Halotolerant Cyanobacterium *Aphanothece halophytica* Contains an Na\(^+\)-dependent F\(_{1}\)F\(_{0}\)-ATP Synthase with a Potential Role in Salt-stress Tolerance**§

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*Aphanothece halophytica* is a halotolerant alkaliphilic cyanobacterium that can grow in media of up to 3.0 M NaCl and pH 11. Here, we show that in addition to a typical H\(^+\)-ATP synthase, *Aphanothece halophytica* contains a putative F\(_{1}\)F\(_{0}\)-type Na\(^+\)-ATP synthase (ApNa\(^+\)-ATPase) operon (ApNa\(^+\)-atp). The operon consists of nine genes organized in the order of putative subunits β, ε, I, hypothetical protein, a, c, b, α, and γ. Homologous operons could also be found in some cyanobacteria such as *Synechococcus* sp. PCC 7002 and *Acaryochloris marina* MBIC11017. The ApNa\(^+\)-atp operon was isolated from the *A. halophytica* genome and transferred into an *Escherichia coli* mutant DK8 (Δatp) deficient in ATP synthase. The inverted membrane vesicles of *E. coli* DK8 expressing ApNa\(^+\)-ATPase exhibited Na\(^+\)-dependent ATP hydrolysis activity, which was inhibited by monensin and tributyltin chloride, but not by the protonophore, carbonyl cyanide m-chlorophenyl hydrazone (CCCP). The Na\(^+\) ion protected the inhibition of ApNa\(^+\)-ATPase by *E. coli* ATPase, whereas another type is the primary ATP-driven Na\(^+\)-pumps in the plasma membrane (P-type Na\(^+\)-ATPase) (5, 6). The P-type Na\(^+\)-ATPase is a single component polypeptide found in many organisms, including yeast. However, the presence of P-type Na\(^+\)-ATPase in plants and algae seems to be rare.

To survive at high salinity, living organisms must prevent the excessive Na\(^+\) accumulation in the cytoplasm. To date, two types of Na\(^+\)-transporting mechanisms are known to extrude Na\(^+\) from the cytoplasm. The Na\(^+\)/H\(^+\) antiporters are of one type that utilize energy of the transmembrane proton gradient for Na\(^+\) translocation (4), whereas another type is the primary ATP-driven Na\(^+\)-pumps in the plasma membrane (P-type Na\(^+\)-ATPase) (5, 6). The P-type Na\(^+\)-ATPase is a single component polypeptide found in many organisms, including yeast. However, the presence of P-type Na\(^+\)-ATPase in plants and algae seems to be rare.

*Aphanothece halophytica* is a halotolerant cyanobacterium isolated from the Dead Sea. *A. halophytica* is known to accumulate betaine and can grow in a wide range of salinity (0.25—3.0 M NaCl) as well as at alkaline pH conditions (7). Previous studies have shown that *A. halophytica* has a unique biosynthetic pathway of osmoprotectant betaine consisting of three-step methylation of glycine (8) and novel Na\(^+\)/H\(^+\) antiporters such as Ap-NhaP1, Ap-NapA1—1, Ap-NapA1—2, and Ap-Mrp (9—11). From the shotgun sequencing of the whole genome, we found that *A. halophytica* contains a putative F\(_{1}\)F\(_{0}\)-type Na\(^+\)-ATP synthase (ApNa\(^+\)-ATPase) operon (ApNa\(^+\)-atp). Isolation of the operon and its functional characterization are described below.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions—** *A. halophytica* cells were grown photoautotrophically in the BG11 liquid medium con-
taining 18 mM NaNO₃ and Turk Island salt solution at 30 °C (7, 12). *Synechococcus* sp. strain PCC 7942 cells were also grown in the BG11 liquid medium supplemented with 10 mM HEPES-KOH and bubbled with 3% CO₂ at 30 °C under continuous fluorescent white light (40 µE m⁻² s⁻¹). *Escherichia coli* DH5α and DK8 (Δ*atp*) (13) cells were grown at 37 °C in Luria-Bertani (LB) medium.

