.

KEY WORDS: Cadmium resistance; CadA; topology; soft metal ion pump.

INTRODUCTION

Plasmid pl258 *cadCA* operon confers resistance to cadmium, zinc, and lead in *S. aureus* (Chopra, 1975; Novick and Roth, 1968; Nucifora *et al.*, 1989). The *cadA* gene product catalyzes active efflux of cadmium, zinc, or lead (Rensing *et al.*, 1998; Tsai *et al.*, 1992). Based on its sequence, CadA is a member of the P-type superfamily of cation-translocating ATPases (Nucifora *et al.*, 1989), the first identified member of the subfamily of CPx-type (Solioz and Vulpe, 1996), P1-type (Axelsen and Palmgren, 1998), or soft metal ion-translocating ATPases (Gatti *et al.*, 2000). Soft metal ions are defined as the cations of soft Lewis acids, including the transition metals Zn(II) and Cd(II) and the heavy metal Pb(II). These have high polarizing power (a large ratio of ionic charge to the radius of the ion), and typically form strong bonds with soft Lewis bases such as the sulfur and nitrogen ligands of cysteine and histidine residues in proteins (Lippard and Berg, 1994).

The physiological role of a number of soft metal ion-translocating ATPases has been characterized (Gatti *et al.*, 2000; Solioz and Vulpe, 1996), although only a few have been demonstrated to transport metals: plasmid pl258 CadA catalyzes cadmium transport when expressed in either *Bacillus subtilis* (Tsai *et al.*, 1992) or *E. coli* (Rensing *et al.*, 1998); *Enterococcus hirae* CopB has been shown to transport copper and silver ions (Solioz and Odermatt, 1995); *E. coli* ZntA transports zinc and cadmium (Beard *et al.*, 1997; Rensing *et al.*, 1997, 1998); and *E. coli* CopA transports Cu(I) (Rensing *et al.*, 2000). In addition to typical features shared by all P-type ATPases, the soft metal pumps share unique structures not found in their hard metal relatives (Lutsenko and Kaplan, 1995; Solioz and

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Vulpe, 1996). For example, the soft metal pumps have formed one to six N-terminal conserved GXTCXXC motif(s) that are proposed to participate in the metal binding (DiDonato *et al.*, 1997; Lutsenko *et al.*, 1997a,b). Another unique structure found in this class of P-type ATPases is a CPx motif (where x can be cysteine, histidine, or serine) located in the putative sixth transmembrane segment that may form an ion transduction domain or ion channel within the membrane (Solioz and Vulpe, 1996).

To understand the mechanism of ion translocation requires knowledge of the membrane topology of the pumps. Genetic methods to determine the transmembrane segments (TMs) of bacterial membrane proteins have been developed (Silhavy and Beckwith, 1985). In-frame fusions of the *phoA* gene, which encodes alkaline phosphatase (Manoil and Beckwith, 1986) or the *lacZ* gene, which encodes β-galactosidase (Froshauer and Beckwith, 1984), give topological information in which high alkaline phosphatase activity indicates a periplasmic location of a protein region, and high β-galactosidase activity indicates a cytosolic localization of a region of a membrane protein. Gene fusions with the *blaM* gene for β-lactamase give similar topological results (Broome-Smith, 1990). These methods have given empirically consistent results with a growing number of membrane proteins.

The membrane topology of one hard metal P-type ATPase, the MgtB magnesium pump of *Salmonella typhimurium*, has been determined using gene fusions with *blaM* and *lacZ* (Smith *et al.*, 1993). The data supported a model in which MgtB has 10 TMs with the amino and carboxyl termini residing in the cytosol, and it was proposed that eukaryotic hard metal ion-translocating P-type ATPases similarly have 10 TMs. The crystal structure of the calcium pump of sarcoplasmic reticulum, a hard metal P-type ATPase, has been recently reported (Toyoshima *et al.*, 2000). The structure clearly demonstrates that the calcium pump has 10 transmembrane α-helices. Thus it is likely that most or all hard metal P-type ATPase have 10 TMs.

From sequence similarities, the first six TMs of the sarcoplasmic reticulum calcium pump are homologous to regions in the soft metal pumps, but the last four TMs appear to have no corresponding regions in the latter (Toyoshima *et al.*, 2000). This suggests that the soft metal ATPases may have a different number of TMs than hard metal pumps. Based on hydropathic analysis, soft metal pumps have been predicted to have eight TMs (Solioz and Vulpe, 1996). Recently the membrane topology of two soft metal P-type ATPases from *Helicobacter pylori* was determined using an in vitro transcription/translation method (Melchers *et al.*, 1996, 1998). One is a putative monovalent soft metal ion (Cu(I) and/or Ag(I)) pump (Melchers *et al.*, 1998), and the second is a putative divalent soft metal ion (Zn(II) and/or Cd(II)) pump (Melchers *et al.*, 1996).

The only topological information on soft metal P-type ATPases is for the *H. pylori* proteins. Considering the proposed differences in topology between hard and soft metal pumps (Melchers *et al.*, 1999; Smith *et al.*, 1993), it is of importance to demonstrate the generality of the 8-TM topology. For that reason we undertook a topological analysis of CadA, the first identified soft metal ion P-type ATPase (Nucifora *et al.*, 1989). In this study, we used *phoA* and *lacZ* as reporters to prepare a series of *cadA-phoA* and *cadA-lacZ* fusions throughout the *cadA* gene. From alkaline phosphatase and β-galactosidase activity assays, we demonstrate that the pl258 CadA has 8 TMs, with the N- and C-termini located in the cytosol. These results support the proposition that soft metal ion-translocating P-type ATPases have 8 transmembrane segments.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

E. coli strains and plasmids used in this study are described in Table I. *E. coli* strain JM109 was used as host for plasmid construction. *E. coli* strain LMG194 was used for expression of *cadA-phoA* fusions, and strain MC1000 was used for expression of *cadA-lacZ* fusions. *E. coli* strain RW3110, which has a disruption of the chromosomal *zntA* gene that is responsible for resistance to Zn(II), Cd(II), and Pb(II) (Rensing *et al.*, 1997), was used for expression of *cadA*. In all experiments, cells were grown in LB medium (Sambrook *et al.*, 1989) at 37°C with 200 rpm shaking and supplement with 100 μg/mL of ampicillin (Sigma, St. Louis, MO). A quantity of 400 μg of either 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma, St. Louis, MO) or 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-GAL) (Sigma, St. Louis, MO) were spread onto LB agar plates for screening colonies for expression of alkaline phosphatase or β-galactosidase.

