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An Alkaline Phosphatase/Phosphodiesterase, PhoD, Induced by Salt Stress and Secreted Out of the Cells of *Aphanothece halophytica*, a Halotolerant Cyanobacterium▽†

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Alkaline phosphatases (APases) are important enzymes in organophosphate utilization. Three prokaryotic APase gene families, PhoA, PhoX, and PhoD, are known; however, their functional characterization in cyanobacteria largely remains to be clarified. In this study, we cloned the *phoD* gene from a halotolerant cyanobacterium, *Aphanothece halophytica* (*phoD*$_{Ap}$). The deduced protein, PhoD$_{Ap}$, contains Tat consensus motifs and a peptidase cleavage site at the N terminus. The PhoD$_{Ap}$ enzyme was activated by Ca^{2+} and exhibited APase and phosphodiesterase (APDase) activities. Subcellular localization experiments revealed the secretion and processing of PhoD$_{Ap}$ in a transformed cyanobacterium. Expression of the *phoD*$_{Ap}$ gene in *A. halophytica* cells was upregulated not only by phosphorus (P) starvation but also under salt stress conditions. Our results suggest that *A. halophytica* cells possess a PhoD that participates in the assimilation of P under salinity stress.

Phosphorus (P) is an essential nonmetal nutrient for all living cells. Despite its relative abundance in the ecosystem, P is sometimes a limiting factor for organisms in terrestrial, oceanic, and freshwater environments (1, 11). This is because while all organisms can utilize soluble inorganic phosphate (P_i), the P-containing organic compounds generally found in nature are complex insoluble forms that are not readily available to cells (1, 11). Alkaline phosphatases (APases) release free P_i from organic compounds. To date, three prokaryotic APase gene families—PhoA, PhoX, and PhoD—have been documented (7). In addition to different levels of homology among these APases, dissimilar metal requirements for their activities have been reported (7). A recent metagenomics analysis revealed that PhoX, a recently characterized phosphatase, is more abundant in marine bacteria than the previously considered classical PhoA (12). However, it has also been shown that PhoD is more abundant in marine bacteria than PhoA or PhoX (7), suggesting an important role for PhoD in marine bacteria. PhoD encompasses a family of phosphatase/phosphodiesterases (APase/APDase). Except for *Bacillus subtilis* PhoD (PhoD$_{Bs}$) (3), little is known on other PhoD proteins. PhoD$_{Bs}$ was shown to be secreted into extracellular medium by the Tat pathway, which recognizes targeted proteins by their N-terminal twin-arginine signal peptides containing the Tat consensus (SRRXFLK) motif (3, 9).

Cyanobacteria inhabit a broad range of ecosystems and play a vital role in the global cycling of nutrients, including P. Hitherto, extensive studies on the regulation of gene expression during phosphate stress have been carried out (14). In contrast, functional properties of cyanobacterial APases have been reported only on classical PhoA (8), atypical APase (11), PhoV (21), and PhoX (5), and there is no report on the cyanobacterial PhoD. Moreover, little is known on the role of APase under high-salinity conditions, and this encouraged us to examine the role of cyanobacterial PhoD under high-salinity conditions.

Aphanothece halophytica is a halotolerant cyanobacterium isolated from the Dead Sea and can grow in a wide range of salinity conditions (0.25 to 3.0 M NaCl), as well as at alkaline pH (17). Previous studies have shown that *A. halophytica* has a unique biosynthetic pathway of the osmoprotectant betaine, three-step methylation of glycine (19) and novel Na^+/H^+ antiporters NhaP1$_{Ap}$, NapA1-1$_{Ap}$, NapA1-2$_{Ap}$, and Mrp$_{Ap}$ (4, 18, 23). In the present study we investigated the presence of novel APases in *A. halophytica* and report a gene homologous to PhoD$_{Bs}$, termed PhoD$_{Ap}$. Our data reveal that PhoD$_{Ap}$ is a secreted APase/APDase and is activated by Ca^{2+}. Finally, we demonstrate the salt-inducible expression of *phoD*$_{Ap}$, suggesting that PhoD$_{Ap}$ is involved in P assimilation under salinity stress.

MATERIALS AND METHODS

Culture conditions for cyanobacteria. The cyanobacteria *Synechococcus elongatus* PCC7942 and *A. halophytica* were grown photoautotrophically in BG11 alone and in BG11 plus Turk Island salt solution containing 0.5 M NaCl, respectively, as described previously (20), under continuous cool white fluorescent light intensity of 70 $\mu E\ m^{-2}\ s^{-1}$ at 30°C. For P starvation, *A. halophytica* cells collected by centrifugation were resuspended into the medium in which KH_2PO_4 was replaced with KCl (0.18 mM). For salinity stress, collected cells were resuspended into the medium containing 2.5 M NaCl.

