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The *pacL* Gene of *Synechococcus* sp. Strain PCC 7942 Encodes a Ca^{2+}-Transporting ATPase†

TOM BERKELMAN,* PHILLIP GARRET-ENGELE,‡ AND NEIL E. HOFFMAN

Department of Plant Biology, Carnegie Institution of Washington, Stanford, California 94305

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An ATP-dependent Ca^{2+} uptake activity was identified in plasma membrane vesicles prepared from *Synechococcus* sp. strain PCC 7942. This activity was insensitive to agents which collapse pH gradients and membrane potentials but sensitive to vanadate, indicating that the activity is catalyzed by a P-type Ca^{2+}-ATPase. A gene was cloned from *Synechococcus* sp. strain PCC 7942 by using a degenerate oligonucleotide based on a sequence conserved among P-type ATPases. This gene (*pacL*) encodes a product similar in structure to eukaryotic Ca^{2+}-ATPases. We have shown that *pacL* encodes a Ca^{2+}-ATPase by demonstrating that a strain in which *pacL* is disrupted has no Ca^{2+}-ATPase activity associated with its plasma membrane. In addition, Ca^{2+}-ATPase activity was restored to the Δ*pacL* strain by introducing *pacL* into a second site in the *Synechococcus* sp. strain PCC 7942 chromosome.

Ca^{2+}-ATPases belong to a superfamily of cation transporters called P-type ATPases that are found in both prokaryotes and eukaryotes. These enzymes are distinguished by formation of a phosphorylated intermediate during the reaction cycle, inhibition by vanadate ion, and a requirement for Mg^{2+} as well as the ion(s) transported (2). P-type ATPases share several regions with a high degree of sequence identity. These conserved sequences have been used by several groups in the design of degenerate oligonucleotide probes used to clone these enzymes (7, 11, 18).

In eukaryotic organisms, Ca^{2+} serves as a signaling ion. Ca^{2+}-ATPases play an integral role in this system by maintaining steep Ca^{2+} gradients across cellular membranes (12). In addition, Ca^{2+}-ATPases are thought to protect against the toxic effects of calcium in the cytoplasm. Cytoplasmic Ca^{2+} is generally maintained at submicromolar concentrations, whereas extracellular Ca^{2+} can be several orders of magnitude higher. In some cases, disruption of a gene encoding a Ca^{2+}-ATPase results in a sensitivity of the organism to elevated Ca^{2+} in the medium (6, 11).

Ca^{2+} transport is less well studied in prokaryotic organisms. It is known that bacteria maintain cytoplasmic Ca^{2+} levels in the submicromolar range (9). Most studied species appear to pump Ca^{2+} out of the cytoplasm by secondary transport mediated by a Ca^{2+}/H^+ or Ca^{2+}/Na^+ antiport (25); however, primary Ca^{2+} transport catalyzed by Ca^{2+}-ATPases has been described in *Streptococcus faecalis* (19) and *Flavobacterium odoratum* (8) as well as the cyanobacterium *Anabaena variabilis* (20). The possible role of Ca^{2+} in intracellular signaling is also less well understood in bacteria. Intracellular Ca^{2+} has been shown to be involved in *Escherichia coli* chemotaxis (30). In cyanobacteria, Ca^{2+} is implicated to play a role in motility responses (1, 16) and in heterocyst differentiation (29).

In this report, we describe a calcium-transporting ATPase associated with plasma membranes from *Synechococcus* sp. strain PCC 7942. In addition, we describe the cloning of the gene encoding this activity (recently also cloned by another group [18]) and confirm that this gene encodes the Ca^{2+}-ATPase by demonstrating that disruption of the gene results in loss of activity and that reinsertion of the gene results in restoration of activity in the disrupted strain.

* Corresponding author. Carnegie Institution of Washington, Department of Plant Biology, 290 Panama St., Stanford, CA 94305. Phone: 415-325-1521 ext. 331. Fax: 415-325-6857. Electronic mail address: tom@andrew.stanford.edu.

† Carnegie Institution of Washington publication no. 1210.

‡ Present address: Department of Biology, Stanford University, Stanford, CA 94305.

MATERIALS AND METHODS

Reagents. $^{45}CaCl_2$ was obtained from NEN. Solid medium for cyanobacterial growth was prepared by using Bacto-Agar from Difco. The ATP (Na_2ATP, Sigma catalog no. A-5394, low vanadium content) and other reagents were purchased from Sigma. DNA modification reagents (polymerases and restriction enzymes, etc.) were obtained from New England Biolabs, U.S. Biochemical Corp., Stratagene, or Pharmacia. Inorganic reagents were reagent grade or better. All aqueous solutions were prepared with distilled, deionized water. Solutions used in Ca^{2+} transport assays were prepared and stored in plastic containers.