Construction of a CadA Expression System

Gene cloning was performed using standard procedures. Polymerase Chain Reaction (PCR) was performed using Takara Ex Taq polymerase (Takara, Japan) in a PTC-200 thermocycler (MJ Research, Waltham, MA). Plasmids and fragment DNAs, including PCR products and digested DNA, were purified using NucleoSpin Plus Plasmid Miniprep Kits and NucleoSpin Extraction Kits (Clontech, Palo Alto, CA), respectively. Restriction enzymes used in this study were purchased from New England Biolabs

Table I. Bacterial Strains and Plasmids

Strain/plasmid	Genotype/description	Reference
JM109	F′ *traD36 lacIq* Δ*(lacZ)M15 proA*$^+$*B*$^+$/e14$^-$(McrA$^-$) Δ*(lac-proAB) thi gyrA96* (Nal^r) *endA1 hsdR17*(r_K^- m_K^+) *recA1 relA1 supE44*	Promega
LMG194	F$^-$ Δ*(lacIPOZY)X74 galE galK thi rpsL* Δ*phoA ra714*	Invitrogen
MC1000	F$^-$*araD139* Δ*(araABC-leu)*7679 *galU galK* Δ*(lac)*X74 *rpsL thi*	Ellis *et al.*, 1995
RW3110	F$^-$*mcrA mcrB IN(rrnD-rrnE)1 lambda-ZntA::km*	Rensing *et al.*, 1998
pKJ3	2.6-kilobase pair *Xba*I fragment containing the 3′ end of *cadC* and the complete *cadA* gene from *S. aureus* in pET11a	This lab
pFU3K	Entire *cadA* gene, 2184 base pair *Nco*I-*EcoR*I fragment without internal *Nco*I and *Eco*RI, ligated into pBADMycHisA	This lab
pSE380	An expression vector offering *trc* promoter, *lacO* operator, *lacI^q* repressor, and Amp^R selection marker	Invitrogen
pMM1	A transposon delivery plasmid carrying a mini-transposon TnTAP and Tn5 transposase	Guan *et al.*, 1999
pMLB1069	pBR322 Δ(tet^r) *lac′ZY′*	Ellis *et al.*, 1995
pKJ100	*Nco*I-*Xba*I fragment of entire *cadA* gene in vector plasmid pSE380	This study
pXxP series	Fusions of *phoA* to various PCR fragments of *cadA* gene	This study
pXxL series	Fusions of *lacZ* to various PCR fragments of *cadA* gene	This study

(Beverly, MA). DNA ligation reactions were performed using a T4 DNA ligase (Promega, Madison, WI). Transformation was done by electroporation using a Gene Pulser II (Bio-Rad, Hercules, CA). The *Xba*I and *Hin*dIII fragment from plasmid pSE380 (Invitrogen, Carlsbad, CA) was replaced by a 368-bp *Xba*I-*Hin*dIII fragment from pET11a to yield plasmid pSE380a, in which the *Spe*I site from pSE380 was eliminated. A 2.2-kb *Nco*I-*EcoR*I fragment containing the *cadA* gene from plasmid pFU3K was used to replace the *Nco*I-*EcoR*I fragment of pSE380a to produce plasmid pSE-FU3K. The 1.7-kb *Spe*I-*Xba*I fragment of pSE-FU3K was replaced by a *Spe*I-*Xba*I fragment from pKJ3 (Tsai *et al.*, 1992) to eliminate the internal *Nco*I site of *cadA* gene, for constructing the reporter gene fusion, to generate the plasmid pKJ100. In this construct, *cadA* is controlled by the *trc* promoter. The sequence of the *cadA* gene in each clone was determined using an ABI PRISM™ Dye Terminator Cycle Sequencing System in a core facility laboratory at Chung Shan Medical University. The primers used for sequencing are listed in Table II.

Cadmium Resistance Assays

Cadmium resistance assays were performed as described (Nucifora *et al.*, 1989). RW3110 cells harboring pKJ100 or pSE380 were cultured overnight in LB medium in the presence of 100 μg of ampicillin at 37°C. Aliquots were diluted 50-fold with fresh LB containing 0, 10, 20, 40, and 80 μM $CdCl_2$, respectively, and grown for another 6 h. Growth was estimated from the absorbance at 600 nm, and growth in the presence of cadmium was normalized to the absorbance of cells grown in the absence of cadmium.

Construction of *lacZ* and *phoA* Fusions

A series of *phoA* or *lacZ* gene fusions were constructed with various 5′ regions of the *cadA* gene. Plasmid

Table II. Primers

	Sequence[a,b]
Primers for *cadA-phoA* and *cadA-lacZ* constructions	
NcoI(+)	5′ GTGAAGGT*CcATGg*CTGAACAAAAG 3′
BamHI327(−)	5′ GTGTG*GgAtcC*AGCAATGTACTATG 3′
BamHI390(−)	5′CATGGAA*GgatCC*AGGTTATCTTCTCC 3′
BamHI465(−)	5′ AATCAAA*GgGatcC*AAATTTTGAAA 3′
BamHI543(−)	5′ ACAACAATA*GgatCC*TCTGCCC 3′
BamHI612(−)	5′ GAACGTATG*GgaTcc*CTTGATCTGTCC 3′
BamHI690(−)	5′ CCCACAGCGATA*ggaTCC*ACATGG 3′
BamHI765(−)	5′ GCCGACAAG*ggATcC*ACAATGATTCC 3′
BamHI849(−)	5′ TCGTTAAGC*GgAtCc*GCAAATACTTC 3′
BamHI978(−)	5′ CGCAAAT*ggATCc*ACGAATGCTTGGG 3′
BamHI1068(−)	5′ AAACCCAT*GgATCC*CAACTG 3′
BamHI1164(−)	5′ TTTTTTCGCT*GgATcc*CCAATTGCCG 3′
BamHI1242(−)	5′ TTCCTGTT*ggATCc*AATGCGACTGTC 3′
BamHI1335(−)	5′ CTAAAGCT*GgAtcc*ATAGAGAATAGCTC 3′
BamHI1431(−)	5′ CGAAGTGAAT*gaaTCC*ACTTGTAC 3′
BamHI1527(−)	5′ GGCTAAAATCG*GgAtCc*TTTAATTCC 3′
BamHI1626(−)	5′ CATCTGCAACG*GgAtcc*ACGCCGAGAA TTG 3′
BamHI1731(−)	5′ GCATTTGCA*GgAtCc*TGATTATCACC 3′
BamHI1833(−)	5′ GCTACATTA*ggATcC*TCCGATTGC 3′
BamHI1938(−)	5′ ATCAGCT*GgaTCc*ATTGCAGTATCCG 3′
BamHI2043(−)	5′ TTTAATTCCG*ggAtCc*AAAGTGATG 3′
BamHI2109(−)	5′ ATATCGGAA*gGAtcC*GCTATCCAAAG 3′
BamHI2178(−)	5′ TCTACCTAT*ggATCC*TTCACTC 3′
Primers for DNA sequencing	
SE204F	5′ CAATTAATCATCCGGCTCG 3′
AP113R	5′ GCAGTAATATCGCCCTGAGCAGC 3′
LAC1362R	5′ GGGGATGTGCTGCAAGGCG 3′

