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ATP-Dependent Cadmium Transport by the *cadA* Cadmium Resistance Determinant in Everted Membrane Vesicles of *Bacillus subtilis*

KAN-JEN TSAI,[1] KYUNG PYO YOON,[2]† AND ANITA R. LYNN[1]*

Department of Biochemistry, Wayne State University School of Medicine, Detroit, Michigan 48201,[1] and Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, Illinois 60680[2]

Received 24 June 1991/Accepted 25 October 1991

Resistance to cadmium conferred by the staphylococcal plasmid pI258 occurs by means of energy-dependent efflux, resulting in decreased intracellular accumulation of cadmium. Recent sequence information suggested that efflux is mediated by a P-type ATPase. The *cadA* gene was previously expressed in *Bacillus subtilis*, conferring resistance to cadmium. Everted membrane vesicles were prepared from *B. subtilis* cells harboring either a plasmid containing the *cadA* system or the vector plasmid alone. $^{109}Cd^{2+}$ transport into the everted membranes was measured in the presence of various energy sources. Cadmium transport was detected only in the presence of ATP as an energy source. The production of an electrochemical proton gradient ($\Delta\mu_{H^+}$) by using NADH or phenazine methosulfate plus ascorbate was not able to drive transport. Reagents which dissipate ΔpH abolished calcium transport due to the Ca^{2+}/H^+ antiporter but only partially inhibited cadmium transport. Inhibition of transport by the antibiotic bafilomycin A_1 occurred at concentrations comparable to those which inhibit P-type ATPases. A band corresponding to the *cadA* gene product was identified on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and antibodies to the protein were prepared.

Plasmids from clinical isolates of *Staphylococcus aureus* which confer resistance to penicillin often contain genes which code for resistance to a variety of toxic ions, such as arsenic, lead, mercury, zinc, and cadmium (13). Cadmium probably enters cells of gram-positive bacteria as an alternative toxic substrate for the manganese active transport system (16, 23, 26). Two distinct cadmium resistance determinants, *cadA* and *cadB*, have been identified on *S. aureus* penicillinase plasmids (22). The *cadB* gene product may confer resistance by enhancing binding of cadmium to the cell, but the mechanism is not clear (16). The presence of the *cadA* determinant decreases the intracellular accumulation of cadmium (23, 26), suggesting that resistance is due to an active efflux of the toxic cation. Earlier studies have indicated that resistance is mediated by an energy-dependent efflux mechanism (24). The sensitivity of cadmium efflux to agents, such as DCCD (*N*,*N*′-dicyclohexylcarbodiimide) or nigericin, which prevent the development of a ΔpH led to the suggestion that efflux was mediated by an electroneutral $Cd^{2+}/2H^+$ antiporter (24, 25).

Recently, the *cadA* cadmium resistance determinant was subcloned and expressed in *Bacillus subtilis* (14, 28). The DNA sequence has two open reading frames: *cadC*, coding for a protein of 122 amino acids, and *cadA*, coding for a protein of 727 amino acids (14). Comparison of the predicted amino acid sequence of CadA with those of other proteins identified several regions of significant sequence similarity to members of a class of cation-translocating ATPases known as E_1E_2 or P-type ATPases (19, 20). These regions of sequence similarity include the putative ATP-binding site and a conserved region with an aspartate residue which has been demonstrated to be phosphorylated during the enzyme cycle in some P-ATPases (7). Because of these regions of sequence similarity, it was reported that this protein also may be a P-ATPase (14, 20).

In this paper, we report the assay of cadmium transport in everted membrane vesicles of *B. subtilis*. Using this assay, we have demonstrated that cadmium efflux is energized only by ATP, supporting the model of cadmium resistance mediated by a cadmium-transporting ATPase. Reagents that affect the proton-motive force only partially inhibited transport, whereas the Ca^{2+}/H^+ antiporter was completely inhibited. A protein band corresponding to the *cadA* gene product was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and polyclonal antibodies to that protein band have been prepared.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. subtilis* 1A46 harboring either plasmid pGN114 (14), containing the cadmium resistance determinant, or the parent vector pBD64 (8) were obtained from Simon Silver, University of Illinois, Chicago. Both plasmids were also transformed into *B. subtilis* IS75 (21) generously provided by Issar Smith, Public Health Research Institute of the City of New York. Plasmid pKPY7 was constructed by insertion of a *Nhe*I triple nonsense codon linker into the *Xho*I site of *cadA* as described previously (28). This plasmid was also transformed into strain IS75. Strains were maintained on Luria-Bertani (LB) plates (12) containing chloramphenicol (10 μg/ml) with or without 10 μM $CdCl_2$.

