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Functional Expression in *Escherichia coli* of Low-Affinity and High-Affinity $Na^+(Li^+)/H^+$ Antiporters of *Synechocystis*

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***Synechocystis* sp. strain PCC 6803 has five genes for putative Na^+/H^+ antiporters (designated *nhaS1*, *nhaS2*, *nhaS3*, *nhaS4*, and *nhaS5*). The deduced amino acid sequences of NhaS1 and NhaS2 are similar to that of NhaP, the Na^+/H^+ antiporter of *Pseudomonas aeruginosa*, whereas those of NhaS3, NhaS4, and NhaS5 resemble that of NapA, the Na^+/H^+ antiporter of *Enterococcus hirae*. We successfully induced the expression of *nhaS1*, *nhaS3*, and *nhaS4* under control of an Na^+-dependent promoter in *Escherichia coli* TO114, a strain that is deficient in Na^+/H^+ antiport activity. Inverted membrane vesicles prepared from TO114 *nhaS1* and TO114 *nhaS3* cells exhibited $Na^+(Li^+)/H^+$ antiport activity. Kinetic analysis of this activity revealed that *nhaS1* encodes a low-affinity Na^+/H^+ antiporter with a K_m of 7.7 mM for Na^+ ions and a K_m of 2.5 mM for Li^+ ions, while *nhaS3* encodes a high-affinity Na^+/H^+ antiporter with a K_m of 0.7 mM for Na^+ ions and a K_m of 0.01 mM for Li^+ ions. Transformation of *E. coli* TO114 with the *nhaS1* and *nhaS3* genes increased cellular tolerance to high concentrations of Na^+ and Li^+ ions, as well as to depletion of K^+ ions during cell growth. To our knowledge, this is the first functional characterization of Na^+/H^+ antiporters from a cyanobacterium. Inverted membrane vesicles prepared from TO114 *nhaS4* cells did not have Na^+/H^+ antiport activity, and the cells themselves were as sensitive to Na^+ and Li^+ ions as the original TO114 cells. However, the TO114 *nhaS4* cells were tolerant to depletion of K^+ ions. Taking into account these results and the growth characteristics of *Synechocystis* mutants in which *nhaS* genes had been inactivated by targeted disruption, we discuss possible roles of NhaS1, NhaS3, and NhaS4 in *Synechocystis*.**

High salinity is a major environmental factor that limits the growth and productivity of plants, eukaryotic microorganisms, and bacteria. Control of membrane permeability to Na^+ ions and the counteracting K^+ ions is the most important aspect of the acclimation of these organisms to high-salt conditions. Na^+/H^+ antiporters are membrane proteins that are essential for maintenance of the balance between Na^+ and K^+ ions in plant, fungal, and bacterial cells, in particular when the organism lacks primary Na^+ pumps or when the Na^+ pumps are not operative (8, 33).

Escherichia coli has at least three genes for Na^+/H^+ antiporters: *nhaA* (14, 23), *nhaB* (34), and *chaA* (19, 31). The presence of a primary Na^+ pump has been suggested (4), but *E. coli* mutants deficient in all three of these genes are hypersensitive to Na^+ and Li^+ ions (31, 35). *Saccharomyces cerevisiae* has Na^+-ATPases (17) and an $Na^+(K^+)/H^+$ antiporter, Nha1, in the plasma membrane (5). In addition, it has been suggested that an Na^+/H^+ antiporter in yeast, designated Nhx1, functions to remove Na^+ ions from the cytosol by sequestering these ions in a prevacuolar compartment (28, 29). In contrast, it is well established that high-affinity K^+ channels that restrict the influx of Na^+ ions determine the capacity of plant cells to tolerate high-salt stress (38). Moreover, Apse et al. (1) demonstrated that a vacuolar Na^+/H^+ antiporter in *Arabidopsis thaliana*, AtNHX1, which is homologous to Nhx1 of *S. cerevisiae*, also participates in the acclimation of *A. thaliana* to high-salt conditions. Shi et al. (40) proposed recently that SOS1 of *A. thaliana*, a homolog of Na^+/H^+ antiporters in plasma membranes, might play a role in Na^+/K^+ homeostasis.

