Halotolerant Cyanobacterium *Aphanotothece halophytica* Contains an Na\(^+/\)H\(^+\) Antipporter, Homologous to Eukaryotic Ones, with Novel Ion Specificity Affected by C-terminal Tail*

Recently, a cyanobacterium *Synechocystis* sp. PCC 6803 has been shown to contain an Na\(^+/\)H\(^+\) antipporter gene homologous to plants (SOS1 and AtNHX1 from Arabidopsis) and mammals (NHEs from human) but not to *Escherichia coli* (nhaA and nhaB). Here, we examined whether a halotolerant cyanobacterium *Aphanotothece halophytica* has homologous genes. It turned out that *A. halophytica* contains an Na\(^+/\)H\(^+\) antipporter homologous to plants, mammals, and some bacteria (nhnP from *Pseudomonas* and synnhaP from *Synechocystis*) but with novel ion specificity. Its gene product, ApNHnP (Na\(^+/\)H\(^+\) antipporter from *Aphanotothece halophytica*), exhibited the Na\(^+/\)H\(^+\) antipporter activity over a wide pH range between 5 and 9 and complemented the Na\(^+\)-sensitive phenotype of the antipporter-deficient *E. coli* mutant. The ApNHnP had virtually no activity for the Li\(^+/\)H\(^+\) antipporter but showed high Ca\(^{2+}/\)H\(^+\) antipporter activity at alkaline pH. The ApNHnP complemented the Ca\(^{2+}/\)H\(^+\)-sensitive phenotype of the *E. coli* mutant but not the Li\(^+\)-sensitive phenotype. The replacement of a long C-terminal tail of ApNHnP with that of *Synechocystis* altered the ion specificity of the antipporter. These results suggest that the ion specificity of an Na\(^+/\)H\(^+\) antipporter is partly determined by the structural properties of the C-terminal tail, which was well exemplified in the case of *A. halophytica*.

The Na\(^+/\)H\(^+\) antipporters catalyze the exchange of Na\(^+\) for H\(^+\) across membranes and play a variety of functions, such as the regulation of internal pH, cell volume, and sodium level in the cytoplasm (1–3). In *Escherichia coli*, three antipporters (NhaA, NhaB, and ChaA) are known, and their functional characteristics have been well described (1). Six homologous Na\(^+/\)H\(^+\) antipporters (exchangers) (NHE1–6) have been found in animals (2, 10). These facts suggest that the cyanobacterial antipporters would provide a model system for the study of the structural and functional properties of eukaryotic Na\(^+/\)H\(^+\) antipporters.

To date, only a few functional residues, especially the residues involved in the cation transport, have been identified in Na\(^+/\)H\(^+\) antipporter proteins. The importance of Asp\(^{138}\) in SynNHnP (9) and Asp\(^{163}\), Asp\(^{165}\), and Asp\(^{164}\) in NhaA (11, 12) has been reported. The Na\(^+/\)H\(^+\) antipporter has been thought to exchange specifically between H\(^+\) and Na\(^+\) or Li\(^+\), but some antipporters exhibited low exchange activity between H\(^+\) and Li\(^+\) (13). The *E. coli* ChaA has been reported to have an exchange activity between Ca\(^{2+}\) and H\(^+\) as well as Na\(^+\) and H\(^+\) at alkaline pH (14), but this has not been examined in detail. It is not clear by which factors ion specificity is determined. To identify the conserved sequences for the cation transport in Na\(^+/\)H\(^+\) antipporters, the cloning of an antipporter with novel ion specificity is a prerequisite.

*Aphanotothece halophytica* is a halotolerant cyanobacterium that can grow in a wide range of salinity conditions from 0.25 to 3.0 M NaCl (15, 16). *A. halophytica* accumulates an osmoprotectant glycine betaine at high salinity (15). The DnaK protein of *A. halophytica* has been shown to contain a longer C-terminal segment than other DnaK/Hsp 70 family members (17) and has been shown to enhance the tolerance for salt (18). Transformation of tobacco with the DnaK from *A. halophytica* was shown to enhance the tolerance for salt (19).