**Construction of Expression Plasmids**—The ApNa⁺-atp operon of *A. halophytica* was amplified by PCR using the primer set ApATPaseBamHI-F/ApATPaseSalI-R. The sequences of ApATPaseBamHI-F and ApATPaseSalI-R are 5'-GGATCCT-GGAGTTAGGCGGATGTTACAAT-3' and 5'-GTGCAG-GGATGAAGGATGATCATC-3', respectively. The amplified fragment was ligated into the EcoRV site of pBSK/H11001 (Stratagene) and sequenced. The insert was then cloned in the BG11 liquid medium supplemented with 10 mM HEPES-KOH, pH 7.5, containing 42 mM Na₂HPO₄, 2.2 mM KH₂PO₄, 8.5 mM NaCl, 2 mM MgSO₄, 0.1 mM CaCl₂, 15 mM thiamine, a carbon source (35 mM glucose or 35 mM succinate), and 1 mM isopropyl-β-d-thiogalactopyranoside (0.5 mM) was used to induce the ApNa⁺-atp expression under the trc promoter.

**Preparation of Inverted Membrane Vesicles from *Synechococcus* sp. PCC 7942**—Cells were harvested at exponential growth phase and washed with 20 mM Tris-HCl, pH 7.6, containing 1.0 mM succrose. The collected cells were suspended in extraction buffer (20 mM Tris-HCl, pH 7.6, 1 mM DTT, 1 mM PMSF, and 5 mM MgCl₂) with a ratio (g ml⁻¹) of 1.0:2.0. After homogenization, the cells were disrupted by two passages through a French pressure cell at 400 kilopascals. Unbroken cells and large debris were removed by centrifugation at 9800 × g for 15 min. The inverted membrane vesicles were sedimented by centrifugation at 100,000 × g for 30 min and resuspended in 50 mM HEPES-KOH, pH 7.0, to a final protein concentration of 1 mg ml⁻¹.

**Isolation of Thylakoid and Cytoplasmic Membranes from *Synechococcus* sp. PCC 7942**—The above prepared membrane vesicles (total membrane fraction) were used for the separation of thylakoid and cytoplasmic membranes by sucrose gradient centrifugation at 110,000 × g for 16 h (14). The gradient consisted of four layers: 5.5 ml of 58% sucrose, 4.5 ml of 40% sucrose, 4.5 ml of 30% sucrose, and 4 ml of 10% sucrose. Thylakoid membranes remained at the 40%/58% sucrose interface, whereas cytoplasmic membranes moved into the 30% sucrose layer that was essentially free of chlorophyll (14).

**Other Methods**—The nucleotide sequences were determined using an ABI310 genetic analyzer (Applied Biosystems, Foster City, CA). Cellular ions were determined with a Shimadzu PIA-1000 personal ion analyzer (9). Protein concentration was measured using the Bradford’s method. SDS-PAGE and Western blot analysis were carried out as described previously (15). Antibody raised against the His₆ tag was obtained from R&D Systems (Minneapolis, MN).

**Nucleotide Sequence Accession Number**—Nucleotide sequence data of the ApNa⁺-atp operon from *A. halophytica* are deposited to the DDBJ database under the accession nos. AB602793, AB602794, and AB602795.
RESULTS