Fractionation of the cyanobacterial cells. In order to study the subcellular localization of PhoD$_{Ap}$, extracellular, cell-bound, periplasmic, and total soluble

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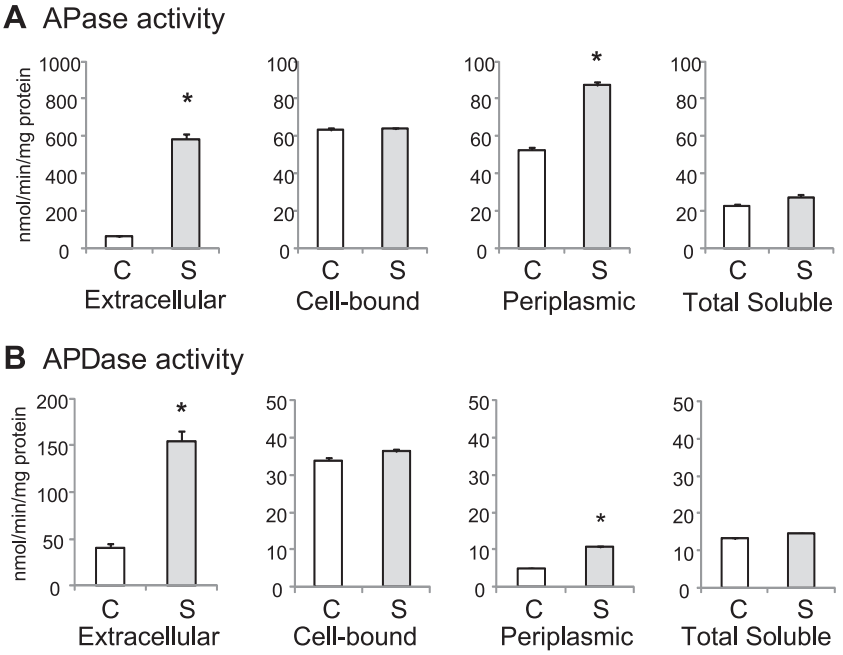

FIG. 1. APase (A) and APDase (B) activity in *A. halophytica* cells. *A. halophytica* cells grown in nutrient solution containing 0.5 M NaCl were transferred to medium containing 0.5 M NaCl (C columns) or 2.5 M NaCl (S columns) and incubated under standard growth conditions for 6 h. The APase and APDase activities of concentrated extracellular medium, whole cells (cell-bound), periplasmic fractions, and soluble protein extract were determined. Each value is an average of three independent measurements. Means ± the standard errors of the mean (SEM) are shown ($n = 3$). An asterisk indicates significant difference ($P < 0.05$) from the control (C).

fractions from *A. halophytica* cells were prepared. To obtain the total soluble fraction, cyanobacterial cells (50 ml, $A_{730} = 0.8$) were harvested and resuspended in 1 ml of cold extraction buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$ [pH 10]). The suspended cells were lysed by sonication and centrifuged at 2,290 × *g* for 10 min at 4°C, followed by centrifugation twice at 22,000 × *g* for 30 min at 4°C. The resulting supernatant was used as soluble fraction, from which 20-μl aliquots were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide gel. Proteins transferred onto polyvinylidene difluoride membranes and subjected to Western blot analysis. Pellets representing the total membrane fraction were also subjected to Western blot analysis; pellets were dissolved in 500 μl of SDS sample buffer, and then 10-μl aliquots were separated by SDS-PAGE. The periplasmic fraction of these cyanobacterial cells was obtained as described earlier (1), except that the pH of the buffer was maintained at 10. Approximately 1 ml of periplasmic fractions was obtained; 20 μl of the aliquot was subjected to Western blot analysis.

For analyzing the proteins present in extracellular medium, 50 ml of the cyanobacterial cell culture was centrifuged at 2,290 × *g* for 5 min at 4°C. The cell-free supernatant was filtered through a 0.22-μm-pore-size membrane (Millipore, Billerica, MA), and the resulting solution was concentrated by using YM-3 membrane (Millipore) to ~1 ml, 30 μl of which was used for measurement of the APase/APDase activity or for Western blot analysis.