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SOS1) (7, 8) Na\(^+/\)H\(^+\) antipporters have been reported. The homology between *E. coli* antipporters and eukaryotic ones was very low, suggesting the independent evolution among Na\(^+/\)H\(^+\) antipporter genes.

In a previous study (9), we showed that a cyanobacterium *Synechocystis* sp. PCC 6803 contains an Na\(^+/\)H\(^+\) antipporter, SynNHnP, homologous to eukaryotic and prokaryotic (NhaP from *Pseudomonas aeruginosa*) ones but not to the NhaA, NhaB, and ChaA. It was also shown that the SynNHnP contains a conserved Asp\(^{138}\) in the transmembrane (TM)-spanning region and a relatively long C-terminal hydrophilic tail, which are important for the carrier activity. The long C-terminal tails are believed to play a role in the regulation of transport activity in animals (2, 10). These facts suggest that the cyanobacterial antipporters would provide a model system for the study of the structural and functional properties of eukaryotic Na\(^+/\)H\(^+\) antipporters.
isolates an Na\textsuperscript{+}/H\textsuperscript{+} antiporter gene, homologous to eukaryotic ones but with novel ion specificity. It was also shown that the ion specificity was affected by the C-terminal tail.

**MATERIALS AND METHODS**

*A. halophytica Culture Conditions—*A. halophytica cells were grown phototrophically in BG11 medium plus 18 mM NaCl, and Turk Island salt solution as described previously (15–17) except that the NaCl concentration of the culture medium was adjusted to a range from 0.25 to 2.5 M as desired. Cotton-plugged 500-ml conical flasks containing 200 ml of medium each were used and shaken on a reciprocal shaker without supplementation of condensed CO\textsubscript{2} gas. The culture flasks were incubated at 28 °C under continuous fluorescent white light (30 microeinstens m\textsuperscript{-2} s\textsuperscript{-1}).

**Isolation of A. halophytica Na\textsuperscript{+}/H\textsuperscript{+} Antiporter—**A. halophytica genomic DNA was isolated as described (17). Partially degenerate oligonucleotides were designed based on two highly conserved polypeptide regions among several Na\textsuperscript{+}/H\textsuperscript{+} antiporters (9). The forward primer, NP-F, was designed after the polypeptide stretch Glu-Gly-Glu-Ser-Leu-Phe-Asn-Asp-Gly (residues 160 to 168 in SynNhaP). The reverse primer, NP-R, was designed after the polypeptide stretch Phe-Leu-Pro-Pro-Leu-Glu-Ala (residues 73 to 81 in SynNhaP). The reverse primer, ANP-N-R, was the complementary sequence of SNP-C-F. The amplified fragment resulting plasmid, pANhaP, encodes the ApNhaP fused in-frame to six histidines and was transferred first to *E. coli* DH5α cells and then to TO114 cells in which *nhaA*, *nhaB*, and *chaA* genes were deleted (21).