Gene Organization in ApNa\(^{+}\)-atp Operon of A. halophyta—

From the shotgun sequence analysis of the A. halophyta genome, we found a putative F\(_{1}\)F\(_{0}\)-type ApNa\(^{+}\)-atp operon in addition to the typical H\(^{+}\)-ATPase (ApH\(^{+}\)-ATPase) operon (ApH\(^{+}\)-atp). The ApNa\(^{+}\)-atp operon had nine ORFs, each with a putative start codon. These genes were organized in the order atpD, atpE, atpC (I), atpA (b), and atpB (c) (Fig. 1A). By contrast, gene organization of ApH\(^{+}\)-atp splits into two clusters, encoding lacbb’bocx and 7be (Fig. 1B). The gene organization of ApNa\(^{+}\)-atp is similar to those of the atp operons in E. coli (EcH\(^{+}\)-atp) (13, 16) and Ilyobacter tartaricus (ItNa\(^{+}\)-atp) (16) that encode F-type H\(^{+}\)– and Na\(^{+}\)-ATPases, respectively. But, in ECH\(^{+}\)-atp and ItNa\(^{+}\)-atp, the operon starts from gene I and does not contain the hypothetical gene (Fig. 1, C and D). A Blast search revealed the presence of a similar operon in some cyanobacteria, such as Synechococcus sp. PCC 7002, Acaryochloris marina MBIC11017, and Cyanothecae sp. ATCC 51142 (Fig. 1E). Among them, Synechococcus sp. PCC 7002 had the same gene organization to that of ApNa\(^{+}\)-atp, whereas in A. marina MBIC11017 and Cyanothecae sp. ATCC 51142, additional gene(s) were found.

The deduced protein sequences of the nine genes, except the gene encoding hypothetical protein, were compared with the corresponding sequences of ATPase subunits from different species. The protein encoded by ApNa\(^{+}\)-atpI showed the least homologous (4–7% identity) compared with the corresponding subunit of H\(^{+}\)-transporting ATPase from E. coli and Na\(^{+}\)-transporting ATPase from I. tartaricus, Propionigenium modestum, Acetobacterium woodii, and Clostridium paradoxum. The \(\alpha\) and \(\beta\) subunits of ApNa\(^{+}\)-ATPase showed greater similarity to the corresponding subunits from other species (40–51% identity) than the remaining subunits, which showed ~15–39% identity.

\(\beta\) Subunit of ApNa\(^{+}\)-ATPase Exhibits Higher Homology to Those of ItNa\(^{+}\)-ATPase and EcH\(^{+}\)-ATPase than That of ApH\(^{+}\)-ATPase—Fig. 2 shows the phylogenetic tree of several ATPases. The \(\beta\) subunit of ApH\(^{+}\)-ATPase exhibited high homology to the cyanobacterial H\(^{+}\)-ATPases from Synechococcus sp. PCC 7002, A. marina MBIC11017, and Cyanothecae sp. ATCC 51142. It also exhibited high homology to that of spinach H\(^{+}\)-ATPase, supporting the view that the plant chloroplast originated through endosymbiosis from a cyanobacterium. The \(\beta\) subunit of ApNa\(^{+}\)-ATPase exhibited high homology to the Na\(^{+}\)-ATPases from Synechococcus sp. PCC 7002, A. marina MBIC11017, and Cyanothecae sp. ATCC 51142, but relatively low homology to subunit B of A-type ATPase from Methanosarcina mazei G01 and V-type ATPase from Enterococcus hirae. This result suggests that cyanobacterial Na\(^{+}\)-ATPases are F-type ATPases, and evolved earlier than the separation between cyanobacteria, E. coli, and I. tartaricus.

ApNa\(^{+}\)-ATPase Has Putative Na\(^{+}\)-binding Site in Subunit c—

The membrane-spanning helices of the multiple copies of subunit c in the F\(_{0}\) complex are the rotor ring of H\(^{+}\) (or Na\(^{+}\))-translocating ATP synthases (1). Suplemental Fig. S1A shows the phylogenetic tree of subunit c in several ATPases. Subunit c of ApH\(^{+}\)-ATPase exhibited high homology to that of cyanobacterial H\(^{+}\)-ATPase and spinach H\(^{+}\)-ATPase but was considerably different from that of ApNa\(^{+}\)-ATPase, which is similar to the phylogenetic tree of \(\beta\) subunit. Suplemental Fig. S1B shows the alignment of subunit c of several F-type ATPases. Based on x-ray crystallography, cryo-electron microscopy, and atomic force microscopy studies, the binding sites for H\(^{+}\) and Na\(^{+}\) have been identified (1, 17). The binding site of H\(^{+}\) in subunit c in spinach H\(^{+}\)-ATPase has been assigned as Gln (Gln-34, num-