Expression of PhoD$_{Ap}$ in *Escherichia coli* and its purification. The coding region of *phoD$_{Ap}$* was amplified from the genomic DNA of *A. halophytica* by PCR using specific primers (see Table S1 in the supplemental material). PCR products were subcloned into vector pCR2.1 (Invitrogen, California) and digested with NcoI and SalI. The resulting fragments were ligated into the digested NcoI and SalI sites of pTrcHis2C (Invitrogen). The generated plasmid, pTrcHis2C-ApphoD, was transferred to *E. coli* BL21. Transformed cells were grown in 3 ml of LB medium at 37°C for 4.5 h, and the culture was transferred to 1 liter of LB medium and subcultured at 37°C for 3 h. IPTG (isopropyl-β-D-thiogalactopyranoside) was added at a final concentration of 0.5 mM, and the culture was allowed to grow for a further 3 h. The cells were harvested and subjected to affinity purification by using an Ni-NTA spin kit column (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The eluate containing the protein of interest was subjected to Superdex 75 gel filtration column chromatography using a 50 mM Tris-HCl (pH 8.0) buffer containing 100 mM NaCl and 0.1 mM $CaCl_2$. PhoD$_{Ap}$ containing fractions were concentrated using an YM-3 membrane and subjected to anion-exchange chromatography (Resource Q; Amersham, Little Chalfont, United Kingdom) using a buffer containing 50 mM Tris-HCl (pH 10.0) and 0.1 mM $CaCl_2$. PhoD$_{Ap}$ was eluted using a 0 to 100% NaCl gradient. All purification steps were carried out at 4°C. Gel filtration and anion-exchange chromatography was carried out using an AKTA prime liquid chromatography system (GE Healthcare Life Science, Little Chalfont, United Kingdom).

Measurement of APase/APDase activities. The bacterial APases identified thus far are metalloenzymes requiring specific metal ions, i.e., Mg^{2+}, Zn^{2+}, and Ca^{2+}, for their activities (7). We therefore examined the metal requirement for the APase/APDase activity of PhoD$_{Ap}$-His$_6$. Portions (20 μl) of fractionated solutions from cyanobacterial cells or purified recombinant PhoD$_{Ap}$ was mixed with 40 μl of 50 mM Tris-HCl (pH 10.0) containing various concentrations of metal and *p*-nitrophenyl (*p*NP) phosphate (*p*NPP) or *bis*-*p*NPP at a final concentration of 13.5 μM. The mixture was incubated at 37°C for 20 min. The reaction was stopped by the addition of 240 μl of 13% KH_2PO_4, and the absorbance of *p*NP was measured at 400 nm. Enzyme activity (expressed as nmol min^{-1} mg of $protein^{-1}$) was calculated by using the molar extinction coefficient (ε), i.e., 18,900 M^{-1} cm^{-1} for *p*NP at 400 nm (2). The total protein concentration in the sample solution was measured by the Bradford method (19).

For kinetic studies of recombinant PhoD$_{Ap}$ purified from *E. coli*, the same methods were used except that various amounts of substrates in the reaction mixtures were used (i.e., 0.43, 0.85, 1.7, 3.4, 6.8, and 13.5 μM). The concentration of the PhoD$_{Ap}$ protein in the reaction mixture was kept constant at 8.3 μg/ml.

Reverse transcriptase PCR (RT-PCR) analysis. *A. halophytica* cells (25 ml) were cultured to achieve a turbidity (A_{730}) of 0.8. The cells were harvested and resuspended in 500 μl of extraction buffer (100 mM Tris-HCl, 10 mM EDTA, 100 mM LiCl, 1% SDS [pH 8.0]). After the addition of 500 μl of Tris-buffered phenol, the mixture was incubated on ice for 5 h, followed by centrifugation at 10,000 × *g* for 30 min at 4°C. To the aqueous fraction, 250 μl of 8 M LiCl was added, followed by incubation on ice overnight. The supernatant was removed after centrifugation at 10,000 × *g* for 1 h at 4°C. The resulting RNA precipitation was solubilized in sterile distilled water and re-extracted with phenol-chloroform.

The cDNA was synthesized from 1 μg of total RNA by using RT M-MLV (Takara, Otsu, Japan). Portions (20 μl) of synthesized cDNA were diluted two times with double-distilled H_2O, and 2-μl aliquots were subjected to PCR in a reaction volume of 50 μl. Specific primer sets (see Table S1 in the supplemental material) were at a final concentration of 0.4 μM in the PCR mixture. RT-PCR