Materials. $^{109}CdCl_2$ was obtained from New England Nuclear (Boston, Mass.). $^{45}CaCl_2$ was from ICN Biomedicals, Inc. (Costa Mesa, Calif.). Freund's complete and incomplete adjuvants were obtained from Difco Laboratories (Detroit, Mich.). Horseradish peroxidase-conjugated mouse anti-rabbit immunoglobulin G was purchased from Sigma Chemical Co. (St. Louis, Mo.). Bafilomycin A_1 was a

* Corresponding author.
† Present address: Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125.

gift from K. Altendorf (Universität Osnabrück, Osnabrück, Germany).

Inhibition of growth by cadmium. Single colonies of strain IS75 alone or transformed with pBD64, pGN114, or pKPY7 were inoculated into 1 ml of LB medium containing 10 μg of chloramphenicol per ml (omitted for IS75 without a plasmid) and grown at 37°C with shaking at 200 rpm for 8 h. Tubes containing 5 ml of LB medium with 0 to 1 mM $CdCl_2$ were inoculated with 0.05 ml of the stationary-phase cultures and incubated at 37°C with shaking at 250 rpm for 16 h. Growth was measured as optical density at 600 nm.

Everted membrane vesicles. *Bacillus* cells containing pBD64, pGN114, or pKPY7 were grown at 37°C with shaking at 200 rpm to mid-exponential phase in LB medium containing chloramphenicol (5 μg/ml). Cadmium chloride was added as described below in each experiment. Cells were harvested by centrifugation and washed twice in 0.1 volume of 10 mM MOPS (morpholinepropanesulfonic acid)-KOH, pH 7.0. The washed cells were suspended in sucrose buffer (50 mM MOPS-KOH [pH 7.0], 250 mM sucrose, 200 mM KCl, 10 mM $MgSO_4$) at 5 ml/g of wet cells. Lysozyme was added to a final concentration of 1 mg/ml of buffer, and cells were shaken at 200 rpm at 37°C for 1 h. The protoplasts formed were lysed by passage through a French pressure cell at 10,000 lb/in^2 to obtain everted membrane vesicles. Cell debris and unbroken cells were removed by two sequential centrifugations at 12,000 × *g*. Membranes were pelleted by centrifugation of the low-speed supernatant at 160,000 × *g* for 90 min. The pellets were suspended in 100 mM MOPS-KOH (pH 7.0) at a concentration of 30 to 50 mg of protein per ml. The membrane vesicles were stored at −70°C until assayed. Protein concentration was estimated by a modification of the Lowry assay (3, 17). Membrane vesicles were solubilized at room temperature in sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.0125% bromphenol blue, and 5% β-mercaptoethanol, and the proteins were analyzed by SDS-PAGE (11).

Transport assays. Cadmium transport was measured as uptake of ^{109}Cd into the everted vesicles. Membranes (1 mg of total protein per ml) were assayed at room temperature in 20 mM bis-Tris-propane-HCl (pH 6.0)–200 mM KCl–50 μM $^{109}CdCl_2$ (0.4 μCi/ml). Reactions were started with the addition of 5 mM $MgSO_4$ with or without 5 mM ATP (sodium salt). Aliquots were removed at timed intervals and filtered onto 0.2-μm-pore-size cellulose nitrate filters. Filters were washed with 6 ml of 20 mM MOPS-KOH (pH 7.0)–200 mM KCl–10 mM $MgSO_4$–20 mM $CdCl_2$, dissolved in 5 ml of scintillation cocktail, and counted in a liquid scintillation counter.

Calcium transport was used as a control in some experiments and was measured in a fashion similar to that for cadmium transport as uptake of $^{45}Ca^{2+}$ into the everted vesicles. Membranes (1 mg of total protein per ml) were assayed in 20 mM MOPS-KOH (pH 7.0)–200 mM KCl–50 μM $^{45}CaCl_2$ (20 μCi/ml). Reactions were started with the addition of 5 mM $MgSO_4$ with or without 5 mM ATP, and assays were carried out as described above for cadmium transport but without $CdCl_2$ in the wash buffer.