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**FIGURE 1. Schematic structures of gene organization of several ATP synthases.** A, ApNa\(^{+}\)-ATPase (accession no. AB602793); B, ApH\(^{+}\)-ATPase (AB602794, AB602795); C, EcH\(^{+}\)-ATPase (E. coli, J01594); D, ItNa\(^{+}\)-ATPase (I. tartaricus, AF522463); and E, four kinds of cyanobacterial Na\(^{+}\)-ATPases. The corresponding sequences of ATPase subunits from different species. Subunit c in the F\(_{0}\)-F\(_{1}\) complex of H\(^{+}\)-ATPase/Na\(^{+}\)-ATPase, supporting the view that the plant chloroplast originated through endosymbiosis from a cyanobacterium. The \(\beta\) subunit of ApNa\(^{+}\)-ATPase exhibited high homology to the Na\(^{+}\)-ATPase from Cyanobacterium sp. PCC 7002, A. marina MBIC11017, and Cyanotheraceae sp. ATCC 51142. It also exhibited high homology to that of spinach H\(^{+}\)-ATPase, supporting the view that the plant chloroplast originated through endosymbiosis from a cyanobacterium. The \(\beta\) subunit of ApNa\(^{+}\)-ATPase exhibited high homology to the Na\(^{+}\)-ATPase from Synechococcus sp. PCC 7002, A. marina MBIC11017, and Cyanotheraceae sp. ATCC 51142, but relatively low homology to subunit B of A-type ATPase from Methanosarcina mazei G01 and V-type ATPase from Enterococcus hirae. This result suggests that cyanobacterial Na\(^{+}\)-ATPases are F-type ATPases, and evolved earlier than the separation between cyanobacteria, E. coli, and I. tartaricus.

**FIGURE 2. Phylogenetic trees for \(\beta\) subunit of H\(^{+}\)- and Na\(^{+}\)-translocating ATPases from several species.** F-type Na\(^{+}\)-translocating \(\beta\) subunits, AB602793 (ApNa\(^{+}\)), cce_1512 (cceNa\(^{+}\)), SYNPCC7002_G0144 (7002H\(^{+}\)), AM1_D0157 (AM1Na\(^{+}\)), and AF522463 (IlNa\(^{+}\)), F-type H\(^{+}\)-translocating \(\beta\) subunits, AB602795 (ApH\(^{+}\)), cce_1512 (cceH\(^{+}\)), SYNPCC7002_G0144 (2002H\(^{+}\)), AM1_D0157 (AM1H\(^{+}\)), and spinach \(\beta\) subunit (spii\(^{+}\), NP_054943). An A-type \(\beta\) subunit of Methanosarcina mazei, Metanob (NP_632803.1) and V-type \(\beta\) subunit of Enterococcus faecalis, Enterob (NP_815220.1) are shown.
boring in subunit c of ApNa\(^+\)-ATPase, Phe (Phe-65), Glu (Glu-67), Thr (Thr-70), and Tyr (Tyr-72), whereas the binding site of Na\(^+\) in subunit c of ItNa\(^+\)-ATPase has been assigned as Gln (Gln-34), Val (Val-65), Glu (Glu-67), Ser (Ser-68), and Tyr (Tyr-72). The H\(^+\) binding site (Gln-34, Phe-65, Glu-67, Thr-70, and Tyr-72) was conserved in all cyanobacterial H\(^+\)-translocating ATPases (supplemental Fig. S1B). For the Na\(^+\) binding site, Glu-67, Ser-68, and Tyr-72 were conserved in all cyanobacterial Na\(^+\)-translocating ATPases. However, the Gln-34 changed to Glu (Glu-34) in all cyanobacterial Na\(^+\)-ATPases. Val-65 did not change, but Glu-67, Ser-68, and Thr-69 motif were conserved. These results indicate that ApH\(^+\)-ATPase presumably H\(^+\)-translocating ATPases, although ion specificity of ApNa\(^+\)-ATPase need to be tested experimentally.