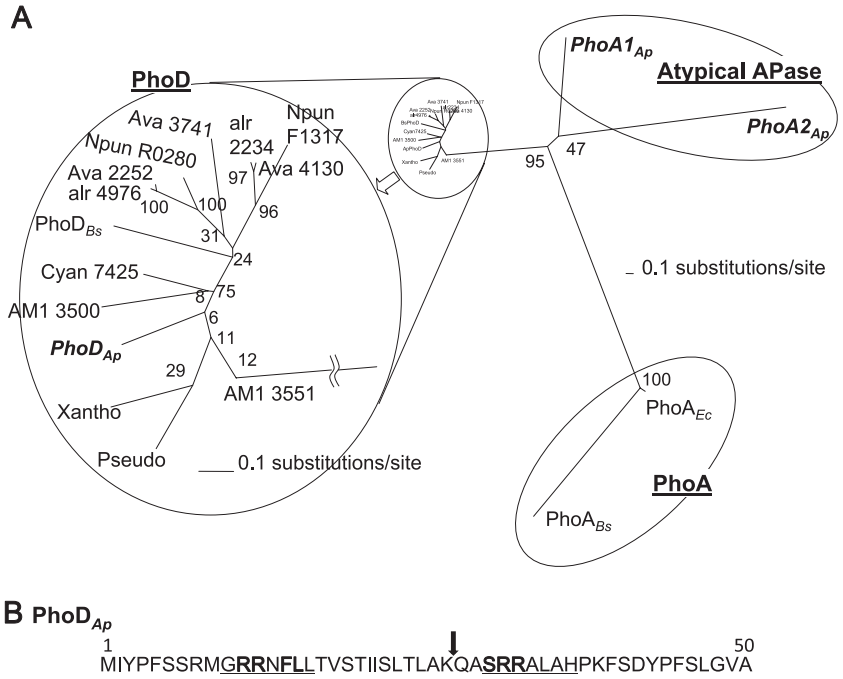

FIG. 2. (A) Phylogenetic analysis of APases. A radial tree of the APases shows PhoA1$_{Ap}$ (accession number, AB602344), PhoA2$_{Ap}$ (AB602345), and PhoD$_{Ap}$ (AB602343) from *A. halophytica*; AM1 3550 (AM1_3550) and AM1 3551(AM1_3551) from *Acaryochloris marina* MBIC11017, Cyan7425 (Cyan7425_1386) from *Cyanothece* sp. strain PCC 7425, Ava 2252 (Ava_2252), Ava 3741 (Ava_3741), and Ava 4130 (Ava_4130) from *Anabaena variabilis* ATCC 29413, alr 2234 (alr2234) and alr 4976 (alr4976) from *Anabaena* sp. strain PCC 7120, Npun R0280 (Npun_R0280) and Npun F1317 (Npun_F1317) from *Nostoc punctiforme* PCC 73102, Pseudo (EFW82021) from *Pseudomonas syringae* pv. glycinea strain B076, Xantho (YP_001905643) from *Xanthomonas campestris*, PhoA$_{Bs}$ (BSU09410) and PhoD$_{Bs}$ (BSU02620) from *Bacillus subtilis*, and PhoA$_{Ec}$ (b0383 JW0374) from *Escherichia coli*. The bars represent evolutionally distance. The scale bar is 0.1 expected changes per amino acid site. The reliability of the tree was measured by bootstrap analysis with 1,000 replicates. The branches of the PhoD proteins were magnified for clarity. (B) Predicted signal peptides for PhoD$_{Ap}$. Underlined text denotes Tat consensus motifs. An arrow at the position between 26 and 27 denotes the peptide cleavage site as predicted by SignalP, version 3.0.

was initiated with AmpliTaq Gold (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The PCR conditions used were as follows: denaturation at 95°C for 3 min and then 27 amplification cycles of denaturation for 15 s at 95°C, annealing for 30 s at 55°C, and extension for 45 s at 72°C. The resulting reaction mixtures were subjected to 1% agarose gel electrophoresis and visualized by staining with ethidium bromide. The intensity of the PCE product as a clear band on the gel was quantified by ImageJ software and was normalized by setting the signals from nonstressed cells to 1.0.

Expression of PhoD$_{Ap}$ in the cyanobacterium *Synechococcus elongatus*. The region containing both the *trc* promoter and the coding region of *phoD$_{Ap}$* was amplified from pTrcHis2C-ApphoD by PCR using a specific primer set (see Table S1 in the supplemental material). PCR products were subcloned into vector pCR2.1 and digested with XhoI. The resulting fragments were ligated into XhoI-digested pUC303, which can replicate independently in *S. elongatus* cells (6). Ten micrograms of generated plasmid was added to 1 ml of *S. elongatus* culture (optical density at 730 nm of 0.5), followed by culture with shaking for 12 h in the dark. Transformants were selected using streptomycin (25 μg/ml) on 1.5% agar BG11 medium.

Measurement of cellular P contents. *A. halophytica* cells grown at 0.5 M NaCl were harvested by centrifugation and suspended in medium containing 0.5 or 2.5 M NaCl. After 6 h growth, the cells were collected and washed twice with water. The cells were dried by vacuum evaporation at 65°C, followed digestion in a boiling mixture of nitric acid (HNO_3) and sulfuric acid (H_2SO_4) (5:1), and incubated at ambient temperature for 10 min with H_2SO_4, antimony potassium tartrate, ammonium molybdate, and ascorbic acid at final concentrations of 0.47 M, 75 μM, 1.7 mM, and 11 mM, respectively. The mixture was centrifuged at 10,000 × *g* for 5 min at 4°C. The supernatant was used for measurement of the cellular P contents in *A. halophytica* cells using the absorption at 880 nm.