**Construction of Chimera between ApNhaP and SynNhaP—**A chimera gene that encodes the TM region of ApNhaP (residues Met\textsuperscript{1}–Ile\textsuperscript{400}) and the C-terminal cytosolic region of SynNhaP (residues Glu\textsuperscript{504}–Ser\textsuperscript{603}) was constructed as follows (Fig. 1). The nucleotides corresponding to the TM region of ApNhaP and C-terminal region of SynNhaP were amplified by the forward/reverse primer sets, ANP-NcoI/ANP-N-R and SNP-C-F/SNP-C-R, respectively. The template for the former nucleotide was the pANhaP and that for the latter was the pSANhaP in which the synnhaP gene was ligated into the NeoI/EcoRI site of pTrcHis2C (9). The forward primer, SNP-C-F, contains the sequence corresponding to the polypeptide stretches Glu\textsuperscript{494}–Thr-Val-Ile\textsuperscript{493} of ApNhaP and Glu\textsuperscript{502}–Phe-Leu-Val-Leu\textsuperscript{501} of SynNhaP, and the reverse primer, ANP-N-R, is the complementary sequence of SNP-C-F. The reverse primer, SNP-C-R, contains the EcoRI site just before the stop codon of pSanhaP. Upon mixing both PCR products, a chimera gene *apynhaP* was amplified using the forward ANP-NcoI and reverse SNP-C-R primers and ligated into the *NeoI/EcoRI*-digested sites of pTrcHis2C, which generated the plasmid pSANhaP. For the construction of a chimeraic gene that encodes the TM region of SynNhaP (residues Met\textsuperscript{1}–Thr-Ile\textsuperscript{499}) and the C-terminal region of ApNhaP (residues Glu\textsuperscript{502}–Glu\textsuperscript{521}), the respective nucleotides were amplified by the following forward/reverse primer sets, SNP-N-F/SNP-N-R and ANP-C-F/ANP-C-R. The reverse primer, ANP-C-R, contains the EcoRI site just before the stop codon of pSanhaP. Upon mixing both PCR products, a chimera gene *synapnhaP* was amplified using the forward SNP-N-F and reverse SNP-N-R primers and ligated into the *NeoI/EcoRI*-digested sites of pTrcHis2C, which generated the plasmid pSANhaP. For the construction of a chimeraic gene that encodes the TM region of SynNhaP (residues Met\textsuperscript{1}–Thr-Ile\textsuperscript{499}) and the C-terminal region of ApNhaP (residues Glu\textsuperscript{502}–Glu\textsuperscript{521}), the respective nucleotides were amplified by the following forward/reverse primer sets, SNP-N-F/SNP-N-R and ANP-C-F/ANP-C-R. The reverse primer, ANP-C-R, contains the EcoRI site just before the stop codon of pSanhaP. Upon mixing both PCR products, a chimera gene *synapnhaP* was amplified using the forward SNP-N-F and reverse SNP-N-R primers and ligated into the *NeoI/EcoRI*-digested sites of pTrcHis2C, which generated the plasmid pSANhaP. For the construction of a chimeraic gene that encodes the TM region of SynNhaP (residues Met\textsuperscript{1}–Thr-Ile\textsuperscript{499}) and the C-terminal region of ApNhaP (residues Glu\textsuperscript{502}–Glu\textsuperscript{521}), the respective nucleotides were amplified by the following forward/reverse primer sets, SNP-N-F/SNP-N-R and ANP-C-F/ANP-C-R. The reverse primer, ANP-C-R, contains the EcoRI site just before the stop codon of pSanhaP. Upon mixing both PCR products, a chimera gene *synapnhaP* was amplified using the forward SNP-N-F and reverse SNP-N-R primers and ligated into the *NeoI/EcoRI*-digested sites of pTrcHis2C, which generated the plasmid pSANhaP.

**Na\textsuperscript{+}/H\textsuperscript{+} Antiporter Activity—**The Na\textsuperscript{+}/H\textsuperscript{+} antiporter activity was examined on everted membrane vesicles prepared from the cells grown in LBK medium (Luria broth with KCl instead of NaCl) as described (9, 22). Briefly, *E. coli* cells were harvested by centrifugation at 3,000 × g for 10 min at 4 °C and then washed with a suspension buffer TCDS (10 mM Tris–HCl (pH 7.5), 0.14 M choline chloride, 0.5 mM dithiothreitol, and 0.25 mM sucrose). The pellet was suspended in 10 ml of TCDS buffer and applied to a French pressure cell (4,000 p.s.i.). Then, the solution was centrifuged at 12,000 × g for 10 min at 4 °C. The supernatant was centrifuged at 110,000 × g for 60 min at 4 °C, and the pellet was suspended in 600 μl of TCDS buffer. The antiporter activity was esti-
Figure 2: Genomic organization of the apnha locus of A. halophytica. A, genes and restriction sites. B, nucleotide sequence of the junction region between apnhaP and cytochrome _c_550. The putative −10 (ATGAACT) and −35 (TACTAC) consensus sequences of _apnhaP_ and the amino acid sequences deduced from the nucleotide sequences of _apnhaP_ and cytochrome _c_550 are also shown.