Inhibitors or ionophores were added to assays to the following concentrations: DCCD, 50 μM; sodium *ortho*-vanadate, 50 μM; nigericin, 0.2 μM; valinomycin, 0.5 μM; FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone), 10 μM. Bafilomycin A_1 at several different concentrations from 5 to 200 μM was added to the assays. After addition of the inhibitor, the assays were incubated at room temperature for 10 min before the addition of ATP. Transport assays with energy sources other than ATP were performed as described above with the substitution of 5 mM NADH or 20 mM phenazine methosulfate plus 20 mM sodium ascorbate for ATP.

Induction of *cadA*. Cells containing plasmid pBD64, pGN114, or pKPY7 were grown in LB medium with 10 μg of chloramphenicol per ml without cadmium to an optical density at 600 nm of approximately 1.0. $CdCl_2$ at various concentrations was added to the cultures. Aliquots of the cultures were removed at timed intervals, and membrane vesicles were prepared as described above. Induction of the *cadA* gene product was measured by the presence of $^{109}Cd^{2+}$ transport and the appearance of a unique band on SDS–7% polyacrylamide gels.

Preparation of antibodies to CadA and immunoblot analysis. The CadA band identified by SDS-PAGE was cut out of preparative gels and electroeluted (Elutrap; Schleicher & Schuell, Inc., Keene, N.H.). The protein concentration of the electroeluted material was determined with bovine serum albumin as a standard (18). A total of 120 μg of eluted protein was emulsified in complete Freund's adjuvant (1:1, vol/vol) and injected subcutaneously into a rabbit. Two booster injections with the same amount of protein in incomplete Freund's adjuvant (1:1, vol/vol) were given at intervals of 10 days. Antiserum was preabsorbed with SDS-lysed whole-cell protein of *B. subtilis* containing the vector plasmid and used for immunoblot analysis.

Membrane proteins were electrophoresed on SDS–7% polyacrylamide gels as described above and transferred to nitrocellulose membrane by electroblotting overnight at 0.1 A in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol). Blots were washed once with 5% nonfat dry milk in phosphate-buffered saline (14.7 mM KH_2PO_4, 108 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl). Preabsorbed antibody was added to the final wash at 1:5,000 dilution and incubated at 37°C for 1 h. Blots were washed three times with milk and then incubated with a 1:2,500 dilution of goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase for 1 h at 37°C. After being washed with milk to remove unbound antibody, the blots were incubated with 20 ml of developer (0.5 mg of 4-chloro-1-naphthol per ml, 15% methanol, 0.03% H_2O_2 brought to volume with phosphate-buffered saline) until bands appeared. The reaction was stopped by addition of 7.5 mM NaOH.

RESULTS

Inhibition experiments demonstrate the degree of resistance conferred in *B. subtilis* by the presence of the plasmid containing *cadA*. Strains containing no plasmid, the vector plasmid pBD64 or pKPY7 (containing the *cadA* mutation) were unable to grow in LB containing 25 μM $CdCl_2$. Cells containing plasmid pGN114 continued to grow in cadmium concentrations as high as 0.5 mM (data not shown).

Transport assays. Cadmium transport measured as uptake of radioactive cadmium into everted membrane vesicles is shown in Fig. 1B. The data are plotted as net uptake (cadmium bound in the absence of ATP was subtracted at each time point). Everted membrane vesicles prepared from cells with plasmid pGN114 take up 2.5- to 5-fold more Cd^{2+} after 5 min in the presence of ATP than in its absence. Membranes prepared from cells containing the vector plasmid pBD64 or pKPY7 (containing the *cadA* mutation) took up no more $^{109}Cd^{2+}$ in the presence of ATP than in its absence.