Other methods. The nucleotide sequences were determined by using an ABI310 genetic analyzer (Applied Biosystems, Foster City, CA). The protein concentration was measured by using the Bradford method. SDS-PAGE and Western blot analysis were carried out as described previously (23). Antibody-raised against a His_6 (hexahistidine) tag was obtained from R&D Systems (Minneapolis, MN). For the phylogenetic analysis, CLUSTAL W (15) was used to generate a phylogenetic tree of amino acid sequences of bacterial APases/ADPases.

Nucleotide sequence accession numbers. Nucleotide sequence data for *phoA1$_{Ap}$*, *phoA2$_{Ap}$*, and *phoD$_{Ap}$* from *A. halophytica* are available in the DDJB database under accession numbers AB602344, AB602345, and AB602343, respectively.

RESULTS

APase/APDase activity in *A. halophytica*. In a previous study (10), we reported that under conditions of high salinity, the intracellular P content and P uptake in *Anabaena doliolum* cells decreased, whereas the extracellular APase activity increased. Similar results were obtained with *A. halophytica* cells (data not shown). Therefore, we first investigated the effect of salinity stress on APase and APDase activities of *A. halophytica* cells (Fig. 1). To do so, we prepared extracellular, cell-bound, periplasmic, and total soluble fractions from *A. halophytica* cells exposed to salinity stress for 6 h and measured the APase and APDase activities by using *p*NPP and *bis-p*NPP, respectively, as substrates. Exposure of *A. halophytica* to salinity stress enhanced the extracellular and periplasmic APase activities by 9- and 1.7-fold, respectively (Fig. 1A). Salinity stress also enhanced the extracellular and periplasmic APDase activities by 3.8- and 2.1-fold, respectively (Fig. 1B). These results

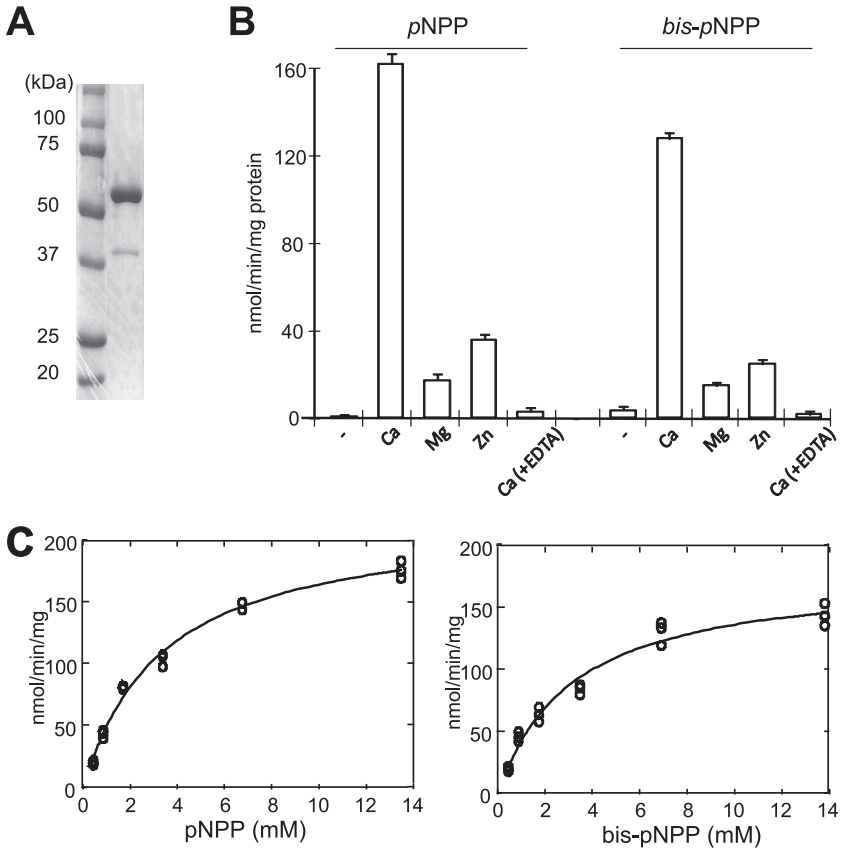

FIG. 3. Characterization of $PhoD_{Ap}$. (A) Purified $PhoD_{Ap}$-His_6 was subjected to SDS-PAGE and stained with Coomassie brilliant blue. (B) Effect of metal ions on APase/APDase activity of $PhoD_{Ap}$-His_6. Activity was measured with each metal ion and EDTA at a final concentration of 5 mM. Each value represents an average of three independent measurements. Means ± the SEM are shown. (C) Determination of kinetic values for APase and APDase activities of $PhoD_{Ap}$. The values of kinetic constants were computed using the freeware program SIMFIT (http://www.simfit.man.ac.uk).

indicated that the extracellular APase and APDase activities of *A. halophytica* cells increased under high-salinity conditions.