Figure 3: Analysis of the hydropathy plot of the _ApNhaP_ protein (1). The Asp139 in _ApNhaP_ was conserved (61% identity in amino acids). Analysis of the hydropathy plot revealed the presence of 10 putative transmembrane domains (putative _TMs_). The _TMs_ are numbered from N to C. The predicted gene spanned positions 796 to 2361. The predicted _gene_ was ligated into the expression vector pTrcHis2C, and _ApNhaP_ was expressed in _E. coli_. The resulting _rATG_ band (data not shown). These results indicate that _ApNhaP_ could be expressed and assembled in _E. coli_ membranes.

**RESULTS**

Cloning of Na⁺/H⁺ Antiporter from _A. halophytica_—Using the mixed oligonucleotides of two highly conserved regions among several _NhaP_-type antiporters, a fragment of the _Na⁺/H⁺_ antiporter gene was amplified and sequenced. Then, the upstream and downstream regions of the fragment were amplified by the inverse PCR method (10). The resulting nucleotide sequence of the entire _2431 bp_ was determined (Fig. 2). The sequence analysis revealed one open reading frame, _apnhaP_, spanning positions 796 to 2361. The predicted gene product consists of 521 amino acids with a molecular mass of 58,881 Da (ApNhaP). The upstream sequence of the first _Met_ revealed the presence of −10 (ATGAACT) and −35 (TACTAC) consensus sequences (Fig. 2B). At further upstream regions, _ApNhaP_ contained a long hydrophilic C-terminal tail, which is homologous to eukaryotic _Na⁺/H⁺_ antiporters but not in the _NhaP_ gene from _P. aeruginosa_. These data indicate that a halotolerant cyanobacterium _A. halophytica_ contains an _Na⁺/H⁺_ antiporter homologous to eukaryotic ones.

_ApNhaP Complements Na⁺-sensitive E. coli Mutant_—To characterize the molecular properties of _ApNhaP_, the _apnhaP_ gene was ligated into the pTrcHis2C plasmid. The resulting plasmid, pANhaP, as well as pSNhaP, was expressed in the _E. coli_ host cells (TO114) in which _NhaA_, _NhaB_, and _ChaA_ were absent (9, 21). Western blotting analysis of the membrane fractions revealed that the _E. coli_ cells transformed with pANhaP and pSNhaP exhibited a single cross-reaction band corresponding to ∼53 kDa, whereas the _E. coli_ cells transformed with the vector alone (pTrcHis2C) did not show any band (data not shown). These results indicate that the _ApNhaP_ could be expressed and assembled in _E. coli_ membranes.

Fig. 4A shows that the _E. coli_ TO114 cells transformed with pANhaP can grow with a similar rate to that of the TO114 cells transformed with pTrcHis2C and pSNhaP in LBK medium at pH 7.0 and 37 °C. However, because of the absence of _Na⁺/H⁺_ antipporter genes _nhaA_, _nhaB_, and _chaA_ in TO114 cells, the _E. coli_ cells transformed with pTrcHis2C could not grow in the presence of 0.2 M NaCl (Fig. 4B). In contrast, the _E. coli_ cells transformed with pANhaP or pSNhaP could grow (Fig. 4B). Interestingly, the TO114 cells transformed with pANhaP grow slightly faster than those transformed with pSNhaP (Fig. 4B).