Cell growth. *Synechococcus* sp. strain PCC 7942 was kindly supplied by Jackie Collier. It was grown at 32°C under continuous illumination in BG-11 medium either in liquid culture or on plates containing 1.2% agar (4). Liquid cultures were bubbled with 3% CO_2. Transformed strains were grown under continuous antibiotic selection. Spectinomycin was used at 5 μg/ml for liquid culture and 20 μg/ml for plates. Kanamycin was used at 10 μg/ml for liquid culture and 25 μg/ml for plates.

Plasma membrane purification. Plasma membranes were prepared as described by Murata and Omata (21), with the following modifications. Cells were grown in 2.5-liter batches, which yielded 2 to 3 g of cells after 3 days of growth. The cells were washed as described previously (21), suspended in 40 ml of 10 mM sodium *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES-NaOH) (pH 7.0)–600 mM sucrose–2 mM EDTA–0.075% lysozyme and incubated at 37°C for 2 h. The cells were washed again as described previously (21), resuspended in 20 ml of 20 mM TES-NaOH–600 mM sucrose, and passed twice through a French pressure cell at 40 MPa. Subsequent manipulations and sucrose step-gradient separation were carried out as described previously (21). The purified membranes were resuspended in 500 mM sucrose–10 mM TES-NaOH (pH 7.0)–10 mM NaCl to a concentration of 4 to

12 mg of protein/ml. The protein concentration was determined by A_{275} as described by Murata and Omata (21). The membranes were frozen in liquid nitrogen and stored at −80°C.

Ca^{2+} transport assays. Ca^{2+} transport assays were conducted similarly to those previously described (3) in an assay medium consisting of 250 mM sorbitol–50 mM *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid)-1,3-bis[tris(hydroxymethyl)-methylamino]propane (HEPES-BTP) (pH 8.0)–20 mM NaCl–20 mM KCl–5 mM $MgCl_2$–1 mM ATP–500 μM dithiothreitol–20 μM $CaCl_2$ containing 0.5×10^7 to 1×10^7 cpm of ^{45}Ca per ml at room temperature (24°C). Negative control reactions were also routinely run without ATP. In experiments in which the effect of ethanol-soluble potential inhibitors (i.e., valinomycin, nigericin, or carbonyl cyanide *m*-chlorophenylhydrazone [CCCP]) was tested, these substances were added by 1:1,000 dilution of ethanolic stocks. In these cases, the positive control reaction contained an equivalent amount of ethanol. *Synechococcus* sp. strain PCC 7942 plasma membranes were thawed on ice, and the reactions were started by the addition of membranes to a concentration of 50 μg of protein/ml of reaction mixture. Membranes used for transport assays were thawed only once. Calcium uptake was assayed at various times by pipetting duplicate 100-μl samples onto 25-mm-wide 0.45-μm-pore-size mesh filters (Millipore) prewetted in wash medium (250 mM sorbitol, 50 mM HEPES-KOH [pH 8.0], 20 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$) on a vacuum manifold. Vacuum was applied, and the filters were rinsed twice with 5 ml of wash medium. The filters were then placed in scintillation vials and allowed to dry overnight. Associated ^{45}Ca was determined by liquid scintillation counting. Radioactivity in a portion of the unfiltered reaction was also determined to allow calculation of the amount of Ca^{2+} taken up.

Purification of *Synechococcus* sp. strain PCC 7942 DNA. A DNA purification procedure which incorporated elements from references 13 and 27 was used. Cells were grown to late log phase, and 10- to 50-ml samples were centrifuged 5 min at $3,600 \times g$. The pellet was resuspended in 25 ml of 150 mM NaCl–50 mM EDTA (pH 8.0) and repelleted as described above. This pellet was resuspended in 10 ml of 25% sucrose–50 mM Tris-HCl (pH 8.0)–100 mM EDTA–0.2% lysozyme and incubated at 37°C. After 1 h, 50 μl of 20 mg of proteinase K per ml and 200 μl of 25% sarkosyl were added, and the lysate was incubated at 55°C. After 30 min, 10 ml of 1.5 M NaCl–100 mM Tris-HCl (pH 8.0)–20 mM EDTA–2% hexadecyltrimethylammonium bromide (CTAB) was added. The lysate was mixed thoroughly by inversion and incubated another 10 min at 65°C. An equal volume of chloroform-isoamyl alcohol (24:1) was then added, and the mixture was centrifuged for 10 min at $5,200 \times g$ after thorough mixing. The upper phase was transfered to a new tube, and the nucleic acids were precipitated by the addition of a 0.7 volume of isopropanol and recovered by centrifugation for 2 min at $3,600 \times g$. The pelleted nucleic acids were resuspended in 4 ml of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA and 16 μl of 10 mg of RNase A per ml was added. After 30 min, 4 μl of 20 mg of proteinase K per ml and 20 μl of 20% sodium dodecylsulfate (SDS) were added, and the mixture was incubated at 55°C for a further 30 min. After three extractions with phenol-chloroform-isoamyl alcohol (25:24:1) saturated with Tris-HCl (pH 8.0), the DNA was precipitated by the addition of 0.1 volume sodium acetate (pH 5.2) and 2 volumes of ethanol and recovered by brief centrifugation. This pellet was washed with 70% ethanol, air-dried, and resuspended in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. This DNA preparation was found to digest well with every restriction endonuclease tested.