Transport of cadmium and calcium by using various

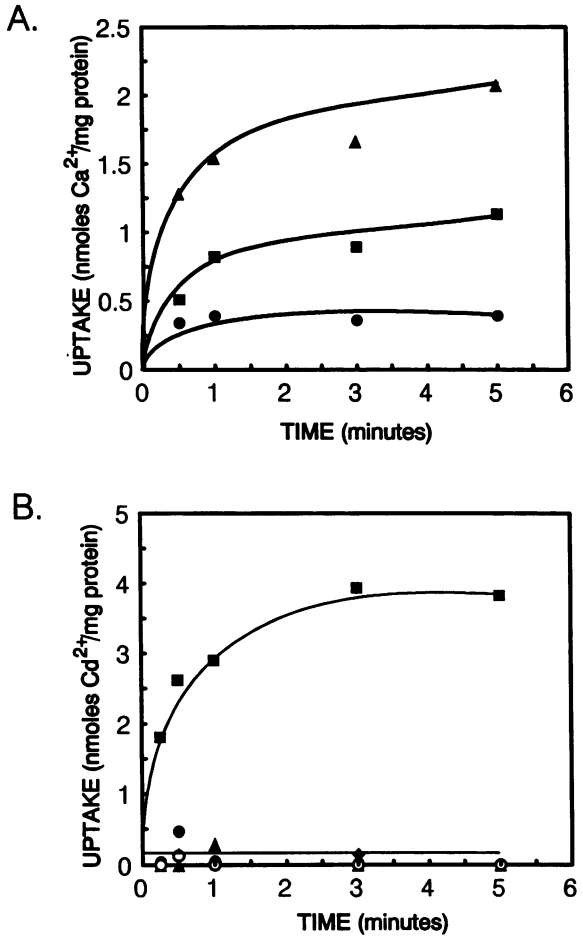

FIG. 1. $^{45}Ca^{2+}$ (A) and $^{109}Cd^{2+}$ (B) transport. Transport assays were performed with membrane vesicles of strain 1A46 containing pGN114 (cadmium and calcium) or either pBD64 or pKPY7 (cadmium only), as described in the text. ■, pGN114 with ATP as energy source; ○, pBD64 with ATP; ◆, pKPY7 with ATP; ●, pGN114 with NADH; ▲, pGN114 with phenazine methosulfate plus sodium ascorbate.

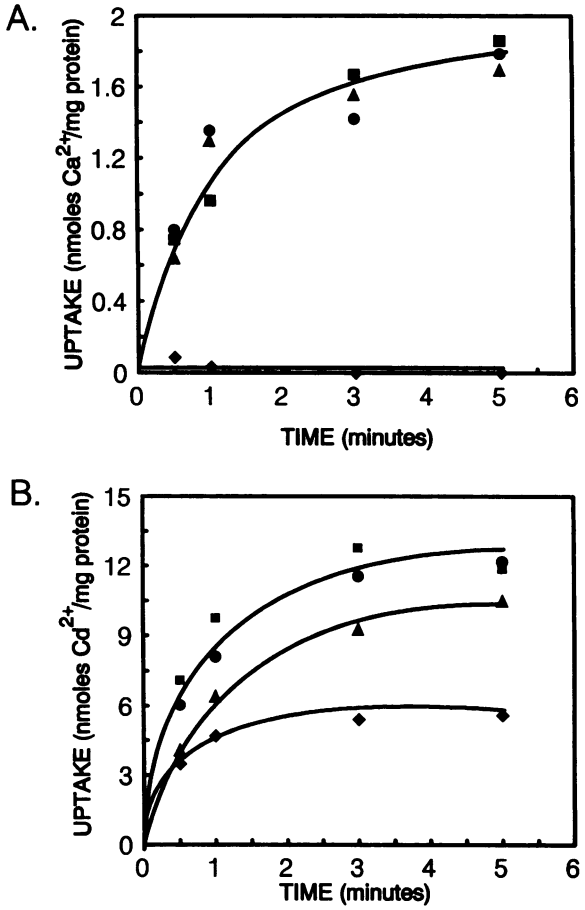

FIG. 2. Effect of inhibitors and ionophores on calcium (A) and cadmium (B) transport. Transport assays were performed with ATP as described in the text. ▲, no inhibitor; ◆, 50 μM DCCD, 0.2 μM nigericin or 10 μM FCCP; ●, 0.5 μM valinomycin; ■, 50 μM sodium *ortho*-vanadate.

energy sources is shown in Fig. 1. Transport of calcium by the Ca^{2+}/H^+ antiporter (6) was used as a control for the effects of energy source, inhibitors, and ionophores. Calcium transport can be driven by ATP, NADH, or phenazine methosulfate plus sodium ascorbate (Fig. 1A). However, cadmium transport was energized only by ATP (Fig. 1B).