Fig. 4B suggests the different complementation ability between _ApNhaP_ and _SynNhaP_. Thus, we examined other conditions that cause more clear differences in the complementation ability in these two antiporters. Fig. 5A shows that at the growth temperature of 30 °C, the TO114 cells transformed with pANhaP could grow slightly faster than those transformed with pSNhaP at alkaline pH in LBK medium. Under these conditions, _ApNhaP_ could complement the salt-sensitive phenotype of TO114 cells when the growth medium contained 0.2 M NaCl, whereas _SynNhaP_ could not (Fig. 5B). TO114 cells transformed with pANhaP could even grow in the presence of 0.5 M NaCl (Fig. 5C). These differences in complementation ability were not observed at lower pH values (data not shown). The above results indicate that _ApNhaP_ could complement more efficiently than _SynNhaP_ at alkaline pH.

**Novel Ion Specificity of ApNhaP Measured by Activity in the Everted Membrane Vesicles**—To examine directly the antiporter activity of _ApNhaP_, the everted membrane vesicles were prepared, and their antiporter activities were monitored by measuring the lactate-induced fluorescence quenching (Q) and salt-induced fluorescence dequenching (ΔQ). As shown in the
upper panel of Fig. 6, the dequenching ($\Delta Q \times 100/Q$) was observed upon the addition of NaCl in the ApNhaP-expressing cells but not in the control (pTrcHis2C) cells, indicating that ApNhaP has $Na^+/H^+$ antiporter activity. Fig. 6A shows that the dequenching of fluorescence by ApNhaP was observed over a wide pH range between 5 and 9, which is similar to dequenching in Fig. 3.

**FIG. 3.** Comparison of the deduced amino acid sequences of $Na^+/H^+$ antiporters. A, alignment of the deduced amino acid sequences of $Na^+/H^+$ antiporters from eight organisms. The sequences were aligned by the program ClustalW. The alignment is based on the N-terminal 550-amino acid residues in the cases of SOS1, human NHE1, and yeast NHX1. The amino acid residues conserved in all sequences are highlighted in black, and conservative substitutions are shown by dots. The conserved Asp (Asp139 in ApNhaP) is shown by the asterisk. Predicted membrane-spanning regions were marked above the alignment. B, phylogenetic analysis of eight $Na^+/H^+$ antiporters. Multiple sequence alignment and generation of the phylogenetic tree were performed with ClustalW and TreeView software, respectively.

**FIG. 4.** Effects of NaCl on the growth rates of three kinds of E. coli cells. The control and transformant cells at logarithmic phase in LBK medium were transferred to the fresh LBK medium containing the indicated concentrations of NaCl at pH 7.0. A, time courses of growth in LBK medium. B, time courses of growth in LBK containing 0.2 M NaCl. Circles, control cells; squares, ApNhaP-expressing cells; triangles, SynNhaP-expressing cells. Each value shows the average of three independent measurements.
ing by SynNhaP (9) but quite different from that by E. coli NhaA. In E. coli NhaA, the antiporter activity could not be observed below pH 7.5, whereas the activity increased with increasing pH (1).

To examine the ion specificity of ApNhaP, the dequenching was measured upon the addition of different cations. In contrast with most Na+/H+ antiporters, which could catalyze the exchange between Li+/H+ and H+/H+, ApNhaP showed virtually no activity of the Li+/H+ antiporter at various pH values (Fig. 6B). Moreover, it was found that ApNhaP could exhibit Ca2+/H+ antiporter activity at neutral or alkaline pH (Fig. 6C), whereas only small or no antiporter activity was observed for K+/H+ or Mg2+/H+, respectively (data not shown). These results indicate that ApNhaP is an antiporter with novel ion specificity.