Cloning of *pacL*. A genomic library was constructed from *Synechococcus* sp. strain PCC 7942 DNA by cloning fragments from a partial *Sau*3AI digest in the range of 1 to 4 kb into the *Xho*I site of λZAP (Stratagene) by 2-base fill-in. This library was screened with a ^{32}P-labeled degenerate oligonucleotide, obtained from Alan Bennett, which had previously been used to clone P-type ATPases from *Lycopersicon esculentum* (7, 32). The inosine-containing 29-mer [5′-GGIGCA(G)TCG(A)TTI ACICCA(G)TCICCIGTCAT-3′] was derived from the sequence MTGDGVNDAP, which is conserved in many P-type ATPases and is thought to form the ATP-binding site (2). Hybridization was performed overnight at 47°C in 6× standard saline citrate (SSC) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS–0.1% bovine serum albumin–0.1% polyvinylpyrrolidone–0.1% Ficoll–0.05% $NaPP_i$–100 μg of sheared, denatured salmon sperm DNA per ml. Washes were carried out in 6× SSC–0.05% sodium pyrophosphate at 47°C (28). Plasmid was recovered from the λZAP clones according to Stratagene instructions. Sequencing of these plasmids and appropriate subclones was done with a *Taq* polymerase thermal cycle sequencing kit (Promega).

PCR cloning of a portion of URF5. A portion of the *Synechococcus* sp. strain PCC 7942 homolog of the *Synechococcus* sp. strain PCC 6301 URF5 was amplified by PCR with oligonucleotide primers derived from the published sequence of URF5 (5). Primer 1 (sense) had the sequence 5′-CGCCT GCCTTAGAAGCTGATAGTCG-3′ and corresponded to nucleotides 4687 to 4711 of the published sequence. The last 4 bases of this primer overlap a *Sal*I site. Primer 2 (antisense) had the sequence 5′-CAAGAATTCGGGTAGGACTCAT CGG-3′ and corresponded to nucleotides 3748 to 3771 of the published sequence. The sixth base of primer 2 was an A rather than the T found in the published sequence in order to facilitate cloning by creating an *Eco*RI site in the PCR product. A standard 35-cycle PCR reaction was carried out with 2 U of *Taq* polymerase, 25 pmol of each primer, and 100 ng of purified *Synechococcus* sp. strain PCC 7942 DNA in a volume of 100 μl (28). Each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 58°C, and 1.5 min of extension at 72°C. The product was gel purified, digested with *Eco*RI and *Sal*I, and ligated into Bluescript SK+ (Stratagene).

Preparation of genetic constructs. The construct used for the insertional inactivation of *pacL* was designated pPacL::spec (Fig. 1). The parent plasmid for pPacL::spec was pAN1, which came from a λZAP library and consisted of a 1,297-bp portion of *pacL* (from 2,214 to 3,511 in the sequence as it appears in GenBank) in pBluescript SK+ (Stratagene). pPacL::spec was prepared by ligating a 2,056-bp *Bam*HI fragment containing the spectinomycin resistance gene from pHP45Ω (23) into the *Xho*I site of pAN1 following 2-base fill-in. The construct used for the insertional inactivation of URF5 was designated pURF5::spec (Fig. 1). The parent plasmid for pURF5::spec was the cloned PCR product described above. pURF5::spec was prepared by ligating a 2,066-bp *Sma*I fragment containing the spectinomycin resistance gene from pHP45Ω into the *Msc*I site of the PCR product.

The pPGR4 vector used to insert genes into the chromosome of *Synechococcus* sp. strain PCC 7942 consisted of a kanamycin resistance gene and a multiple cloning site flanked by portions of the *Synechococcus* sp. strain PCC 7942 *phoA* gene (Fig. 1). It was prepared by using portions of the pSP72 vector (Promega), the *phoA* gene (24), and the kanamycin resistance gene from Tn*903* (22). A 2,810-bp *Sal*I-*Bam*HI