ApNa\(^+\)-ATPase and ApH\(^+\)-ATPase Do Not Have Cys Residues Involved in Redox-induced Activation of Plant ATPase—The plant F-type H\(^+\)-ATPase has a unique regulatory mechanism, which is caused by a redox change of two cysteine residues located in the γ subunit. Under light, the reducing equivalents produced by photosynthetic electron transport reduce thioredoxin, which in turn reduces the disulfide bond formed within the γ subunit, yielding the active enzyme. The two cysteine residues forming the disulfide bond in the spinach H\(^+\)-ATPase were assigned to Cys-199 and Cys-205 (18). Supplemental Fig. S2A shows the alignment of the γ subunit of several F-type ATPases. Cys-199 and Cys-205 of spinach H\(^+\)-ATPase are shown in supplemental Fig. S2A in closed boxes. These cysteines were not conserved in all F-type ATPases from cyanobacteria, E. coli, and I. tartaricus. The spinach γ subunit is unique in having an extra domain around the two cysteine residues as shown by the dotted line box. In the cyanobacterial H\(^+\)-ATPase, the extradomain was nine residues smaller and did not contain the two cysteine residues. By contrast, in the cyanobacterial Na\(^+\)-ATPase, that type of extradomain was missing as is the case also in E. coli and I. tartaricus. The phylogenetic tree in supplemental Fig. S2B showed that the γ subunit of spinach H\(^+\)-ATPase was separated from that of cyanobacterial H\(^+\)-ATPase, indicating the evolution of unique regulating system in plant H\(^+\)-ATP synthetases.

Cloning of ApNa\(^+\)-atp and Expression in E. coli DK8 Cells—To clone the ApNa\(^+\)-atp operon from A. halophytica, PCR amplification was performed resulting in a 6832-bp PCR product in which the GTG start codon in atpD was changed to ATG. The PCR product was cloned into the EcoRV restriction site of pBSK+ vector and sequenced. Although one nucleotide change from GAG (Glu-41) to GAT (Asp-41) was found in the β subunit, this clone was used for further experiments because the substitution is rather conserved. Then, the insert was digested with BamHI and SalI and subcloned into the BamHI/SalI sites of pTrcHis2C expression vector. The last gene, atpg, was fused in frame with His\(_6\) tag.

To examine the functional properties of ApNa\(^+\)-atp, these genes were expressed in the E. coli mutant DK8 lacking the unc operon encoding ATPase (Δatp). As shown in Fig. 3A, the γ subunit of ApNa\(^+\)-ATPase could be detected in the E. coli DK8 cells transformed with pTrcHis2C-ApNa\(^+\)-atp but not with the vector alone. The expression was maximum at 2–5 h, and therefore, the 3-h induction time point was employed hereafter, unless otherwise stated. The expression level of γ subunit increased with increase in NaCl concentrations (Fig. 3B). To examine the localization of γ subunit in DK8, E. coli cells expressing ApNa\(^+\)-atp were fractionated into the cytoplasmic, periplasmic, and membrane fractions, followed by SDS-PAGE and immunoblot analyses. The γ subunit was detected in the membrane fraction (Fig. 3C). These results suggest that the γ subunit and whole complex are assembled into the cytoplasmic membranes of E. coli cells.

As a next step, the E. coli membrane fraction was solubilized with Triton X-100, fractionated by 40–65% (g/v) ammonium sulfate, and applied to an ion exchange column chromatography. The active fractions were then used for activity measurements.