Phylogenetic analysis of *A. halophytica* APases. From shotgun sequencing data, several putative APase genes were found in the *A. halophytica* genome. In Fig. 2A, three genes, $phoA1_{Ap}$, $phoA2_{Ap}$, and $phoD_{Ap}$, are shown. $PhoA1_{Ap}$ and $PhoA2_{Ap}$ do not belong to the PhoA family and thus could be classified as atypical APases. However, we found that $phoD_{Ap}$ encodes a protein homologous to the *Bacillus* PhoD protein. $phoD_{Ap}$ encodes 526 amino acid residues. The genes homologous to $phoD_{Ap}$ were found in several cyanobacteria, including *Bacillus*, *Pseudomonas*, and *Xanthomonas* spp., but not in many whole-genome-sequenced cyanobacteria such as *Synechocystis* sp. strain PCC6803, *Synechococcus elongatus* PCC7942, and *Prochlorococcus* spp. Among the 14 PhoD proteins shown in Fig. 2A, $PhoD_{Ap}$ exhibited the highest homology with *Acaryochloris marina* MBIC11017 (AM1_3551, 56% identity), *Cyanothece* sp. strain PCC7425 (53% identity), *Acaryochloris marina* MBIC11017 (AM1_3550, 52% identity), and then other cyanobacteria (47 to 49% identity). The identities to PhoD from *Bacillus*, *Pseudomonas*, and *Xanthomonas* spp. ranged between 44 and 45%. Interestingly, three putative PhoD genes were found in *Anabaena variabilis* ATCC 29413, but only two genes were found in *Anabaena* sp. strain PCC7120, *Acaryochloris marina* MBIC11017, and *Nostoc punctiforme* PCC73102. One putative PhoD gene was found in *A. halophytica*, *Cyanothece* sp. strain PCC7425, *Pseudomonas syringae* pv. *glycinea* strain B076, *Xanthomonas campestris*, and *Bacillus licheniformis* ATCC 14580. Among the 14 PhoD proteins shown in Fig. 2A, 12 PhoD proteins contain 520 to 540 amino acid residues, whereas *Acaryochloris* PhoD (AM1_3551) contains 509 amino acid residues (due to the missing N-terminal region), and *Bacillus* PhoD contains 583 amino acids (due to the extra C-terminal amino acid residues). These data indicate that the homology of PhoD is highly conserved among several bacterial species. Although the distribution of PhoD in cyanobacteria is restricted to certain species, there is no tendency of distribution among them (e.g., freshwater and ocean, unicellular and filamentous, or diazotrophic and nondiazotrophic).

All of the PhoD proteins shown in Fig. 2A, except for *Acaryochloris* PhoD (AM1_3551), contain the predicted Tat consensus motif and peptidase cleavage site in the N-terminal region (data not shown). We speculate that the PhoD proteins are secreted proteins. Interestingly, $PhoD_{Ap}$ contains two Tat consensus motifs (Fig. 2B). However, the physiological role of two Tat consensus motifs remains to be clarified. In contrast, $PhoA1_{Ap}$ and $PhoA2_{Ap}$ do not contain the deduced signal peptide (data not shown). These findings prompted us to fur-