[a] The added restriction sites are underlined.
[b] Altered nucleotides from *cadA* gene are written in lowercase letters.

pKJ3 was used as template with the indicated PCR primers (Table II). The *cadA-phoA* gene fusions were prepared first by insertion a 1.9-kb *BamH*I-*Hin*dIII fragment containing the translation signal-sequenceless *phoA* gene from pMM1 (Ehrmann *et al.*, 1997; Guan *et al.*, 1999) to replace the *BamH*I-*Hin*dIII fragment of pSE380, producing plasmid pSE-phoA. Next, six PCR products containing truncated *cadA* genes were treated with both *Nco*I and *BamH*I enzymes to generate *Nco*I-*BamH*I fragments and used to replace the *Nco*I-*BamH*I fragment of plasmid pSE-phoA, producing plasmids pL109P, pL130P, pL155P, pE181P, pR204P, and pV326P. Another 16 PCR products were digested with *Spe*I and *BamH*I, and the truncated *cadA* genes were inserted into *Spe*I-*BamH*I digested plasmid pV326P, creating 16 *cadA-phoA* fusions. The DNA sequences at fusion junction of each of the 22 *cadA-phoA* gene fusions were determined to confirm that no other mutations had been introduced. The resulting plasmids have been designated as pXxP, where X represents the CadA residue, x represents the CadA residue number at the fusion junction of *cadA-phoA* fusions and P indicates that it is a *phoA* fusion.

The *cadA-lacZ* gene fusions were prepared in a similar manner as the *cadA-phoA* fusions. The *BamH*I-*Msc*I region of plasmid pSE380 was replaced with a 3.3-kb *BamH*I-*Msc*I *lacZ* fragment from pMLB1069 (Ellis *et al.*, 1995), producing plasmid pSE-lacZ. A total of 22 truncated *cadA* PCR fragments were cloned separately into this vector using either *Nco*I-*BamH*I or *Spe*I-*BamH*I digestion, as described, and their DNA sequences were verified. The resulting plasmids were designated pXxL, where X represents the CadA residue, x represents the CadA residue number at the fusion junction of *cadA-lacZ* fusions and P indicates that it is a *lacZ* fusion.

Hybrid Protein Expression

For hybrid protein expression, pXxP or pXxL plasmids were transformed into *E. coli* strains LMG194 or MC1000, respectively. Transformants were grown with shaking overnight in 5 mL of LB media in the presence of 100 μg of ampicillin at 37°C. The cultures were diluted into 200 mL of fresh LB media and grown to an optical density at 600 nm of 0.5–0.6, at which time isopropyl β-D-thiogalactopyranoside (IPTG) was added to 0.1 mM. The cultures were incubated for an additional 2 h at 30°C, following which the cells were harvested for assay of reporter enzyme activity and for Western blot analysis. Plasmids isolated from a portion of each culture were digested with the appropriate restriction enzymes and analyzed by electrophoresis to confirm the presence of each hybrid gene.

Enzyme Activity Assays

Alkaline phosphatase activity was assayed essentially as described (Michaelis *et al.*, 1983). Cells bearing pXxP series plasmids were harvested, washed once with 0.1 M Tris-HCl, pH 8.0, and suspended in a 1 mL of the same buffer. One drop each of 0.01% SDS and chloroform was added with vortexing. A portion (0.1 mL) of the sample was mixed with 0.85 mL of a buffer consisting of 0.1 M Tris-HCl, pH 9.5, containing 0.1 M NaCl and 5 mM $MgCl_2$. To each sample 50 μl of 0.4% ρ-nitrophenyl phosphate (PNPP) was added, and the absorbance was monitored at 600 nm over 10 min. Alkaline phosphatase activity was calculated as $(\Delta A_{420\ nm} \cdot 1000)/(\min \cdot mL \cdot A_{600\ nm})$.

β-Galactosidase activity was assayed by a modification of the method described by Miller (1992). Cells bearing pXxL series plasmids were grown as described above for alkaline phosphatase activity. Following harvesting, the cells were washed with 0.1 M sodium phosphate, pH 7.5, and suspended in the same buffer for the assays. One drop each of 0.01% SDS and chloroform was added with vortexing to the cell suspension, and 0.1 mL of each sample was mixed with 0.85 mL of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol, pH 8.0). To each sample 50 μl of 0.4% *o*-nitrophenyl-β-D-galactoside (ONPG) dissolved in 0.1 M sodium phosphate, pH 8.0, was added, and the change in absorbance at 600 nm was measured over 10 min. Activity was calculated using the same formula as for alkaline phosphatase. All data presented in this study represent the average of results from three to five different experiments.

Cell Fractionation

Cell expressing either *cadA-phoA* or *cadA-lacZ* gene fusions were grown and harvested as described above. The cells were washed twice with 10 mL of 10 mM MOPS, pH 7, suspended in a buffer consisting of 50 mM MOPS, pH 7, 0.25 M sucrose, 0.2 M KCl, and 10 mM $MgCl_2$) and lysed by a single passage through a French Press cell at 10,000 psi. The lysates were centrifuged at 4000 $g \times 15$ min at 4°C to remove cell debris and then at 100,000 $g \times 90$ min at 4°C. The supernatant solutions containing the cytosolic proteins were removed, and the pelleted membranes were suspended in 1.0 mL of 10 mM MOPS, pH 7. Protein concentrations were estimated using a D_C protein assay kit (Bio-Rad), with bovine serum albumin as a standard.

Electrophoresis and Immunoblot Analysis

Cell fractions were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) using 7% acrylamide gels and analyzed by Western blot analysis (Gershoni and Palade, 1983) using mouse antibody to alkaline phosphatase or rabbit antibody to β-galactosidase (Biogenesis) coupled with either polyclonal goat antimouse or antirabbit alkaline phosphatase conjugates (Leinco Technologies or Santa Cruz Biotechnology).