ATP Hydrolysis Activity in Inverted Membrane Vesicles of E. coli DK8 Cells—We next examined whether the expressed ApNa\(^+\)-ATPase has ATP hydrolysis activity. The ATP hydrolysis activity in the inverted membrane vesicles of ApNa\(^+\)-ATPase-expressing E. coli DK8 cells increased with increasing NaCl concentrations (Fig. 4A). The apparent K\(_m\) value for Na\(^+\) was determined to be 3.0 mM. The ATP hydrolysis activity of control cells (the empty vector transformants) was very low and did not increase upon the increase of NaCl concentration (data not shown). The ATP hydrolysis activity of ApNa\(^+\)-ATPase-expressing E. coli DK8 cells increased with increasing NaCl concentrations (Fig. 4B). The apparent K\(_m\) value for ATP was determined to be 3.0 mM. The Na\(^+\) content inside the ApNa\(^+\)-ATPase-expressing E. coli DK8 cells decreased when cells were transferred to a new Na\(^+\)-free medium, but no change in the Na\(^+\) content was observed inside the control cells (data not shown). These results suggest that the E. coli DK8 cells expressing ApNa\(^+\)-ATPase can extrude Na\(^+\) from inside the cells to the external medium.

Effect of Inhibitors on ATP Hydrolysis Activity in Inverted Membrane Vesicles of ApNa\(^+\)-ATPase-expressing E. coli DK8 Cells—The ATP hydrolysis activity in inverted membrane vesicles of ApNa\(^+\)-ATPase-expressing E. coli DK8 cells was strongly inhibited by azide (F\(_1\) inhibitor; 55%), DCCD\(^2\) (F\(_0\) carbonyl cyanide m-chlorophenyl hydrazone).

![FIGURE 3. Expression of ApNa\(^+\)-ATPase in E. coli DK8 cells. A, effects of induction time. B, effects of NaCl. C, localization of ApNa\(^+\)-ATPase.](image-url)
inhibitor; 70%), tributyltin chloride (F0 inhibitor; 80%), and monensin (Na+/H+-gradient dissipator; 70%), but not by CCCP (a protonophore) and KNO3 (a permeant anion) (Fig. 5A). Moreover, Na+/H+-provided protection against inhibition by DCCD.

When NaCl was added into the individual reaction medium, the DCCD-inhibited ATPase activity was decreased to 70, 40, and 30% in the presence of 1, 10, and 50 mM NaCl, respectively (Fig. 5B).

ATP Synthesis in Na+-loaded Inverted Membrane Vesicles of E. coli DK8 Cells—To know whether the expressed ApNa+-ATPase in DK8 cells has ATP synthesis activity, the ATP synthesis activity was assayed using the luciferin/luciferase reaction. The Na+/H+-loaded inverted membrane vesicles of E. coli DK8 cells were prepared and incubated with the reaction medium (0.5 ml) containing 5 mM potassium phosphate, pH 7.5, 5 mM MgCl2, 0.2 mM KCl, and 0.1 mM ADP. The ATP synthesis was observed in the membrane vesicles of ApNa+-ATPase-expressing E. coli DK8 cells (Fig. 6A, closed circles), but not in the empty vector transformants (data not shown).

To examine the requirement of ΔΨ on ATP synthesis, inhibitors and ionophores were added to the reaction medium. Results showed that membrane potential-generating K+-valinomycin and protonophore CCCP stimulated ATP synthesis (Fig. 6A), implying that ATP synthesis requires ΔΨ but not ΔpH. In contrast, the Na+-gradient dissipator monensin and the F0 inhibitor DCCD inhibited ATP synthesis, suggesting that ATP synthesis by F1F0-ATPase requires Na+/H+-. Furthermore, application of external Na+ (20 and 50 mM NaCl) into the reaction medium decreased the ATP synthesis (Fig. 6B). In other words, decrease in ΔpH due to external Na+ causes decrease in the ATP synthesis.