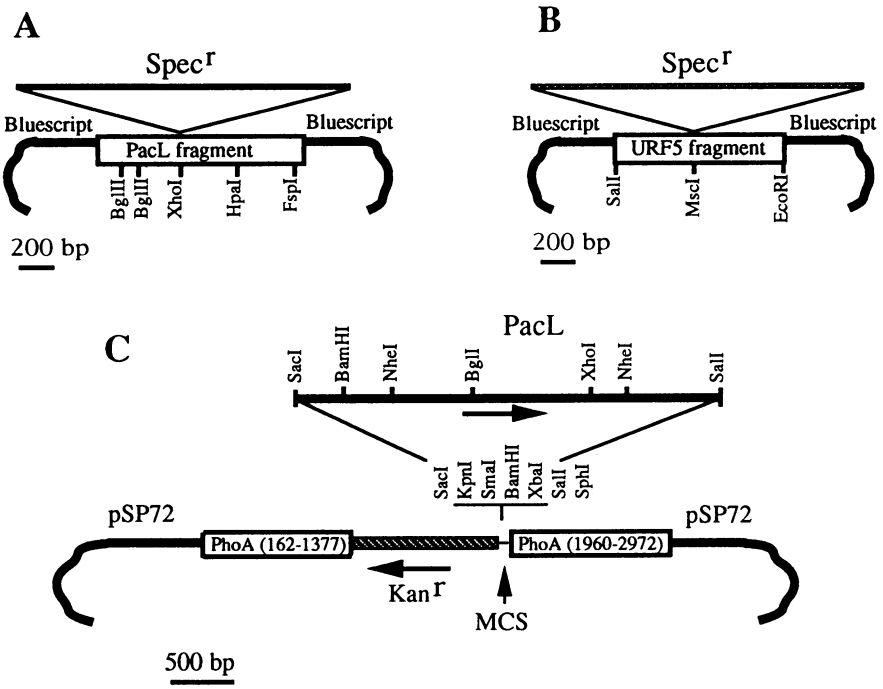

FIG. 1. Constructs used in this investigation. (A) Diagram of pPacL::spec, the construct used for the disruption of *pacL*. (B) Diagram of pURF5::spec, the construct used for the disruption of "URF5." (C) Diagram of pPGR-PacL, the construct used for the second-site complementation of the ΔPacL strain. Selected restriction sites are indicated. Spec^r and Kan^r indicate spectinomycin and kanamycin resistance genes, respectively. MCS indicates the partial pSP72 multiple cloning site. Arrows indicate the direction of transcription.

fragment of *phoA* was ligated into pSP72 cut with *Bgl*II and *Xho*I, creating the plasmid pPhoA. The kanamycin resistance gene was ligated as an approximately 1,300-bp blunt-ended fragment into the *Eco*RV site of pSP72 with the 5′ end of the *kan* gene towards the SP6 promoter, creating the plasmid pKAN1. pPGR4 was created by digesting pPhoA with *Ecl*136II and *Bsp*EI (resulting in the excision of 585 bp of the *phoA* gene), filling in the ends with a Klenow fragment, and ligating in a similarly blunted *Pvu*II-*Bgl*II fragment from pKAN1 containing the kanamycin resistance gene and a portion of the multiple cloning site. A *pacL*-containing derivative of pPGR4 (pPGR-PacL) was created by ligating a 3,553-bp *Sac*I-*Sal*I fragment containing the *pacL* gene into the multiple cloning site of pPGR4 (Fig. 1).

Transformation of *Synechococcus* sp. strain PCC 7942. Transformation of *Synechococcus* sp. strain PCC 7942 was done essentially as previously described (14).

Southern blotting. DNA prepared from *Synechococcus* sp. strain PCC 7942 was digested to completion with either *Bgl*II or *Nhe*I. Samples consisting of 0.5 to 1 μg of DNA were electrophoresed through 0.7% agarose and capillary blotted onto Hybond N+ membranes (Amersham) according to the manufacturer's instructions. The blots were probed with ^{32}P-labeled restriction fragments (28). Low-stringency hybridizations were carried out at 47°C, with a final wash in 2× SSC at 47°C. High-stringency hybridizations were carried out at 65°C, with a final wash in 0.1× SSC at 65°C. The probe for *pacL* was either a 251-bp *Bgl*II-*Xho*I fragment or a 989-bp *Bgl*I-*Xho*I fragment from the *pacL* gene. The probe for URF5 was the cloned PCR product described above.

Sequence analysis. Polypeptide sequence analysis employed various programs of the Genetics Computer Group software package, including TFASTA for database homology searches, GAP for pairwise comparison of individual sequences, and PILEUP for multiple sequence comparisons.

RESULTS

Ca^{2+} transport activity in plasma membrane vesicles from *Synechococcus* sp. strain PCC 7942. Plasma membrane vesicles from *Synechococcus* sp. strain PCC 7942 prepared according to the method of Murata and Omata (21) exhibited ATP-dependent sequestration of the Ca^{2+} ion. The results of a typical Ca^{2+} uptake experiment are shown in Fig. 2. When assayed in the presence of ATP as described in Methods and Materials, these vesicles reproducibly sequestered 7 to 15 nmol of Ca^{2+}/mg of protein over the course of 40 min (Fig. 2 to 5 and data not shown). This rate of Ca^{2+} uptake was linear over the first 8 to 12 min of the assay (data not shown) and was found to vary between 0.3 and 1 nmol of Ca^{2+} min^{-1} mg of $protein^{-1}$. Although some Ca^{2+} associated with the membranes in the absence of ATP, the amount of Ca^{2+} associated did not increase significantly with time and was typically severalfold less than that seen in the presence of ATP after 40 min. Scavenging ATP with hexokinase and glucose or chelating extravesicular Ca^{2+} with ethyleneglycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid (EGTA) caused Ca^{2+} uptake to cease and allowed observation of passive Ca^{2+} efflux from loaded vesicles. The addition of the divalent ionophore A23187 resulted in Ca^{2+} efflux to below background levels within 1 min.