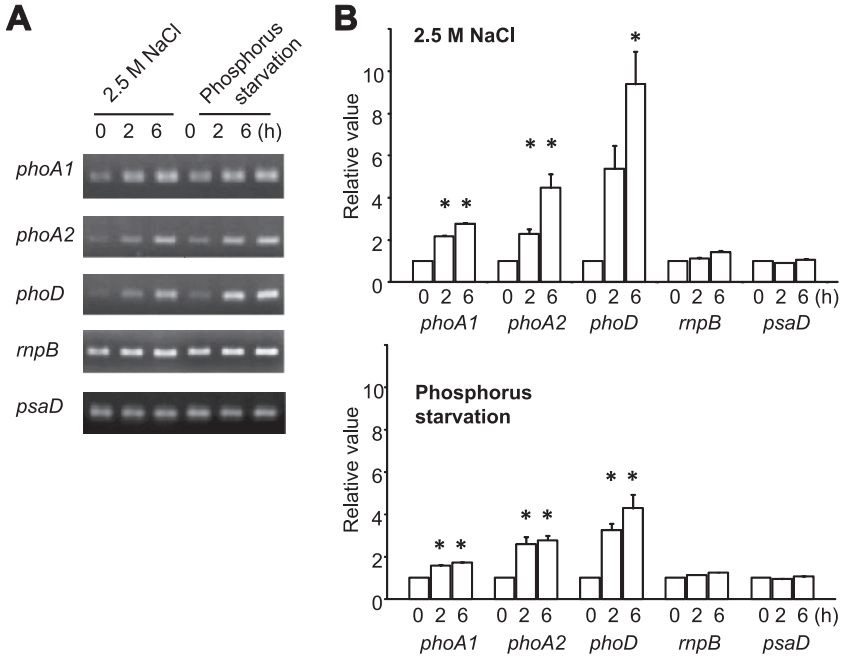

FIG. 4. Expression of APase genes in *A. halophytica* cells. Cells were collected at 0, 2, and 6 h of exposure to salinity stress or P starvation. RT-PCR analysis was performed as described in Materials and Methods. (A) PCR products were subjected to electrophoresis. (B) Relative values of the amount of DNA fragments. RNase P (*rnpB*) and photosystem I subunit II (*psaD*) genes were used as controls whose mRNA abundance remained unchanged under salinity stress or P starvation. The values at time zero of each gene were set to 1. Each value represents an average of three independent measurements. Means ± the SEM are shown. Asterisks indicate significant difference ($P < 0.05$) from the values at time zero.

ther investigate molecular mechanisms in the regulation of $phoD_{Ap}$.

Enzymatic characterization of PhoD$_{Ap}$. To characterize the enzymatic properties of PhoD$_{Ap}$, we purified recombinant PhoD$_{Ap}$ with a His$_6$ tag fused to its C-terminal tail. SDS-PAGE analysis revealed a protein band of ~60 kDa, which matched well the calculated molecular mass, 61.21 kDa, of PhoD$_{Ap}$-His$_6$ (Fig. 3A).

Next, we examined the metal requirement for APase/APDase activity of PhoD$_{Ap}$-His$_6$ (Fig. 3B). The results revealed that Ca^{2+} was required to obtain the maximal activity. The addition of EDTA strongly inhibited the enzyme activity probably by chelating Ca^{2+}. A requirement for Ca^{2+} has been reported for the activity of PhoD from *Bacillus* (3), PhoX from *Pasteurella multocida* strain X73 (22), and cyanobacteria (5), but not for PhoA. In the presence of Ca^{2+} and at pH 10.0, the K_m values for *p*NPP and *bis-p*NPP were 3.38 and 3.13 mM, respectively, while the V_{max} values were 219 and 178 nmol min^{-1} mg of $protein^{-1}$ (Fig. 3C). These results indicated that PhoD$_{Ap}$ is a Ca^{2+}-activated APase/APDase.

Expression of *A. halophytica* APases is upregulated upon salinity stress. As a next step, we examined the effects of salt on APases gene expression by RT-PCR analysis. As shown in Fig. 4, the expression of $phoA1_{Ap}$, $phoA2_{Ap}$, and $phoD_{Ap}$ was induced by 2.8-, 4.5-, and 9.4-fold, respectively, after a 6-h exposure of *A. halophytica* to salinity stress. On the other hand, the mRNA expression level of *rnpB* (encoding ribonucleoprotein) and *psaA* (photosystem I) did not change. We also observed that phosphate starvation of *A. halophytica* cells for 6 h upregulated the expression of $phoA1_{Ap}$, $phoA2_{Ap}$, and $phoD_{Ap}$ to 1.7-, 2.8-, and 4.2-fold, respectively. These results indicated that the levels of mRNA for putative APases, i.e., $phoA1_{Ap}$, $phoA2_{Ap}$, and $phoD_{Ap}$, increase upon salt stress, as well as upon phosphate depletion. It should also be mentioned that the induction of $phoD_{Ap}$ gene expression was much higher those that of $phoA1_{Ap}$ and $phoA2_{Ap}$.