The effect of several inhibitors and ionophores on cadmium and calcium transport is shown in Fig. 2. Vanadate, usually an inhibitor of P-ATPases, had no effect on either calcium or cadmium transport. The addition of DCCD, an inhibitor of the F_0F_1 ATPases, reduced cadmium transport by about 50% (Fig. 2B), while calcium transport was completely abolished (Fig. 2A). Nigericin, which acts to dissipate the ΔpH by electroneutrally exchanging K^+ and H^+, completely eliminated calcium transport and significantly inhibited cadmium transport. Elimination of the electrical potential with valinomycin had no effect on either cadmium or calcium transport. Calcium transport was also completely inhibited, and cadmium uptake was reduced to the same extent as that with nigericin when the electrochemical potential gradient ($\Delta\mu_{H^+}$) was eliminated with FCCP.

The effect of the macrolide antibiotic bafilomycin A_1, a potent inhibitor of vacuolar ATPases, on transport activity was tested (Fig. 3). Inhibition to 50% of control activity occurred at approximately 10 μM.

Induction of *cadA*. To determine whether cadmium transport activity is inducible or constitutively expressed, cells containing pGN114 or pBD64 were grown in cadmium-free medium. When cultures reached an optical density at 600 nm of 1.0, an aliquot was removed and the cells were harvested. $CdCl_2$ at concentrations of 1 μM for cultures containing pBD64 and 1 μM or 10 μM for cultures containing pGN114 was added to the cultures. Aliquots were removed periodically, and the cells were harvested. Membrane vesicles were prepared, and transport was assayed (Fig. 4). Transport activity increases significantly within 10 min after exposure to 1 μM $CdCl_2$ and reaches maximum activity within 40 min. Membranes were also analyzed by SDS-PAGE. The appearance of the unique band seen in Fig. 5 occurred concomitantly with the rise in transport activity (data not shown).

Identification of a unique membrane protein. SDS-PAGE of the membranes showed a unique band with an M_r of approximately 70,000 present in membranes from cells induced with cadmium and containing pGN114 (Fig. 5). This band is absent from membranes prepared from uninduced cells and from induced or uninduced cells containing pBD64.

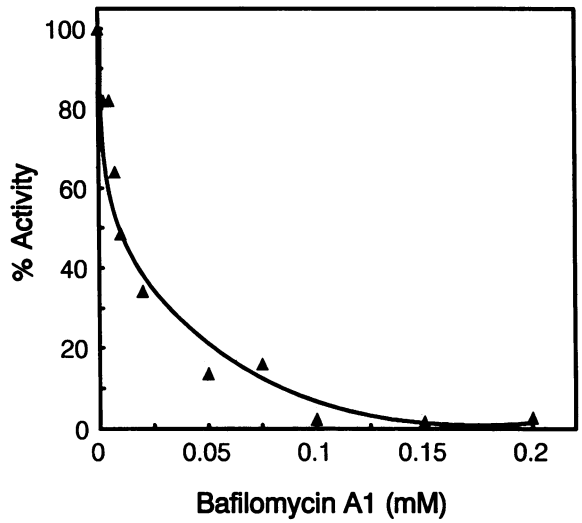

FIG. 3. Inhibition of cadmium transport by bafilomycin. $^{109}Cd^{2+}$ transport was assayed in membrane vesicles prepared from cells of strain 1A46 containing pGN114 in the presence of the antibiotic bafilomycin A_1 as described in the text. Transport activity as a percentage of total activity in the absence of bafilomycin versus the concentration of bafilomycin is plotted.

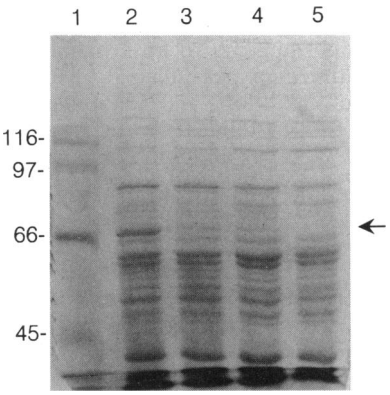

FIG. 5. SDS-PAGE. Strain 1A46 containing the plasmid pBD64 or pGN114 was grown to early-log phase, and then half of the culture was induced with 1 μM $CdCl_2$ for 1 h. Membranes were prepared from uninduced and induced cells as described in the text, electrophoresed on an SDS–7% polyacrylamide gel, and stained with Coomassie blue. Lane 1, molecular weight markers (identified on the left in thousands); lane 2, membrane proteins from cells containing pGN114 induced with $CdCl_2$; lane 3, membrane proteins from cells containing pGN114, uninduced; lane 4, membrane proteins from cells containing pBD64 induced with $CdCl_2$; lane 5, membrane proteins from cells containing pBD64, uninduced. The arrow indicates the unique band occurring concomitantly with the increase in transport activity.