Expression of ApNa+-ATPase Confers Salt-stress Tolerance in E. coli—We next investigated the physiological role of ApNa+-ATPase, namely the NaCl-stress tolerance in E. coli. As shown in Fig. 7, E. coli DK8 cells transformed with pTrcHis2C or pTrcHis2C-ApNa+-atp exhibited similar growth patterns in the absence of NaCl. An increase in the NaCl concentration up to 0.2 and 0.5 mM NaCl resulted in slow growth of all cells. How-
however, the growth rate of ApNa\textsuperscript{+}-ATPase-expressing cells was higher than that of empty vector transformants. Therefore, the effect of NaCl on growth of a salt-sensitive mutant E. coli TO114, in which nhaA, nhaB, and chaa genes were deleted, was determined. The E. coli TO114 cells transformed with pTrcHis2C-ApNa\textsuperscript{+}-atp grew in the growth medium containing 0.2 M NaCl, whereas the E. coli TO114 cells transformed with pTrcHis2C did not (data not shown). To determine whether ApNa\textsuperscript{+}-atp could complement E. coli DK8 (Δatp), empty vector transformant and ApNa\textsuperscript{+}-ATPase-expressing cells were plated onto M13 minimal medium containing succinate as the sole carbon and energy source. Control plates contained glucose in the same amount as succinate. Results showed that empty vector transformants could grow only on glucose, whereas ApNa\textsuperscript{+}-ATPase-expressing cells could grow on both glucose and succinate (data not shown). Hence, the ApNa\textsuperscript{+}-atp operon encoding F\textsubscript{1}F\textsubscript{0}-ATPase and NrtA of nitrate transporter were detected by immunoblotting with anti-His tag and anti-NrtA (19) antibody, respectively.

Expression of ApNa\textsuperscript{+}-atp Operon in the Freshwater Cyanobacterium Synechococcus sp. PCC 7942—The expression plasmid pUC303-ApNa\textsuperscript{+}-atp containing isopropyl-β-D-thiogalactopyranoside-inducible trc promoter was introduced into Synechococcus sp. strain PCC 7942 cells. The expression of γ subunit of ApNa\textsuperscript{+}-ATPase was detected in the membrane vesicles of ApNa\textsuperscript{+}-ATPase-expressing cells (Fig. 8A). Its expression level increased with the increase of NaCl in the growth medium (Fig. 8B). To examine the localization of ApNa\textsuperscript{+}-ATPase, total membrane fractions were applied on the sucrose density gradient as described in “Experimental Procedures” (14). Thylakoid membranes contained ~4.1 μg chlorophyll per
mg protein, whereas cytoplasmic membranes were essentially free of chlorophyll (data not shown). Moreover, data presented in Fig. 8C revealed that a single protein band of the corresponding molecular mass of γ subunit of ApNa⁺-ATPase was found in plasma membrane fraction, which was identified by the presence of the nitrate transporter NrtA (19). This indicated that ApNa⁺-ATPase was localized in the plasma membrane of ApNa⁺-ATPase-expressing cells.

It was shown that all of the transformants could grow at almost the same rate in the BG11 medium (Fig. 9A). At 0.3 M NaCl concentration, the growth rate of both empty vector transformants and ApNa⁺-ATPase-expressing cells decreased but the growth rate of ApNa⁺-ATPase-expressing cells is higher than the growth rate of empty vector transformants (Fig. 9B). At 0.5 M NaCl concentration, empty vector transformants could not grow, whereas ApNa⁺-ATPase-expressing cells could (Fig. 9C), indicating that the ApNa⁺-atp transformation impaired growth at high salinity.