Secretion ability of PhoD$_{Ap}$ in cyanobacteria. It has been reported that in *B. subtilis*, PhoD$_{Bs}$ is secreted into the extracellular medium via the Tat pathway (9). Therefore, we also examined whether PhoD$_{Ap}$ is secreted to the extracellular medium. Since transformation of *A. halophytica* was unsuccessful, a transformant of *S. elongatus* that overexpresses PhoD$_{Ap}$-His$_6$ was prepared. Transformed cells were fractionated, and the proteins were analyzed by Western blotting (Fig. 5). The transformed *S. elongates* cells possessing empty vector (pUC303) did not show any specific band (lanes 1 to 4). In contrast, two forms of PhoD$_{Ap}$ were observed (lanes 5 and 7) in total soluble and membrane fractions of transformed cells overexpressing

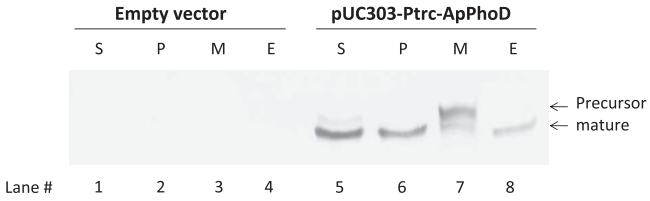

FIG. 5. Secretion of PhoD$_{Ap}$. PhoD$_{Ap}$-expressing cells grown with 1 mM IPTG for 24 h were separated into total soluble (S), periplasmic (P), total membrane (M), and extracellular (E) fractions and subjected to Western blot analysis (lanes 5 to 8). Arrows indicate predicted precursor and mature PhoD$_{Ap}$. *S. elongatus* with empty vector, pUC303, was used as a control (lanes 1 to 4).

$PhoD_{Ap}$. The presence of two bands might be explained as a full-length form (upper, precursor) and a truncated mature form that lacked the signal peptide (lower, mature). Conversely, $PhoD_{Ap}$ was detected as a single mature form in the periplasmic and extracellular portions (lanes 6 and 8). Thus, in *S. elongatus* cells, $PhoD_{Ap}$ is presumably translocated from the cytoplasm to the periplasm and extracellular medium via the inner membrane using the Tat pathway.

DISCUSSION

Hitherto, functional characterization of PhoD has been reported only on $PhoD_{Bs}$. This study is the first report on cyanobacterial PhoD. Our results have revealed that *A. halophytica* contains a gene encoding a protein homologous to $PhoD_{Bs}$ (Fig. 2A). $PhoD_{Ap}$ exhibited hydrolysis activity not only for a monoester (APase activity) but also for a diester (APDase activity), and Ca^{2+} was required for their activities (Fig. 3). Moreover, it was found that $PhoD_{Ap}$ contains the Tat motif at the N-terminal region (Fig. 2B). The results of transformed *S. elongatus* cells supported the secretion ability of $PhoD_{Ap}$ in *A. halophytica* cells (Fig. 5). We cannot rule out the possibility that $PhoD_{Ap}$ might have been detected in extracellular medium after cell lysis. However, we could not detect chlorophyll *a* derived from *S. elongatus* cell lysis in the extracellular medium (data not shown). Moreover, the cross-reacting band from proteins in the extracellular medium in Fig. 5 support the secretion ability of $PhoD_{Ap}$ in cyanobacteria because this signal was obtained as a clear single predicted mature protein band of $PhoD_{Ap}$ lacking the signal peptide (see Fig. 5, lane 8). If this signal was derived from cell lysis, both mature and precursor signals should have appeared in this lane. All of these data indicate that *A. halophytica* contains the PhoD protein that is secreted via the Tat pathway and exhibits the Ca^{2+}-dependent APase and ADPase activities.

One of the most interesting findings of the present study is that the expression of $phoA1_{Ap}$, $phoA2_{Ap}$, and $phoD_{Ap}$ genes was induced under salinity stress conditions (Fig. 4). The induction of these genes under salinity stress was much higher than those of P starvation. Moreover, induction of $phoD_{Ap}$ was higher than that of $phoA1_{Ap}$ and $phoA2_{Ap}$, suggesting an important role for $PhoD_{Ap}$ in the periplasmic and extracellular space of *A. halophytica* cells under salt stress (Fig. 1).

As was the case in *Anabaena doliolum* cells, the cellular P content in *A. halophytica* cells also decreased under salt stress conditions. In this context, we make the following argument. Salt stress triggers the decrease of the intracellular P level, which causes the upregulation of many genes under Pho regulation, such as $phoA1_{Ap}$, $phoA2_{Ap}$, and $phoD_{Ap}$, and phosphate uptake. Indeed, we identified two putative Pho boxes (TTACCC and TTAAGC) in the promoter region of $phoD_{Ap}$ (data not shown) (13). Upregulation of $phoD_{Ap}$ (Fig. 4) could promote the utilization of extracellular organic compounds as a P source. However, the direct effects of salt stress on the induction of Pho-regulated genes cannot be eliminated such as in the case of *isiA*, in which both low Fe and high salt levels induce the transcription of the *isiA* gene (16).

The detailed molecular mechanisms of $PhoD_{Ap}$ secretion and its regulatory mechanism would be very interesting subjects for future investigations.

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