Effects of inhibitors. The effects of ionophores and vanadate were tested in order to determine the nature of this Ca^{2+} transport activity (Fig. 3). Ca^{2+} uptake was insensitive to the combined effects of 5 μM CCCP and valinomycin. As the effect of this combination of ionophores should be the abolition of

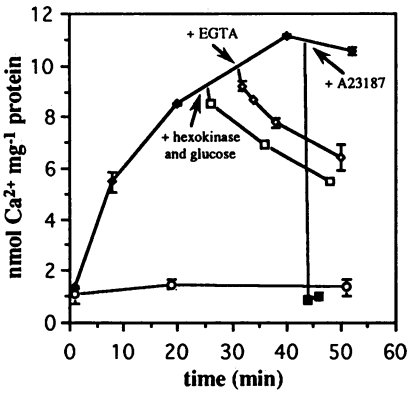

FIG. 2. Calcium transport in plasma membrane vesicles. *Synechococcus* sp. strain PCC 7942 plasma membranes were prepared as described in the text. Ca^{2+} uptake was assayed as described in the presence (◆) and absence (○) of 1 mM ATP. Hexokinase and glucose (□), EGTA (◇), and A23187 (■) were added to separate portions of the reaction mixture with ATP, as indicated, to concentrations of 3 U/ml and 20 mM, 0.5 mM, and 5 μM, respectively. Each datum point is an average of the uptake determined for two replicate samples. Error bars indicate the range of the data. The effects of hexokinase and glucose, EGTA, and A23187 were verified by at least one additional experiment for each of these treatments.

any pH gradient or transmembrane potential difference, it was concluded that the observed Ca^{2+} transport represented primary Ca^{2+} transport catalyzed by an ATPase rather than secondary Ca^{2+} transport catalyzed by an antiport or a channel and dependent upon ΔpH or $\Delta\Psi$. The activity was found to be equally insensitive to 5 μM nigericin (data not shown). The Ca^{2+} transport activity was, however, completely inhibited by

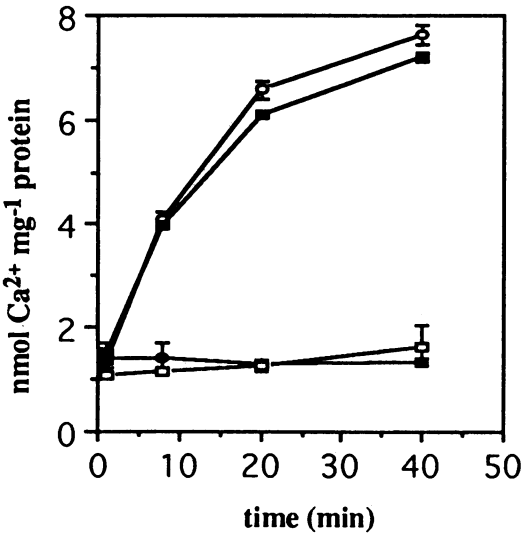

FIG. 3. Ca^{2+} transport in the presence of vanadate or CCCP and valinomycin. *Synechococcus* sp. strain PCC 7942 plasma membranes were prepared as described in the text. Ca^{2+} uptake was assayed as described in the presence (○) and absence (●) of 1 mM ATP and in the presence of 1 mM ATP plus vanadate (□) (250 μM Na_3VO_4) and in the presence of 1 mM ATP plus CCCP and valinomycin (■) (each at 5 μM). Each datum point is an average of the uptake determined for two replicate samples. The effects of the ionophores and vanadate were verified in at least two additional experiments.