RESULTS

Secondary Structure Prediction of CadA

From hydropathic analysis, several soft metal pumps were predicted to have eight TMs (Solioz and Vulpe, 1996). While the membrane topology of a putative divalent soft metal ion-tanslocating P-type ATPases from *Helicobacter pylori* has been experimentally determined (Melchers *et al.*, 1996), from BLAST analysis it is about equally related to *E. coli* ZntA (34% identity, 54% similarity) and to pl258 CadA (31% identity, 55% similarity). ZntA and CadA are more closely related to each other (45% identity, 66% similarity) than to the putative *H. pylori* divalent soft metal cation pump. Although the *H. pylori* homologue is only about 30% identical to CadA, comparison of the secondary structures of the two proteins by the method of Kyte and Doolittle (1982) suggests that they may have a similar membrane topology, with eight regions in CadA that may correspond to the eight transmembrane segments in the *H. pylori* homologue (Fig. 1). The analysis indicated significant differences between the two proteins, so the number of TMs cannot be ascertained from theoretical analysis.

Expression of CadA in *E. coli*

Although plasmid pl258 *cadA* was originally isolated from *S. aureus*, it can also confer soft metal resistance in *E. coli* (Rensing *et al.*, 1998). In this study the *cadA* gene was placed under control of the *trc* promoter in plasmid pKJ100 and expressed in *E. coli* strain RW3110. This strain has a disruption of the *zntA* gene, a *cadA* homologue responsible for resistance to Zn(II), Cd(II), and Pb(II) (Rensing *et al.*, 1997). Cells harboring pKJ100 plasmid were considerably more resistant to cadmium than cells with vector plasmid, pSE380. Furthermore, a protein band with the appropriate mass of CadA was observed with SDS-PAGE analysis of the total membrane protein from RW3110 pKJ100 but was absent in membranes from RW3110 pSE380 (Fig. 2(B)). Functional expression of *cadA* from plasmid pKJ100 implies that native CadA folds and inserts into the membrane properly in *E. coli.*

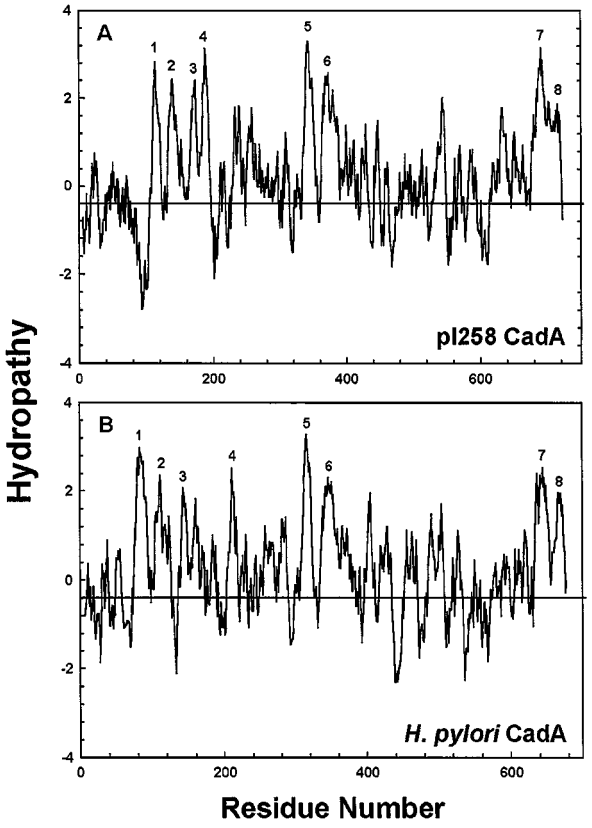

Fig. 1. Hydropathy analysis of CadA. The pl258 (A) and *H. pylori* (B) CadA sequences were analyzed by the method of Kyte and Doolittle (Kyte and Doolittle, 1982) with a window of 11 amino acids. Predicted membrane spanning regions are numbered.

Localization of the First Transmembrane Segment

The cellular location of the first 130 amino acid residues was determined by immunoblotting. *E. coli* strain LMG194 was transformed with either plasmid pL109P or pL130P, in which the *phoA* gene was fused to the C-terminal end of a truncated CadA that contained either the first 109 or 130 amino acid residues. Cell lysates were fractionated into high-speed pellets and soluble components, which represent predominately the membrane and cytosolic plus periplasmic compartments of the cell. The lysates were separated by SDS-PAGE and immunoblotted using antibody to PhoA. Immunoreactive bands were found only in the soluble fraction from cells containing plasmid pL109P or in the membrane fraction from cells

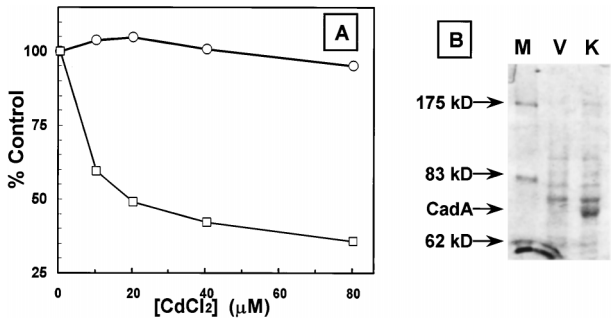

Fig. 2. Cadmium resistance and expression of CadA in *E. coli.* (A) Cadmium resistance assays were performed using *E. coli* strain RW3110 harboring plasmid pKJ100 (*cadA*) or vector plasmid pSE380, as described under Materials and Methods. Relative resistance was calculated from the ratio of the absorbance at 600 nm with and without the indicated concentrations of $CdCl_2$. The SD values in these results were all less than 8%. (B) Membranes were prepared from cells of *E. coli* strain RW3110 harboring plasmid pKJ100 (*cadA*) or vector plasmid pSE380, as described under Materials and Methods, and CadA was visualized by SDS-PAGE using a 7% polyacrylamide gel. M, marker proteins; V, cells harboring vector plasmid pSE380; K, cells harboring plasmid pKJ100.

bearing plasmid pL130P (Fig. 3). The molecular mass of the immunoreactive band from the LMG194/pL109P soluble fraction was approximately 63 kDa, and the reactive band from the membranes from LMG194/pL130P for was approximately 65 kDa. These are close to the masses predicted for these hybrid proteins. The results suggest that