**DISCUSSION**

The sequence of ApNa⁺-atp operon was obtained from the shotgun genome sequencing of *A. halophytica*. Nine ORFs were organized in the order atpD (β), atpC (ε), atpI (I), hypothetical gene (hyp), atpB (a), atpE (c), atpF (b), atpA (α), and atpG (γ) (Fig. 1), which is different from other bacteria. In *C. paradoxa*, *E. coli*, *I. tartaricus*, and *P. modestum*, the atp operon consists of nine structural genes, atpI (I), atpB (a), atpE (c), atpF (b), atpH (δ), atpA (α), atpG (γ), atpD (β), and atpC (ε), which are grouped together to form a single transcriptional unit (16–18, 20, 21). The atp operon in a strictly anaerobic *A. woodii* consists of 11 genes, including nine genes found in the bacterial atp operons and additional two copies of atpE (c) (22). In addition, the structural genes in cyanobacteria *Anabaena* strain PCC 7210, *Synechococcus* strain PCC 6301, and *Synechocystis* sp. PCC 6803 are organized in two separate domains, with one containing atpB (a), atpE(c), atpF (b), atpH (δ), atpA (α), and atpG (γ), whereas the other contained atpD (β) and atpC (ε) (23–25). Although the deduced protein sequences of nine ApNa⁺-ATPase subunits showed low percent identity compared with the corresponding sequences of F-type ATPase subunits from different species, the conserved amino acids Ser68 and Thr69 at the Na⁺-binding site of the ApNa⁺-ATPase subunit c were found (supplemental Fig. S1). The Na⁺-binding site of the *I. tartaricus* ATPase and H⁺-binding site of the spinach chloroplast ATPase subunit c were compared (2). Alanine was present at position 62 of the H⁺-translocating ATPase, whereas a polar serine or threonine was observed in the equivalent position of Na⁺-translocating ATPase. Likewise, the hydrophobic side chain leucine was found in H⁺-translocating ATPase at position 63, but threonine was found at the equivalent position of Na⁺-translocating ATPase. The data presented above clearly indicate that F-type Na⁺-ATPase from *A. halophytica* is a Na⁺-translocating ATPase.

Furthermore, this study examined the biochemical properties and physiological functions of ApNa⁺-ATPase. The expression of ApNa⁺-ATPase in ApNa⁺-ATPase-expressing cells could impair its growth under high salinity (Fig. 7). The ATP hydrolysis activity in inverted membrane vesicles of ApNa⁺-ATPase-expressing *E. coli* DK8 was dependent on NaCl and ATP concentration in the reaction medium (Fig. 4). Moreover, the Na⁺ extrusion from Na⁺-loaded ApNa⁺-ATPase-expressing cells was observed after the cells were energized with glucose (data not shown), suggesting that the Na⁺ extrusion is likely due to the contribution by ApNa⁺-ATPase because glucose can yield ATP during its metabolism. ApNa⁺-ATPase was confirmed as a member of F-type Na⁺-ATPase by using appropriate inhibitors. The ATPase activity was inhibited by azide (F₁ inhibitor), DCCD (F₁ inhibitor), or tributyltin chloride (F₀ inhibitor). In addition, it was found that monensin (Na⁺-gradient dissipator) inhibited ATPase activity, whereas CCCP (a protonophore) and KNO₃ (a permeant anion) did not inhibit activity (Fig. 5A). These data indicate the involvement of ∆pNa⁺ but not ∆pH on ATP hydrolysis. Furthermore, it was found that Na⁺ could protect ATPase activity from DCCD inhibition in a pH-dependent manner (Fig. 5B). Using another approach, ATP synthesis was observed in the Na⁺-loaded membrane vesicles of ApNa⁺-ATPase-expressing *E. coli* DK8 cells in the presence of ∆pNa⁺ and ∆ψ (Fig. 6). Our data revealed that ATP synthesis was stimulated in the presence of K⁺/valinomycin. ATP synthesis decreased when external Na⁺ and monensin (Na⁺-gradient dissipator) were added because both of them reduced ∆pNa⁺ (Fig. 6B). Based on these results, it can be concluded that ATP synthesis catalyzed by ApNa⁺-ATPase requires ∆pNa⁺.

As per the above data, we thought that the introduction of ApNa⁺-atp operon into the freshwater cyanobacterium *Synechococcus* sp. PCC 7942 will confer salt tolerance to this organism. As expected, the ApNa⁺-ATPase-expressing cells could
grow in the BG11 medium containing 0.5 m NaCl, whereas the empty vector transformants could not (Fig. 9). From these results, ApNa⁺-atp transformation could improve salt tolerance in the freshwater cyanobacterium Synechococcus sp. PCC 7942 as well as E. coli DK8 mutant (Δatp) and salt-sensitive mutant E. coli TO114. Western blot analysis revealed that the expression level of ApNa⁺-ATPase increased with increasing NaCl concentration in the growth medium (Fig. 8B). Taken together, our results strongly suggest a role for ApNa⁺-ATPase in the salt-stress tolerance of this photosynthetic organism.

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