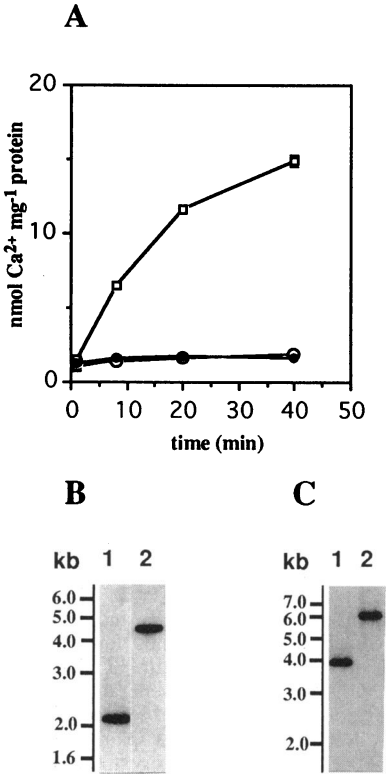

FIG. 4. ATP-dependent Ca^{2+} transport in membranes from strains with disrupted copies of *pacL* or "URF5." (A) *Synechococcus* sp. strain PCC 7942 plasma membranes were prepared as described in the text from the indicated mutated strains. Ca^{2+} uptake was assayed in the presence (+ATP) and absence (−ATP) of 1 mM ATP. □, ΔURF5 + ATP; ●, ΔURF5 − ATP; ■, ΔPacL + ATP (partially obscured by the ΔURF5 − ATP symbols); ○, ΔPacL − ATP (partially obscured by the ΔURF5 − ATP symbols). Each datum point is an average of the uptake determined for two replicate samples. Error bars indicate the range of the data. Similar data were obtained with five separate plasma membrane preparations from ΔPacL and wild-type cells. (B) Southern blot probed with a *pacL* probe. Lanes: 1, *Bgl*II digest of wild-type *Synechococcus* sp. strain PCC 7942 DNA; 2, *Bgl*II digest of ΔPacL DNA. (C) Southern blot probed with "URF5" probe. Lanes: 1, *Bgl*II digest of wild-type *Synechococcus* sp. strain PCC 7942 DNA; 2, *Bgl*II digest of ΔURF5 DNA. The positions of size standards are indicated on the left.

250 μM vanadate. These data indicate that the observed ATP-dependent Ca^{2+} transport is catalyzed by a P-type Ca^{2+}-ATPase.

Cloning of P-type ATPases from *Synechococcus* sp. strain PCC 7942. A screen of a *Synechococcus* sp. strain PCC 7942 genomic library with a degenerate oligonucleotide probe designed to hybridize with P-type ATPases resulted in the isolation of several cross-hybridizing clones. Restriction mapping determined these clones to be overlapping DNA fragments covering a single gene. After this gene had been sequenced, a publication describing a gene, termed *pacL*, encoding a putative P-type ATPase in *Synechococcus* sp. strain PCC 7942 appeared (18). The nucleotide sequence of *pacL* is identical to ours.

Although only one gene was isolated by screening with a degenerate oligonucleotide, a search of GenBank revealed that a portion of another P-type ATPase had been cloned from a closely related strain, *Synechococcus* sp. strain PCC 6301.

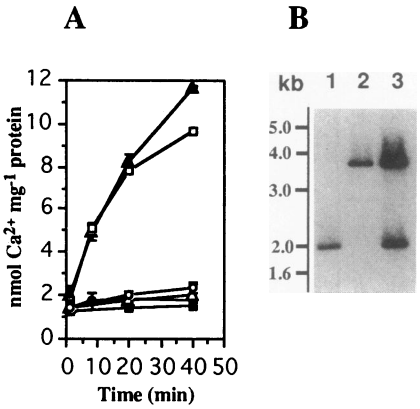

FIG. 5. ATP-dependent Ca^{2+} transport in membranes from wild-type cells, ΔPacL cells, and ΔPacL cells with a wild-type *pacL* gene inserted at a second site. (A) *Synechococcus* sp. strain PCC 7942 plasma membranes were prepared as described in the text from the indicated mutated strains. Ca^{2+} uptake was assayed in the presence (+ ATP) and absence of (− ATP) of 1 mM ATP. □, wild type + ATP; ▲, complemented ΔPacL + ATP; ●, ΔPacL + ATP; △, wild type − ATP; ○, complemented ΔPacL − ATP; ■, ΔPacL −ATP. (The last four sets of symbols partially obscure one another). Each datum point is an average of the uptake determined for two replicate samples. Error bars indicate the range of the data. Similar data were obtained with two different plasma membrane preparations from complemented ΔPacL cells. (B) Southern blot probed with a *pacL* probe. Lanes: 1, *Nhe*I digest of wild-type *Synechococcus* sp. strain PCC 7942 DNA; 2, *Nhe*I digest of ΔPacL DNA; 3, *Nhe*I digest of complemented ΔPacL DNA. The positions of size standards are indicated on the left.

This gene had been termed URF5 (5). With PCR primers designed against URF5, an appropriately sized fragment was amplified from *Synechococcus* sp. strain PCC 7942 DNA. This PCR product had a number of restriction sites in common with the published sequence of *Synechococcus* sp. strain PCC 6301 URF5 including an *Msc*I site predicted to be within the region encoding the putative ATP-binding site. In addition, Southern blots probed with this PCR product revealed bands with sizes consistent with those predicted by the published sequence (data not shown). It was therefore concluded that *Synechococcus* sp. strain PCC 7942 has a gene similar, if not identical, to *Synechococcus* sp. strain PCC 6301 "URF5." This is consistent with the close genetic identity noted between these two strains (31).

pacL and URF5 did not hybridize to each other at low stringency, and Southern blots of *Synechococcus* sp. strain PCC 7942 DNA probed at low stringency with either *pacL* or URF5 did not reveal any additional cross-hybridizing fragments (data not shown).