Membranes from cells containing pKPY7 (28) with the mutation in *cadA* also lack this band (data not shown). Results of an immunoblot of total membrane protein from cells containing pGN114 with *cadA* grown with and without cadmium and from cells containing the vector plasmid pBD64 indicate that only cells containing pGN114 and grown in the presence of cadmium contained a protein which reacted with the antibody (data not shown).

DISCUSSION

In our studies, expression of the staphylococcal *cadA* determinant in *B. subtilis* confers at least 2,000-fold greater resistance to cadmium in organisms containing the plasmid. Disruption of the large open reading frame resulted in a level of resistance comparable to that of the strains containing either the vector plasmid or no plasmid at all. The level of resistance was similar to that reported in *S. aureus* by using a similar method (26). This suggests that expression of resistance is comparable in both species. However, another laboratory using a different strain has reported resistance levels in *B. subtilis* nearly 10-fold lower than that in *S. aureus* (28) and considerably lower than those we report here. The reason for the difference in resistance from one *B. subtilis* strain to the next is not clear.

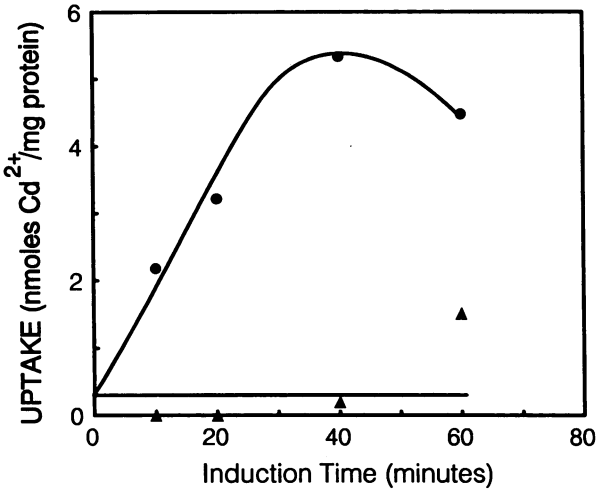

FIG. 4. Induction of cadmium transport. Membrane vesicles were prepared from cells containing the vector plasmid pBD64 (▲) or the cadmium resistance plasmid pGN114 (●), induced with 1 μM $CdCl_2$ and assayed for cadmium transport as described in Materials and Methods.

Attempts to prepare everted membranes which were competent for transport from *S. aureus* were unsuccessful (data not shown). Since resistance was expressed well in *B. subtilis*, transport studies were carried out with membranes from that organism. A Ca^{2+}/H^+ antiporter in *B. subtilis* had been described previously (6), so $^{45}Ca^{2+}$ transport was used as a measure of the capability of membrane preparations to transport and for comparison in the inhibitor studies.

Cadmium uptake in the presence of ATP was 2.5- to 5-fold higher than that in the controls lacking ATP. This indicates that an actively transporting system is operating in these membranes. The level of cadmium uptake with the vector plasmid or the plasmid containing the disrupted gene was similar to that obtained with no added ATP.

Everted membrane vesicle preparations from cells containing the cadmium resistance determinant were capable of transporting $^{109}Cd^{2+}$ in the presence of ATP (Fig. 1B). Membrane vesicles energized by production of a proton motive force by other means, such as with NADH or phenazine methosulfate and sodium ascorbate, were able to transport calcium by the Ca^{2+}/H^+ antiporter but not Cd^{2+}. This suggests that cadmium transport is not mediated by an antiporter as has been previously proposed (24, 25) but is instead due to a primary pump. This is in agreement with the mechanism suggested recently by Nucifora et al. (14) based

on the predicted amino acid sequence of the *cadA* gene. The *cadA* gene product appears to be a member of a class of cation-translocating enzymes called P-ATPases.