We chose cyanobacteria as a model system for studies of the molecular mechanisms of the responses of plants to high-salt stress for the following reasons. (i) Cyanobacteria perform oxygenic photosynthesis using photosystems similar to those in plant chloroplasts. (ii) The structure and the lipid compositions of cyanobacterial membranes resemble those of chloroplasts of higher plants and algae (49). (iii) Cyanobacterial cells exhibit more obvious responses to salt stress than do plant cells, and they can be exposed directly to changes in external salt conditions, demonstrating a pronounced ability to acclimate to new conditions. (iv) Some strains of unicellular cyanobacteria, such as *Synechocystis* sp. strain PCC 6803 (hereafter "*Synechocystis*") and *Synechococcus* sp. strain PCC 7942, are naturally transformable and can easily be modified by transformation and gene targeting (15). (v) The entire nucleotide sequence of the *Synechocystis* genome has been determined (22). Moreover, cyanobacteria themselves are unusual in that they contain thylakoid membranes in addition to the outer and cytoplasmic membranes. The thylakoid membranes provide sites for photosynthesis and a variety of metabolic pathways. The unusual structural and functional features of cyanobacterial cells led us to postulate that the systems that regulate ion fluxes across membranes in cyanobacterial cells might differ from those in other types of cells.

Cyanobacterial cells actively extrude Na^+ ions via the actions of Na^+/H^+ antiporters. They maintain low intracellular concentrations of Na^+ ions and relatively high intracellular concentrations of K^+ ions (36). Therefore, they must have transport systems that discriminate between K^+ and Na^+ ions. When cyanobacterial cells are grown under high-salt condi-

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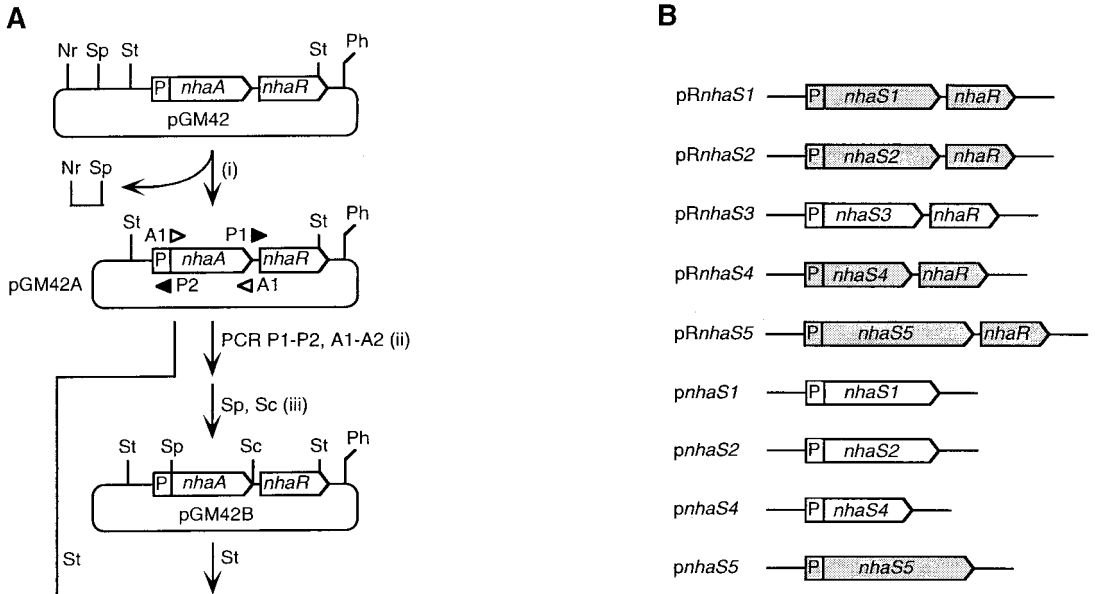

FIG. 1. Plasmids used for expression of *nhaS* genes in *E. coli* TO114. (A) Construction of vector plasmids. Solid lines represent the pBR322 backbone and the flanking regions of the *nhaA* and *nhaR* genes of *E. coli*. P, Na^+-inducible promoter of the *nhaA* gene (*nhaAp*). Restriction sites: Nr, *Nru*I; Sp, *Sph*I; St, *Stu*I; Sc, *Sac*I; Ph, *Psh*AI; Ec, *Ecl*136II. For details, see Materials and Methods. (B) Plasmid characteristics. Plasmids *pnhaS1*, *pnhaS2*, *pRnhaS3*, and *pnhaS4* were successfully introduced into TO114 cells (indicated by the absence of shading).

tions, the pH gradient-dependent (ΔpH-dependent) transport of Na^+ ions across the cytoplasmic membrane is enhanced (7, 30). Respiratory activity and the activity of cytochrome *c* oxidase are also enhanced under high-salt conditions (13, 20, 26). These observations provide circumstantial evidence for the electron transport-driven extrusion of Na^+ ions by an Na^+/H^+ antiporter in cyanobacterial cells. There have been extensive studies of the molecular aspects of salt-inducible proteins (2, 6) and of salt-regulated genes (3, 48). However, Na^+/H^+ antiporters and other transporters involved in the efflux of Na^+ ions have not yet been identified in cyanobacteria.