Ca^{2+} transport properties of mutagenized strains. Constructs for insertional mutagenesis of *pacL* and URF5 were prepared by inserting a spectinomycin gene into cloned fragments of these ATPase genes (Fig. 1). Restriction sites were chosen within or near the regions encoding the putative ATP-binding sites. These constructs were designed to have at least 450 bp of homologous sequence on either side of the resistance marker. Transformation with these constructs and selection on spectinomycin-containing plates resulted in the creation of strains which could be shown by Southern blotting to have an approximately 2,000-bp insertion within the ATPase gene targeted (Fig. 4B and C). These strains are referred to, respectively, as ΔPacL and ΔURF5. Neither of these strains exhibited reduced growth in BG-11 medium under the conditions employed, and neither strain had any easily visible phenotype.

The two insertionally mutagenized strains were grown side by side in the presence of spectinomycin, and plasma membrane vesicles were prepared from them in parallel. ATP-dependent Ca^{2+} transport was assayed in each preparation simultaneously under identical conditions (Fig. 4). Membranes from ΔPacL cells were found to completely lack ATP-dependent Ca^{2+} transport, while membranes from ΔURF5 cells showed levels of Ca^{2+} transport comparable to those of the wild type. This suggested that the observed Ca^{2+} transporter is encoded by *pacL*.

In order to unequivocally establish that *pacL* encodes a Ca^{2+}-ATPase, we reintroduced a wild-type copy of *pacL* into a second site in the chromosome of the ΔPacL strain. This was done by using the pPGR4 vector (Fig. 1), which was designed to introduce the gene of interest and a kanamycin resistance gene into the chromosome of *Synechococcus* sp. strain PCC 7942 by insertion into the *phoA* gene (24). Disruption of *phoA* causes no phenotype when cells are grown in normal BG-11 medium. A 3,559-bp DNA fragment containing the entire open reading frame of *pacL* with 572 bp of untranslated sequence at the 5′ end was inserted into the multiple cloning site of pPGR4 to create the complementation construct termed pPGR-PacL. ΔPacL cells were transformed with pPGR-PacL and plated onto medium containing both kanamycin and spectinomycin. This resulted in a strain in which both a wild-type and a disrupted copy of *pacL* could be detected by Southern blotting (Fig. 5B) and in which the *phoA* gene had an insertion of approximately 4,900 bp (the combined size of the kanamycin resistance gene and the inserted copy of *pacL*) (data not shown). Introduction of *pacL* into the ΔPacL strain in this manner restored wild-type levels of ATP-dependent Ca^{2+} transport to plasma membranes prepared from the transformed cells (Fig. 5).

Experiments to determine ion sensitivity of the ΔPacL strain. On the basis of the results of studies in which Ca^{2+}-ATPase mutants were generated from other microorganisms (6, 11), we expected that the ΔPacL strain would show a Ca^{2+}-sensitive phenotype. We therefore tested the ability of wild-type and ΔPacL cells to grow in BG-11 medium containing additional Ca^{2+} in concentrations up to 150 mM. To our surprise, the ΔPacL cells displayed no noticeable hypersensitivity to elevated Ca^{2+} levels (data not shown). The same conclusion was arrived at independently by Kanamaru et al. (18). They also found no hypersensitivity to Mg^{2+}, Co^{2+}, Zn^{2+}, Hg^{2+}, Cd^{2+}, or Cu^{2+} in their ΔPacL strain. We tested the effects of Mn^{2+} (in concentrations up to 10 mM) and La^{3+} (in concentrations up to 100 μM) on our ΔPacL strain and on wild-type cells and observed no additional sensitivity of the mutant strain to these ions (data not shown).

DISCUSSION

Although Kanamaru et al. noted the similarity between the product of *pacL* and eukaryotic Ca^{2+}-ATPases (18), they did not experimentally verify the function of *pacL*. This report therefore represents the first description of a prokaryotic Ca^{2+}-ATPase for which the primary structure is known. All of the data concerning the ATP-dependent Ca^{2+} uptake activity found in plasma membrane vesicles from *Synechococcus* sp. strain PCC 7942 are consistent with this activity being mediated by a P-type Ca^{2+}-ATPase. The insensitivity of the activity to agents which dissipate pH gradients or membrane potentials indicates that the direct source of energy for the transport is ATP hydrolysis. The sensitivity to vanadate indicates that the

transporter is probably a P-type ATPase (2). The effects of hexokinase, glucose, and EGTA indicate that the activity requires both a continuous supply of ATP and free calcium ions, further supporting the idea that the transport is catalyzed by a Ca^{2+}-ATPase. In addition, the rapid effect of the divalent ionophore A23187 indicates that calcium is being transported into membrane vesicles rather than being bound or sequestered in some other manner. Although leakage is observed from loaded vesicles when ATP is scavenged with hexokinase and glucose or when the amount of free Ca^{2+} is lowered with EGTA, vesicles prepared in this manner appear to be sufficiently sealed to the Ca^{2+} ion to observe ATP-dependent Ca^{2+} transport without the use of a Ca^{2+}-trapping agent such as oxalate.