ApNhaP Complements Ca2+-sensitive but Not Li+-sensitive E. coli Mutant—Since the above results indicate that ApNhaP has an exchange activity between Ca2+ and H+, but not between Li+ and H+, we further examined whether these ion specificities could be demonstrated in the complementation of salt-sensitive phenotypes of E. coli mutant cells. As shown in Fig. 7A, the TO114 cells expressing ApNhaP could not grow in LBK medium containing 4 mM LiCl at pH 8.0, whereas the TO114 cells expressing SynNhaP could grow. In contrast, Fig. 7B indicates that the TO114 cells expressing ApNhaP could grow well in the TrisE medium containing 0.1 M CaCl2 at pH 8.0 (26), but the TO114 cells expressing SynNhaP grew slowly. In both cases, the control cells (vector only) could not grow (Fig. 7A and B). These results are consistent with the results of the
Li\(^{+}/\)H\(^{+}\) and Ca\(^{2+}/\)H\(^{+}\) antiporter activities of ApNhaP and SynNhaP (Fig. 6, B and C), although the growth rate of SynNhaP-expressing cells in Ca\(^{2+}\)-containing medium appeared to be slower than that anticipated from the Ca\(^{2+}/\)H\(^{+}\) exchange activity of SynNhaP (Figs. 7B and 6C).

**C-terminal Domain Plays a Role for Ion Specificity of ApNhaP and SynNhaP**—Previously, it was shown that the partial deletion of the C-terminal tail decreased the Na\(^{-}/\)H\(^{+}\) antiporter activity of SynNhaP (9). Although ApNhaP showed the highest homology to SynNhaP, the charges on the C-terminal tail of these two antiporters are significantly different, 22 basic and 14 acidic amino acids in ApNhaP and 15 basic and 24 acidic amino acids in SynNhaP. Therefore, we constructed the chimeric antiporters, ASNhaP and SANhaP, in which the long C-terminal tails of ApNhaP and SynNhaP were replaced with those of SynNhaP and ApNhaP, respectively, as shown in Fig. 8. The exchange activities of chimeras were measured using the everted membrane vesicles. Fig. 9 shows that the ASNhaP chimera exhibited comparable Na\(^{-}/\)H\(^{+}\), Li\(^{-}/\)H\(^{+}\), and Ca\(^{2+}/\)H\(^{+}\) exchange activities with those of the parental ApNhaP, which were shown in Fig. 6. Interestingly, the Li\(^{-}/\)H\(^{+}\) exchange activity, which was virtually nondetectable in ApNhaP (Fig. 6B), could be clearly observed in the ASNhaP chimera (Fig. 9B). On the other hand, the SANhaP chimera showed reduced exchange activities of both Na\(^{-}/\)H\(^{+}\) and Li\(^{-}/\)H\(^{+}\) as compared with the parental SynNhaP (Fig. 9 versus Fig. 6). Furthermore, the Ca\(^{2+}/\)H\(^{+}\) exchange activity in the SANhaP chimera appeared to be pH-dependent, i.e., increased activity was observed at neutral or alkaline pH (Fig. 9C), which was in contrast with that observed for the parental SynNhaP, showing relatively unchanged activity irrespective of the pH (Fig. 6C). These results suggest that the C-terminal region plays a role for the ion specificity of ApNhaP and SynNhaP, although some activity data do not reconcile with this viewpoint. For example, the SANhaP chimera had low Na\(^{-}/\)H\(^{+}\) exchange activity compared with that of ApNhaP and SynNhaP (Figs. 9A and 6A), and the Ca\(^{2+}/\)H\(^{+}\) exchange activity of ASNhaP chimera was pH-dependent, which is the same as that for the parental ApNhaP but different from that for the SynNhaP (Fig. 9C). These results suggest that the ion specificities of ApNhaP and SynNhaP are also affected by the structures in TM regions, as is assumed in most cases of Na\(^{-}/\)H\(^{+}\) antiporters (1).

The exchange activity data of chimeras were further substantiated by the complementation analysis. As shown in Fig. 10, the ASNhaP chimera could complement the Na\(^{-}\)-, Li\(^{-}\)-, and Ca\(^{2+}\)-sensitive phenotypes of the *E. coli* mutant, whereas the SANhaP chimera could hardly complement the Na\(^{-}\)- and Li\(^{-}\)-sensitive phenotype of the *E. coli* mutant. The growth rate of *E. coli* mutant cells expressing SANhaP was slower than that anticipated from the Ca\(^{2+}/\)H\(^{+}\) exchange activity of the SANhaP chimera (Figs. 10C and 9C).