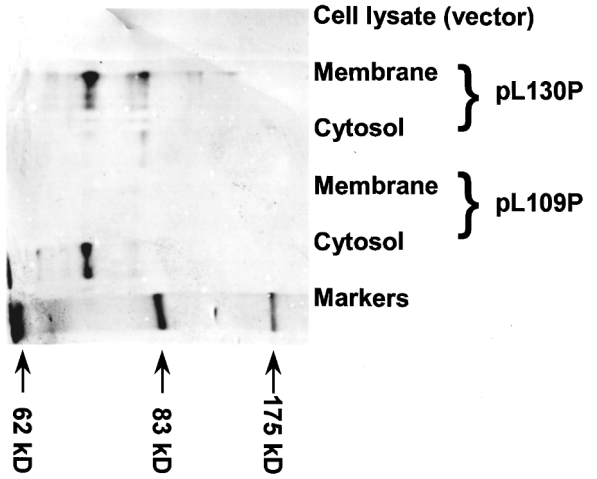

Fig. 3. Determination of the first transmembrane segment of CadA. Cells of *E. coli* strain LMG194 harboring *cadA-phoA* fusion plasmids pL109P or pL130P were induced with 0.1 mM IPTG for 2 h. After the incubation, cells were harvested and used for cytosol and membrane protein preparations, as described under Materials and Methods. Proteins were separated by SDS-PAGE using 7% polyacrylamide gels. Following the electrophoresis, gels were transferred to PVDF membrane and immunoblotting with alkaline phosphatase antibody.

the first N-terminal 109 amino acids of CadA are located in the cytoplasm. A periplasmic location is unlikely because CadA lacks an export signal sequence. When the first 130 residues are present, the peptide is localized in the membrane, indicating that the first TM occurs approximately between CadA residues 109 and 130.

Use of *cadA-phoA* and *cadA-lacZ* Fusions to Determine Additional Transmembrane Segments in CadA

To determine the topology of the remainder of CadA, 44 gene fusions between *cadA* and either *phoA* or *lacZ* were constructed. Cells of *E. coli* strain LMG194 with plasmids carrying each of the *phoA* fusions were grown, and lysates were separated into soluble and membrane fractions. The activity of the enzymatic reporter, alkaline phosphatase or β-galactosidase was measured for each of the 44 fusions (Table III). The CadA-PhoA chimeras could be divided into two groups: fusions at Leu130, Glu181, Trp356, and Ala703 each displayed high alkaline phosphatase activity, ranging from 51 to 200 units. Each of the remaining 18 PhoA chimeras, at Leu109, Leu155, Arg204, Val230, Val255, Aala283, Val326, Gly388, Phe414, Ile445, Val477, Asn509, Val542, Gln577, Glu611, Ile646, Phe681, and Asp726, showed little or no alkaline phosphatase activity. These results suggest that the alkaline phosphatase moiety in Leu130, Glu181, Trp356, or Ala703 fusion clones was located in or near the periplasm, while the other 18 have their alkaline phosphatase reporters localized in or near the cytosol.

While alkaline phosphatase is active only in the periplasm, β-galactosidase is active only in the cytosol and thus provides a topological reporter for the cytosolic regions of membrane proteins. Cells of strain LMG194 bearing each of the 22 *cadA-lacZ* fusions were grown, lysates were separated into soluble and membrane fractions and assayed for β-galactosidase activity (Table III). Almost all of those fusions with a low alkaline phosphatase activity in *cadA-phoA* clones displayed a high β-galactosidases activity when fusions with *lacZ* gene were constructed at the same site. One exception was fusion at the codon for Gly388, in which the *cadA-lacZ* fusion had low β-galactosidase activity and the *cadA-phoA* fusion had low alkaline phosphatase activity. Another anomaly is Glu181, where both types of fusions resulted in high reporter activity.

The lack of enzymatic activity following gene fusion could result from decreased expression or membrane insertion of the chimera. To determine whether the chimeric proteins were produced and membrane associated, the membrane fractions from each strain was separated by

Table III. Activity of CadA-PhoA and CadA-LacZ Fusions[a]

Fusion plasmid	Alkaline phosphatase activity[b]	Deduced location	Fusion plasmid	β-galactosidase activity[b]	Deduced location
pSE380	4 (0)		pSE380	0 (0)	
pL109P	4 (0)	Cytoplasmic	pL109L	27 (4)	Cytoplasmic
pL130P	200 (21)	Periplasmic	pL130L	0 (0)	Periplasmic
pL155P	19 (2)	Cytoplasmic	pL155L	48 (8)	Cytoplasmic
pE181P	105 (7)	Periplasmic	pE181L	28 (9)	Cytoplasmic
pR204P	7 (3)	Cytoplasmic	pR204L	12 (4)	Cytoplasmic
pV230P	0 (0)	Cytoplasmic	pV230L	15 (2)	Cytoplasmic
pV255P	0 (0)	Cytoplasmic	pV255L	18 (1)	Cytoplasmic
pA283P	1 (1)	Cytoplasmic	pA283L	25 (7)	Cytoplasmic
pV326P	3 (2)	Cytoplasmic	pV326L	15 (1)	Cytoplasmic
pW356P	51 (7)	Periplasmic	pW356L	2 (1)	Periplasmic
pG388P	4 (2)	Cytoplasmic	pG388L	4 (1)	Periplasmic
pF414P	0 (0)	Cytoplasmic	pF414L	50 (20)	Cytoplasmic
pl445P	0 (0)	Cytoplasmic	pl445L	35 (12)	Cytoplasmic
pV477P	0 (0)	Cytoplasmic	pV477L	84 (5)	Cytoplasmic
pN509P	3 (3)	Cytoplasmic	pN509L	92 (15)	Cytoplasmic
pV542P	3 (3)	Cytoplasmic	pV542L	93 (31)	Cytoplasmic
pQ577P	0 (0)	Cytoplasmic	pQ577L	52 (10)	Cytoplasmic
pE611P	3 (3)	Cytoplasmic	pE611L	76 (15)	Cytoplasmic
pl646P	1 (1)	Cytoplasmic	pl646L	94 (5)	Cytoplasmic
pF681P	0 (0)	Cytoplasmic	pF681L	32 (2)	Cytoplasmic
pA703P	93 (6)	Periplasmic	pA703L	1 (1)	Periplasmic
pD726P	0 (0)	Cytoplasmic	pD726L	54 (5)	Cytoplasmic

[a] Alkaline phosphatase and β-galactosidase activities were assayed as described under Materials and Methods (A) Alkaline phosphatase activities of *E. coli* strain LMG194 harboring *cadA::phoA* fusions. (B) β-galactosidase activities of *E. coli* strain MC1000 bearing *cadA::lacZ* fusions.

[b] Activity was calculated as $(A_{420} \times 1000)/(\text{min/mL}/A_{600})$ at 23°C. Each value represents the average of three to five different experiments, and the standard deviations (SD) for each data is given in parentheses.

SDS-PAGE and immunoblotted with antibody to alkaline phosphatase (Fig. 4(A)) or β-galactosidase (Fig. 4(B)). The chimeric proteins were found in the membrane fraction in similar amounts, suggesting that the differences in enzymatic activity were not due to differences in protein expression or localization. There was some degradation of the chimerae, but the majority of the activity was found in the band of the predicted molecular mass by activity staining of gels (data not shown). Some of the fusions were found in smaller amounts than others (Fig. 4(A) and (B)), for example the 703P fusion. The relative amounts of the chimeric proteins in the membrane were compared by densitometry, allowing normalization of expression. When the enzymatic activity was normalized by the amount of chimera produced, the conclusions in the Table III were unaltered (data not shown).