This Ca^{2+}-ATPase appears to be similar to one that has been described for *A. variabilis* (20). This, along with the recent report of a gene encoding a putative Ca^{2+}-ATPase in *Synechocystis* sp. strain PCC 6803 (10), suggests that plasma membrane Ca^{2+}-ATPases may be a common feature among cyanobacteria.

Having established that plasma membranes from *Synechococcus* sp. strain PCC 7942 contain Ca^{2+}-ATPase activity, we demonstrated that this activity is encoded by *pacL*. Plasma membranes from the ΔPacL strain completely lacked Ca^{2+}-ATPase activity whereas plasma membranes from the ΔURF5 strain had activity comparable to that of the wild type. Furthermore, we can restore wild-type activity to ΔPacL cells by reintroduction of the *pacL* gene into a second site in the *Synechococcus* sp. strain PCC 7942 chromosome.

In order to observe ATPase-catalyzed transport into membrane vesicles, the active site of the enzyme must be accessible to Ca^{2+} and ATP supplied externally. Since the active site of the enzyme probably faces the cytoplasm, it is likely that the in vivo direction of Ca^{2+} transport catalyzed by the product of *pacL* is from the cytoplasm into the periplasm and extracellular medium. The ability to do these measurements demonstrates that at least some of the plasma membrane vesicles in the preparation used have an inside-out orientation.

We found that ΔPacL cells would grow as well as the wild type with elevated levels of Ca^{2+}. This result was surprising, as we expected the *pacL*-encoded ATPase to pump Ca^{2+} out of the cell in order to maintain cytoplasmic Ca^{2+} at low levels. If this is the case, we would expect cytoplasmic Ca^{2+} to accumulate to high levels when ΔPacL cells are grown in medium containing elevated levels of Ca^{2+}. Possible explanations for the lack of a Ca^{2+}-sensitive phenotype include either a lack of a toxic effect of high cytoplasmic Ca^{2+} or the presence of alternative mechanisms for transporting Ca^{2+} out of the cell. Both Ca^{2+}/H^+ and Ca^{2+}/Na^+ antiports are known in other species of bacteria (25) and may be found in *Synechococcus* sp. strain PCC 7942 as well. Ca^{2+} transport by these mechanisms may not have been detected under the assay conditions used in this study. If such transporters were present, they may either have been inactivated in the membrane preparation or have had too low an affinity for Ca^{2+} to be detectible in the presence of micromolar concentrations of Ca^{2+}. Plasma membranes prepared and assayed in the manner used in this study may also be incapable of maintaining H^+ or Na^+ gradients necessary for antiport function or may have no means of generating such gradients. We tested for the presence of an alternative Ca^{2+}-ATPase induced by high levels of Ca^{2+} by assaying ATP-dependent Ca^{2+} transport in membranes from ΔPacL cells grown in the presence of 40 mM $CaCl_2$. No activity was detected (data not shown); hence, this alternative was ruled unlikely.

When this study was initiated, we thought it likely that a cyanobacterial Ca^{2+}-ATPase would show a high degree of similarity to *PEA1*, a putative Ca^{2+} transporter found in higher plant chloroplasts (17), as chloroplasts are believed to be derived from prokaryotic endosymbionts similar to cyanobacteria. As it turns out, the degree of similarity between the products of *pacL* and *PEA1* is not any higher than that found among Ca^{2+}-ATPases in general. The published sequence most similar to *pacL* is *PMA1*, a putative Ca^{2+}-ATPase from *Synechocystis* sp. strain PCC 6803 (10) (40% identity and 61% similarity, as determined by the GAP program). The next highest degrees of similarity were found to *PMR1*, a putative Ca^{2+} transporter from *S. cerevisiae* involved in secretion (26) (37% identity and 60% similarity) and a mammalian gene encoding an apparent homolog of *PMR1* (15) (38% identity and 59% similarity). *pacL* showed a similar degree of similarity to sarcoplasmic/endoplasmic reticulum-type Ca^{2+}-ATPases. Less similarity was found to *Arabidopsis thaliana PEA1* (17) (32% identity and 55% similarity) and mammalian plasma membrane Ca^{2+}-ATPases.

The identification of a *Synechococcus* sp. strain PCC 7942 homolog of the "URF5" putative ATPase identified in *Synechococcus* sp. strain PCC 6301 (5), along with *pacL* and *pacS* (18), brings the number of P-type ATPase genes identified in *Synechococcus* sp. strain PCC 7942 to three. Although this study did nothing to elucidate the function of the URF5 homolog, the ΔURF5 mutant should allow such a characterization to be possible. The presence of three nonessential P-type ATPases in an easily manipulated model system such as *Synechococcus* sp. strain PCC 7942 should aid both in structure-function studies of this class of enzymes and in understanding their physiological roles. The ΔPacL strain should be a useful tool for studying Ca^{2+} homeostasis and the possible signaling role of Ca^{2+} in prokaryotic cells. The osmotically sensitive phenotype noted by Kanamaru et al. (18) in a *pacL*-disrupted strain suggests a connection between calcium homeostasis and osmoregulation that should be amenable to further study.

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