**DISCUSSION**

We could isolate an Na\(^{-}/\)H\(^{+}\) antiporter gene homologous to eukaryotic ones from a halotolerant cyanobacterium *A. halophyta*. The *A. halophyta* antiporter gene exhibits the highest homology to the synnhaP and encodes a polypeptide consisting of 521 amino acids. The hydropathy plot and TM prediction analysis of ApNhaP suggested the presence of 11 TM segments and a relatively long C-terminal tail in cytosolic space. One of the important ionic amino acids, Asp\(^{139}\), in ApNhaP, was conserved in the membrane-spanning region.

Based on the findings that the antiporter-deficient *E. coli* TO114 mutant cells became salt-tolerant by transformation
with the *apnhaP* gene (Fig. 4) and also by the direct observation of Na\(^+\)/H\(^+\) antiporter activity in the transformant membrane vesicles (Fig. 6), it was concluded that the *apnhaP* encodes the Na\(^+\)/H\(^+\) antiporter ApNhaP. The most striking functional feature of ApNhaP is its novel ion specificity. The ApNhaP did not show the Li\(^+\)/H\(^+\) antiporter activity (Fig. 6B) but did show the Ca\(^{2+}\)/H\(^+\) antiporter activity (Fig. 6C). These conclusions were also substantiated by the observations that the ApNhaP did not complement the Li\(^+\)-sensitive phenotype of the *E. coli* mutant (Fig. 7A) but complemented the Ca\(^{2+}\)-sensitive phenotype of the *E. coli* mutant (Fig. 7B).

It has been reported that the *E. coli* ChaA has proton/cation exchange activity with Na\(^+\) or Ca\(^{2+}\) but not with Li\(^+\) or K\(^+\) (14, 21), which is essentially the same ion specificity as that of ApNhaP. However, the ChaA did not show any homology to the ApNhaP. The ChaA has the acidic motif Glu200-His-Glu-Asp-Asp-Ser-Asp-Asp-Asp\(^{209}\) conserved in several Ca\(^{2+}\)-binding proteins such as calsequerin, calreticulin, and Na\(^+\)/Ca\(^{2+}\) exchanger (14), whereas the ApNhaP lacks the acidic motif. The hydropathy plot of ChaA suggests the absence of a long hydrophilic C-terminal tail seen with the ApNhaP (data not shown). The ApNhaP did not show any homology to the vacuolar Ca\(^{2+}\)/H\(^+\) exchangers from yeast (27) and plants (28). All these data suggest that the ApNhaP is an Na\(^+\)/H\(^+\) antiporter with a different structure from that of ChaA.

The data of Fig. 9 show that the exchange of long C-terminal tails of ApNhaP and SynNhaP with those of SynNhaP and ApNhaP greatly affected the ion specificity of the antiporter, especially that of the Li\(^+\)/H\(^+\) exchange activity. These data suggest that exchange of the long C-terminal tail could disrupt the structural elements of the antiporter that are critical for ion-specific binding. A remarkable difference between the C-terminal tails of ApNhaP and SynNhaP is the net charges; 22 basic and 14 acidic amino acids in ApNhaP as compared with 15 basic and 24 acidic amino acids in SynNhaP. An intriguing possibility is that the negative charges on the C-terminal tail of SynNhaP help the binding of Li\(^+\), which has a smaller ion radius and consequently a higher positive charge density than Na\(^+\), on the membrane-spanning region of the antiporter, thereby allowing the Li\(^+\)/H\(^+\) exchange activity. However, the data of Ca\(^{2+}\)/H\(^+\) exchange activity between parental and chimeric antiporters (Figs. 6C and 9C) do not reconcile with the above viewpoint, suggesting that TM region(s) may also be important for the ion specificity of Na\(^+\)/H\(^+\) antiporters. Since the functional role of the C-terminal domain is relevant to its topology, the topological study on ApNhaP might be interesting. In fact, although the C-terminal domains of Na\(^+\)/H\(^+\) antiporters have generally been assumed to be entirely cytosolic, exposure of at least some portion of C-terminal domains to the periplasm has been demonstrated (29, 30). The construction of various mutants with alteration in the C-terminal hydrophilic region of ApNhaP is needed to elucidate the mechanism underlying the ion specificity of ApNhaP. In addition, the construction of additional chimera in which chaA from *E. coli* that lacks a C-terminal tail serving as a parental antiporter may give further insight into the role of the C-terminal tail.