DISCUSSION

The superfamily of P-type ATPases includes 5 major branches according to substrate specificity (Axelsen and Palmgren, 1998). Members of one branch transports monovalent and divalent cations of hard Lewis acids, including H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+}. A second branch transports monovalent and divalent cations of soft Lewis acids, including Cu(I), Ag(I), Zn(II), Cd(II), and Pb(II). It is likely that members of the hard metal ion-translocating P-type ATPases have 10 TMs (Smith *et al.*, 1993; Toyoshima *et al.*, 2000). The soft metal ion-translocating P-type ATPases have been proposed to have 8 TMs, of which the last six correspond to the first six of the hard metal ion pumps. The 8-TM topology has been experimentally verified for two soft metal P-type ATPases from *H. pylori* (Bayle *et al.*, 1998; Melchers *et al.*, 1996, 1999).

Silver and coworkers were identified the first soft metal ion-translocating P-type ATPase, the plasmid pl258 CadA (Nucifora *et al.*, 1989). They proposed a topological model for CadA baed on a mathematical algorithm and an enzymatic mechanism for its function (Silver and Walderhaug, 1992). During the catalytic cycle, CadA is proposed to bind Cd(II) and ATP in an unknown order, followed by formation of a phosphorylated enzyme at

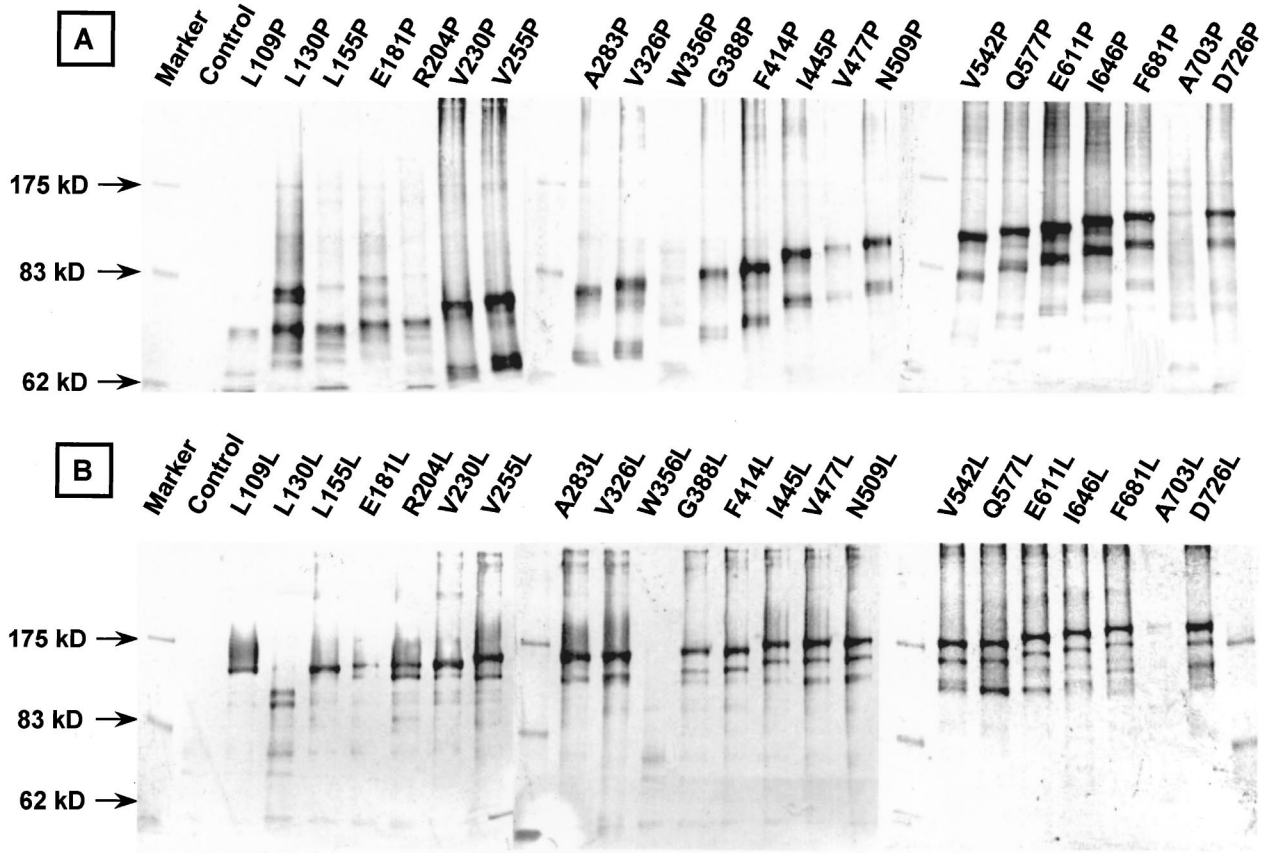

Fig. 4. Expression of CadA-PhoA and CadA-LacZ chimeric proteins. Membrane proteins prepared from *E. coli* strain LMG 194 harboring various *cadA-phoA* fusions (A), or *E. coli* strain MC1000 harboring various *cadA-lacZ* fusions (B) were analyzed by SDS-PAGE using 7% polyacrylamide gels, followed by immunoblotting with a monoclonal antibody against either alkaline phosphatase or β-galactosidase.

conserved Asp415. Phosphorylation causes a conformational change from the E_1 to the E_2 state, with a concomitant decrease of affinity for cadmium. Cd(II) is then transferred from its initial high affinity binding site to a low affinity binding site, possibly containing the conserved Cys_{371}ProCys motif in putative TM6. Finally, Cd(II) is released to the extracellular medium, followed by the release of phosphate from Asp415, and CadA returns to its original conformation to complete the catalytic cycle. Testing of this biochemical mechanism would be greatly facilitated by knowledge of its topology. Even though the topology of several *H. pylori* enzymes has been determined (Bayle *et al.*, 1998; Melchers *et al.*, 1996, 1999), the proposed differences in topology between hard and soft metal pumps (Melchers *et al.*, 1999; Smith *et al.*, 1993) make it important to experimentally determine the membrane topology of a larger number of soft metal ion-translocating ATPases.