In the present study, we attempted to identify the Na^+/H^+ antiporters in *Synechocystis*. This cyanobacterium has five putative genes for homologs of Na^+/H^+ antiporters (22). We used a mutant of *E. coli* that was deficient in Na^+/H^+ antiporters to characterize these cyanobacterial genes by functional complementation. We demonstrate here that *Synechocystis* has at least two genes that encode low-affinity and high-affinity Na^+/H^+ antiporters, respectively.

MATERIALS AND METHODS

Nomenclature of genes. We refer to the putative genes for Na^+/H^+ antiporters in *Synechocystis* as *nhaS1* (slr1727 in the designation system proposed by Kaneko et al. [22]), *nhaS2* (sll0273), *nhaS3* (sll0689), *nhaS4* (slr1595), and *nhaS5* (slr0415).

Bacterial strains and growth conditions. *E. coli* TO114 (W3110 *nhaA*::Km^r *nhaB*::Em^r *chaA*::Cm^r) (31) was generously provided by H. Kobayashi (Chiba University, Chiba, Japan). It was used as the host for complementation tests with cyanobacterial genes. Cells were grown in modified Luria-Bertani medium (39) that consisted of 1.0% tryptone (Difco, Detroit, Mich.), 0.5% yeast extract (Difco), and 100 mM KCl (LBK medium; pH 6.8). For selection and growth of transformed cells, ampicillin was added to 50 μg ml^{-1}.

The cyanobacterial strain *Synechocystis* sp. PCC 6803 was originally provided by J. G. K. Williams (DuPont de Nemours and Co., Wilmington, Del.). Cells were grown at 34°C in BG11 medium (41) supplemented with 20 mM HEPES, and the pH of the medium was adjusted to 7.5 with KOH. Cultures were supplied with illumination from incandescent lamps at 70 μE m^{-2} s^{-1} and aerated with air that contained 1% CO_2. The growth of cells was monitored in terms of optical density at 730 nm (OD_{730}).

Construction of plasmids for expression of *nhaS* genes in *E. coli*. Plasmid pGM42 (14) was kindly provided by E. Padan (Hebrew University of Jerusalem, Jerusalem, Israel). This plasmid is a derivative of pBR322 and includes a 4.2-kbp segment of the chromosomal DNA of *E. coli* that contains the *nhaA* gene under control of the Na^+-inducible promoter *nhaAp* plus the *nhaR* gene for the positive *trans*-acting regulator of the *nhaA* gene (11). Plasmids for expression of each of the five *nhaS* genes in *E. coli* were constructed from pGM42 as shown in Fig. 1A. (i) The *Sph*I site in pGM42 was deleted to yield plasmid pGM42A by digestion with *Sph*I and *Nru*I, blunting with a DNA blunting kit (Takara Shuzo Co. Ltd., Tokyo, Japan), and self-ligation. (ii) A 6.7-kbp fragment that contained the