It is worthwhile to note that there is an apparent disparity of the data between the exchange activity and the growth rate. The difference of growth rates between ApNhaP- and SynNhaP-expressing cells (Fig. 7B) was much greater than the difference of Ca\(^{2+}\)/H\(^+\) antiporter activities between ApNhaP and SynNhaP (Fig. 6B). In the case of the complementation of Ca\(^{2+}\)-sensitive *E. coli* cells (Fig. 7B), Ca\(^{2+}\) must be excluded from the cells against the concentration gradient of Ca\(^{2+}\). This activity was much higher in ApNhaP-expressing cells as compared with SynNhaP-expressing cells. In contrast, for Ca\(^{2+}\)/H\(^+\) exchange, the activity was measured using the everted membrane vesicles subjected to the concentration gradient of Ca\(^{2+}\). The difference in this activity between ApNhaP and SynNhaP was not so high (Fig. 6C). Thus, the experimental conditions are different between the exchange activity measurement and the complementation test, at least on the orientation of vesicles, Ca\(^{2+}\) concentration gradient, and pH at the H\(^+\) binding site. These different experimental conditions might be the cause, at least partly, for the apparent disparity between the exchange activity and the growth rate, although the exact nature of this disparity is still unknown.

The data of Figs. 4 and 5 show that the complementation ability of ApNhaP is more effective than that of SynNhaP. This finding would suggest an interesting application of ApNhaP for the genetic engineering of salt-tolerant plants. Previously, we showed that the DnaK from *A. halophytica* exhibits *in vitro* much higher refolding activity than that of the DnaK from freshwater cyanobacterium (18). The transformation of tobacco plants by *A. halophytica* DNA conferred salt tolerance (19) as well as high temperature tolerance (31). Since it has been reported that the transformation of *Arabidopsis* by the vacuole-type Na\(^+\)/H\(^+\) antiporter AtNHX1 from *Arabidopsis* could confer salt tolerance of *Arabidopsis* (32), further studies aimed at constructing the transgenic plants using ApNhaP would enable us to obtain improved salt-tolerant plants. Transfer of multiple salt-tolerant genes from *A. halophytica* into plants will provide an interesting example to construct the salt-tolerant plants.

In conclusion, we could isolate a eukaryotic Na\(^+\)/H\(^+\) antiporter gene from halotolerant cyanobacterium *A. halophytica*. ApNhaP exhibited the Na\(^+\)/H\(^+\) antiporter activity over a wide pH range. The ion specificity of ApNhaP is unique. The ApNhaP did not show any activity of the Li\(^+\)/H\(^+\) antiporter but had high Ca\(^{2+}\)/H\(^+\) antiporter activity. The replacement of a long C-terminal tail of ApNhaP with that of Synchocystis altered the ion specificity of antiporter. The Na\(^+\)/H\(^+\) antiporter activity was activated by salt shock as well as by an osmoprotectant, betaine. Thus, ApNhaP would provide a unique system for the study of the ion specificity of eukaryotic Na\(^+\)/H\(^+\) antiporters.
and also an application for the genetic engineering of salt-tolerant plants.

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