In this study, CadA topology was examined using either alkaline phosphatase (*phoA*) or β-galactosidase (*lacZ*) fusions. In general the results with the Gram-positive CadA are similar to those of the Gram-negative *H. pylori* soft metal pumps (Bayle *et al.*, 1998; Melchers *et al.*, 1996, 1999), with the major difference in the topological arrangement of TM3 and TM4. The data indicate that CadA spans the membrane at least seven and probably eight times (Fig. 1, Table III, Fig. 5). By immunoblotting, the N-terminal 109 amino acids were in the cytosol, and the first TM was localized between residues 109 and 130 (Fig. 3). The data are consistent with a topology model in which both the N- and C-termini are cytosolic, and CadA traverses the membrane eight times, with three large cytoplasmic domains and four periplasmic loops (Fig. 5). Taking into consideration both the experimental data and hydropathic analyses using SOAP (Klein *et al.*, 1985; Kyte and Doolittle, 1982), Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html), and the positive-inside rule (von Heijne, 1989), we proposed that the eight TMs of CadA are 105–123 (TM1), 131–151 (TM2), 164–192

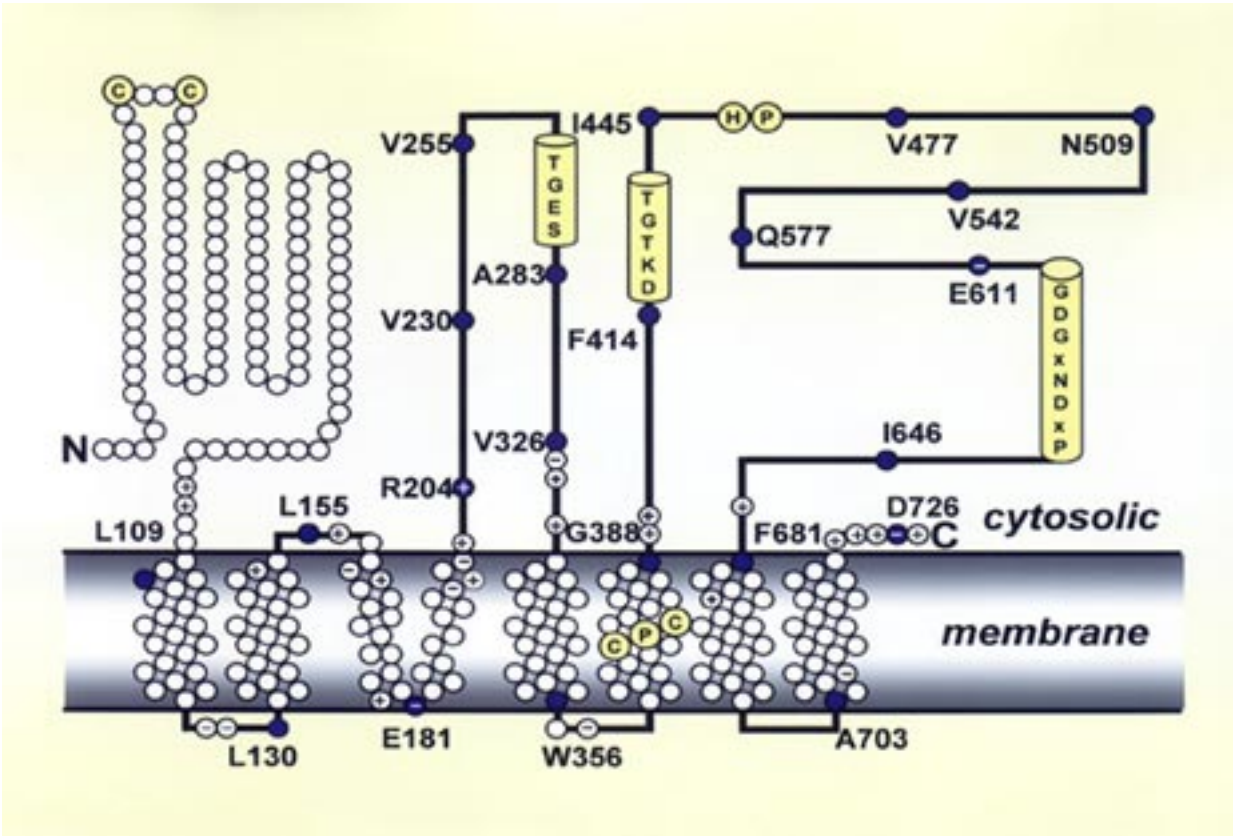

Fig. 5. Membrane topology of the pl258 CadA P-type ATPase. The CadA is similar to that of other soft metal ATPases of the P1-class, with eight transmembrane segments in the membrane (Gatti *et al.*, 2000; Lutsenko and kaplan, 1995; Solioz and Vulpe, 1996). In this model, the cylinders represent motifs conserved in all P-type ATPases. Residues conserved in most soft metal ion-translocating P-type ATPases are also indicated, including the N-terminal $CysX_2Cys$, the CysProCys sequence in TM6, and a His-Pro sequence in the large cytoplasmic domain between TM6 and TM7. The filled circles (blue) indicate the residues identified in this topology using a gene fusion strategy.

(TM3+TM4), 332–356 (TM5), 363–391 (TM6), 677–697 (TM7), and 699–719 (TM8) (Fig. 5).

In the construction of this model there were several ambiguous assignments. First, neither the PhoA (G388P) nor LacZ (G388L) chimera displayed the predicted alkaline phosphatase of β-galactosidase activity (Table III). These reporters were fused immediately following putative TM6, deleting several positive residues. In particular, Lys392, which might anchor TM6, was deleted. Removal of positively charged residues has been shown to prevent the proper membrane insertion of *phoA* or *lacZ* fusions (Franke *et al.*, 1999). Although additional fusions within the third predicted periplasmic loop ranging from amino acids 356–363 would be useful to clarify the confusing results of G388P and G388L, we were unable to generate such a fusion using either transposition or molecular cloning strategy. Possibly production of chimeric proteins within this region are lethal, as is sometimes the case with fusion proteins. In order to make an accurate topological assignment for the region, we extrapolate from the results of Melchers *et al.* (Melchers *et al.*, 1996). In their study the region of the *Helicobacter pylori* CadA (amino acids 328–336) that corresponds to amino acids of 355–363 in the staphylococcal CadA was determined to be a periplasmic loop between TM5 and TM6. Furthermore, when the region of these two proteins are aligned (data not shown), the CPC signature motifs of CPx-ATPase is located at the same position in both proteins (TM6). Since CPC in CPx-ATPases is believed to be within the membrane, the region between 355–363 should be in the third predicted hairpin in our CadA.