The effects of inhibitors and ionophores on cadmium transport did not follow the pattern that would be expected for a P-ATPase. Whereas inhibition of calcium transport by nigericin, FCCP, and DCCD was consistent with the activity of an antiporter, one would not expect these reagents to significantly inhibit the activity of a primary pump. However, these results are in agreement with previous studies in which nigericin was shown to inhibit efflux of cadmium from whole cells (24, 25). It is clear from the studies with alternative energy sources that cadmium transport cannot be mediated by an antiporter such as that for calcium transport in *B. subtilis*. The inhibition of transport by reagents that affect ΔpH was not complete in the case of cadmium, whereas calcium transport was completely abolished under the same conditions. It might be argued that these results suggest a requirement for a pH gradient in addition to ATP for cadmium transport to occur. However, inhibitor studies with crude preparations are frequently ambiguous since there can be multiple effects of the reagents. Perhaps the best approach to resolve this discrepancy will be to reconstitute the activity in artificial liposomes and determine the requirements for transport. These studies are in progress.

Sensitivity to *ortho*-vanadate is characteristic of the enzymes in this class; however, this sensitivity is somewhat dependent upon assay conditions. The degree of inhibition of vanadate on the *Neurospora* plasma membrane H^+-ATPase depends on the ionic concentrations and on the concentration of ATP (4). In addition, the effects of *ortho*-vanadate can be difficult to detect in crude preparations. For example, ATP-dependent calcium transport in streptococci was considerably more sensitive to *ortho*-vanadate when transport was assayed with solubilized and reconstituted proteoliposomes instead of with crude membrane vesicles (1, 9). In one of the earliest reports of the inhibitory effects of vanadate, the Ca^{2+}-ATPase of the sarcoplasmic reticulum was described as insensitive to *ortho*-vanadate (10). Later work showed that the Ca^{2+}-ATPase is inhibited by vanadate but that detection of inhibition is dependent upon assay conditions, especially on whether the effects are measured with membranes or with purified protein (15). It was also reported that the small amounts of calcium present in sarcoplasmic reticulum vesicles provided protection against inhibition of vanadate and the addition of the calcium ionophore A23187 was necessary to detect sensitivity in vesicles (15). The effects of vanadate on the Cd^{2+}-ATPase will be investigated further with more-purified protein in future studies.

Bafilomycin A_1 is a potent inhibitor of the vacuolar ATPases (5). Members of this class of ATPases are inhibited by concentrations of the antibiotic in the nanomolar range. The F_1F_0-ATPases are not affected by this inhibitor at concentrations of up to 1 mM, and the P-type ATPases are intermediate in their sensitivity to this inhibitor with 50% inhibitory values in the micromolar range (5). The 50% inhibitory value for bafilomycin determined for cadmium transport activity was 10 μM, well within the range previously reported for P-ATPases. This supports the view that the CadA protein is a member of the class of P-ATPases.

As shown in Fig. 5, a protein corresponding to the *cadA* gene product was identified by SDS-PAGE as a unique band appearing in membranes prepared from cells containing the gene. The estimated molecular weight of the band is 70,000, somewhat lower than the value of 78,812 based on the predicted amino acid sequence. The faster mobility of the band compared with that which would have been predicted is not an uncommon occurrence in membrane proteins (2).

In the induction experiments, membranes from cells containing *cadA* grown in the presence of chloramphenicol only to maintain the plasmid show no cadmium transport (Fig. 4). The addition of cadmium at concentrations as low as 1 μM resulted in an increase in transport activity in membrane vesicles prepared from cells containing pGN114 with the *cadA* insert but not in membrane vesicles prepared from cells containing the parent plasmid pBD64, thus indicating that *cadA* is inducible. The amount of protein increased concomitantly with an increase in transport activity (data not shown). These data agree with recent studies by Yoon et al., who demonstrated induction of the *cadA* operon with cadmium as well as other cations in growth studies and with a β-lactamase translational fusion to the *cadA* gene (27). Immunoblot analysis indicates that the unique band seen with SDS-PAGE is not present in cells without *cadA*. In cells that do contain the gene, the protein is detectable only when the cells have been induced with cadmium. These experiments along with the appearance of the band in membranes only from cells containing *cadA* grown in the presence of cadmium strongly support the identification of this band as the *cadA* gene product.

In this paper, we have provided evidence that the cadmium resistance determinant, *cadA*, encodes an ATP-dependent cadmium efflux system as previously suggested by sequence data (14, 20). Our data support the proposal that cadmium efflux is mediated by an ATPase, possibly a P-type ATPase, rather than an antiporter. The inhibition of cadmium transport by agents acting on the proton-motive force may suggest that this ATPase has unique properties. The gene product of *cadA* has been identified and purified from denaturing polyacrylamide gels. Attempts to determine the N-terminal amino acid sequence of the purified protein indicate a blocked N terminus (data not shown).

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