Second, our model predicts that TM3 goes from the cytosol at residue 164 to the periplasm at residue 181, with TM4 returning from the periplasm to the cytosol at residues 181–192. However, it is questionable whether the stretch of residues from 164 to 192 is capable of two complete transmembrane spans. In fact, the two fusions at residue 181, E181P and E181L, both resulted in

high reporter gene activity (Table III). We noted a similar anomaly in localization of the first TM of the lysine permease of *E. coli* and postulated that a fusion site near the periplasmic side of a membrane spanning region could result in retardation in translocation of the membrane spanning sequence (Ellis *et al.*, 1995). It may be that the TM3 does not completely traverse the membrane, and that TM4 begins within the membrane. In such a situation, the CadA-PhoA fusion might stretch across the membrane, resulting in high alkaline phosphatase activity, while the larger CadA-LacZ becomes anchored in the cytosol, resulting in high β-galactosidase activity.

There are other minor differences between the 727-residue pl258 CadA and the 686-residue *H. pylori* homologue. The pl258 CadA contains a highly hydrophobic region between residues 626 and 644 that is absent in the H. pylori protein. This extra sequence is predicted to be in a cytosolic domain (Fig. 5). An additional sequence between residues 81 and 102 is found in the N-terminal cystosolic domain of the pl258 CadA. It is not known whether these differences between the two homologues have functional consequences.

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REFERENCES

Axelsen, K. B., and Palmgren, M. G. (1998). *J. Mol. Evol.* **46**, 84–101.
Bayle, D., Wangler, S., Weitzenegger, T., Steinhilber, W., Volz, J., Przybylski, M., Schafer, K. P., Sachs, G., and Melchers, K. (1998). *J. Bacteriol.* **180**, 317–329.
Beard, S. J., Hashim, R., Membrillo-Hernandez, J., Hughes, M. N., and Poole, R. K. (1997). *Mol. Microbiol.* **25**, 883–891.
Broome-Smith, J. K., Tadayyon, M., and Zhang, Y. (1990). *Mol. Microbiol.* **4**, 1637–1644.
Chopra, I. (1975). *Antimicrob. Agents Chemother.* **7**, 8–14.
DiDonato, M., Narindrasorasak, S., Forbes, J. R., Cox, D. W., and Sarkar, B. (1997). *J. Biol. Chem.* **272**, 33279–33282.
Ehrmann, M., Bolek, P., Mondigler, M., Boyd, D., and Lange, R. (1997). *Proc. Natl. Acad. Sci. USA* **94**, 13111–13115.
Ellis, J., Carlin, A., Steffes, C., Wu, J., Liu, J., and Rosen, B. P. (1995). *Microbiology* **141**, 1927–1935.
Franke, C. M., Tiemersma, J., Venema, G., and Kok, J. (1999). *J. Biol. Chem.* **274**, 8484–8490.
Froshauer, S., and Beckwith, J. (1984). *J. Biol. Chem.* **259**, 10896–10903.
Gatti, D., Mitra, B., and Rosen, B. P. (2000). *J. Biol. Chem.* **275**, 34009–34012.
Gershoni, J. M., and Palade, G. E. (1983). *Anal. Biochem.* **131**, 1–15.
Guan, L., Ehrmann, M., Yoneyama, H., and Nakae, T. (1999). *J. Biol. Chem.* **274**, 10517–10522.
Klein, P., Kanehisa, M., and DeLisi, C. (1985). *Biochim. Biophys. Acta* **815**, 468–476.
Kyte, J., and Doolittle, R. F. (1982). *J. Mol. Biol.* **157**, 105–132.
Laemmli, U. K. (1970). *Nature* **227**, 680–685.
Lippard, S. J., and Berg, J. M. (1994). *Principles of Bioinorganic Chemistry*, University Science Books, Mill Valley, CA.
Lutsenko, S., and Kaplan, J. H. (1995). *Biochemistry* **34**, 15607–15613.
Lutsenko, S., Petrukhin, K., Gilliam, T. C., and Kaplan, J. H. (1997a). *Ann. NY Acad. Sci.* **834**, 155–157.
Lutsenko, S., Petrukhin, K., Cooper, M. J., Gilliam, C. T., and Kaplan, J. H. (1997b). *J. Biol. Chem.* **272**, 18939–18944.
Manoil, C., and Beckwith, J. (1986). *Science* **233**, 1403–1408.
Melchers, K., Herrmann, L., Mauch, F., Bayle, D., Heuermann, D., Weitzenegger, T., Schuhmacher, A., Sachs, G., Haas, R., Bode, G., Bensch, K., and Schafer, K. P. (1998). *Acta Physiol. Scand. Suppl.* **643**, 123–135.
Melchers, K., Schuhmacher, A., Buhmann, A., Weitzenegger, T., Belin, D., Grau, S., and Ehrmann, M. (1999). *Res. Microbiol.* **150**, 507–520.
Melchers, K., Weitzenegger, T., Buhmann, A., Steinhilber, W., Sachs, G., and Schafer, K. P. (1996). *J. Biol. Chem.* **271**, 446–457.
Michaelis, S., Guarente, L., and Beckwith, J. (1983). *J. Bacteriol.* **154**, 356–365.
Miller, J. (1992). *A Short Course in Bacterial Genetic*, Cold Spring Harbor Laboratory, New York.
Novick, R. P., and Roth, C. (1968). *J. Bacteriol.* **95**, 1335–1342.
Nucifora, G., Chu, L., Misra, T. K., and Silver, S. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 3544–3548.
Rensing, C., Fan, B., Sharma, R., Mitra, B., and Rosen, B. P. (2000). *Proc. Natl. Acad. Sci. USA* **97**, 652–656.
Rensing, C., Mitra, B., and Rosen, B. P. (1997). *Proc. Natl. Acad. Sci. USA* **94**, 14326–14331.
Rensing, C., Sun, Y., Mitra, B., and Rosen, B. P. (1998). *J. Biol. Chem.* **273**, 32614–32617.
Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Laboratory, New York.
Silhavy, T. J., and Beckwith, J. R. (1985). *Microbiol. Rev.* **49**, 398–418.
Silver, S., and Walderhaug, M. (1992). *Microbiol. Rev.* **56**, 195–228.
Smith, D. L., Tao, T., and Maguire, M. E. (1993). *J. Biol. Chem.* **268**, 22469–22479.
Solioz, M., and Odermatt, A. (1995). *J. Biol. Chem.* **270**, 9217–9221.
Solioz, M., and Vulpe, C. (1996). *Trends. Biochem. Sci.* **21**, 237–241.
Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000). *Nature* **405**, 647–655.
Tsai, K. J., Yoon, K. P., and Lynn, A. R. (1992). *J. Bacteriol.* **174**, 116–121.
von Heijne, G. (1989). *Nature* **341**, 456–458.