nhaAp promoter, the *nhaR* gene, and the pBR322 backbone was amplified by PCR with pGM42A as template, the forward primer P1 (CGTCCATCAGTT TGA*gAGctC*GGTTTACCG, corresponding to nucleotides +1153 to +1182, counted from the site of initiation of translation of the *nhaA* gene, which was designated +1), and the reverse primer P2 (GATGCAGATGTT*gCAtgc*TTTAT TTCTCTTTCAGG, complementary to nucleotides +13 to −19). (Italicized portions represent restriction sites for *Sph*I [GCATGC] and *Sac*I [GAGCTC]; nucleotides that differ from the ones in the template are lowercased.) The *nhaA* gene was also amplified with pGM42A as template, the forward primer A1 (CCTGAAAGAGAAATAAA*gcaTGc*AACATCTGCATC, corresponding to nucleotides −19 to +13, counted from the site of initiation of translation of the *nhaA* gene, which was designated +1), and the reverse primer A2 (CGGTAA ACC*GagCTc*TCAAACTGATGGACG, complementary to nucleotides +1182 to +1153). (iii) The 6.7-kbp fragment and the *nhaA* gene were digested with *Sph*I and *Sac*I and ligated to yield plasmid pGM42B. (iv) A 2.2-kbp *Stu*I-*Stu*I fragment of pGM42A was replaced by the corresponding part of pGM42B. The resultant plasmid, designated pR*nhaA*, was identical to pGM42A except that it contained an *Sph*I site at the site of initiation of translation of the *nhaA* gene (nucleotides −2 to +4) and a *Sac*I site just downstream of the *nhaA* gene (nucleotides +1168 to +1173). (v) The various *nhaS* genes were amplified with the chromosomal DNA isolated from *Synechocystis* as template and the following synthetic oligonucleotides as primers: forward primer CA*gCaTGc*ATACAGCGGTCAACGA (corresponding to nucleotides −4 to +20, counted from the site of initiation of translation of the *nhaS1* gene, which was designated +1) and reverse primer aa*gagctc*CTAGGATGGTTCGGCCACAT (complementary to nucleotides +1584 to +1565) for the *nhaS1* gene, forward primer CT*gCATGc*CTTAAGCT CCCTGTGC (corresponding to nucleotides −4 to +19, counted from the site of initiation of translation of the *nhaS2* gene, which was designated +1) and reverse primer TT*gAGcTC*GTCAGTCATCCTGCAGG (complementary to nucleotides +1632 to +1608) for the *nhaS2* gene, forward primer tt*gcATGc*TTATGAACC CATTGCTCCCTC (corresponding to nucleotides +1 to +25, counted from the site of initiation of translation of the *nhaS3* gene, which was designated +1) and reverse primer tt*gagctc*CTAATCTGGGGTGGGAACTG (complementary to nucleotides +1386 to +1367) for the *nhaS3* gene, forward primer AA*gcATGc*A CACCAATACTTTACTGCTAATT (corresponding to nucleotides −4 to +27, counted from the site of initiation of translation of the *nhaS4* gene, which was designated +1) and reverse primer tt*gaGcTc*TTAATGGGCTGGGGCAGGAT (complementary to nucleotides +1237 to +1214) for the *nhaS4* gene, and forward primer tt*gcATGc*ATGGCCTATTCGCACCAATTC (corresponding to nucleotides +1 to +25, counted from the site of initiation of translation of the *nhaS5* gene, which was designated +1) and reverse primer aa*gagctc*CTAGGCG TAGGGATCGCCA (complementary to nucleotides +2097 to +2079) for the *nhaS5* gene. (vi) The *nhaA* gene in pR*nhaA* was removed by digestion with *Sph*I and *Sac*I, and an amplified *nhaS* gene was inserted. The resultant plasmids were designated pR*nhaS1*, pR*nhaS2*, pR*nhaS3*, pR*nhaS4*, and pR*nhaS5*. (vii) To generate another set of plasmids that did not contain the *nhaR* gene, the plasmids pR*nhaS1*, pR*nhaS2*, pR*nhaS3*, pR*nhaS4*, pR*nhaS5*, and pR*nhaA* were further digested with *Ecl*136II (an isozyme of *Sac*I) and *Psh*A1 and self-ligated. The resultant plasmids were designated p*nhaS1*, p*nhaS2*, p*nhaS4*, p*nhaS5*, and p*nhaA*. We failed to generate p*nhaS3*. All the amplified fragments and the ligated junctions were verified by determination of nucleotide sequences.

Isolation of RNA. *E. coli* cells were grown in LBK medium to the early exponential phase of growth (OD_{600}, 0.4). Aliquots of the culture were withdrawn, mixed immediately with an equal volume of ice-cold ethanol that contained 5% (wt/vol) phenol, and centrifuged at 3,000 × *g* for 10 min. Each pellet was washed with 50 mM Tris-HCl (pH 8.0) and 100 mM EDTA and then resuspended in 600 μl of 50 mM Tris-HCl (pH 8.0)–5 mM EDTA–0.25% sodium dodecyl sulfate (SDS). The suspension was mixed with 600 μl of acid phenol (a mixture of 50% phenol, 48% chloroform, and 2% isoamyl alcohol [vol/vol], buffered with an equal volume of 50 mM sodium acetate, pH 5.2), and the mixture was incubated at 65°C for 5 min to disrupt the cells. Total nucleic acids were extracted three times with acid phenol and precipitated in ethanol. Total RNA was separated from DNA by precipitation twice in LiCl and stored at −80°C.

DNA probes. The DNA fragments used for the preparation of probes for Northern blotting analysis were generated by excision from the *nhaS* genes that had been amplified by PCR as described above: *nhaS1* (with *Hin*cII, nucleotides +13 to +648), *nhaS2* (with *Sph*I and *Nco*I, nucleotides +1 to +545), *nhaS3* (with *Sph*I and *Eco*RI, nucleotides +1 to +652), and *nhaS4* (with *Sph*I and *Bst*EII, nucleotides +1 to +576). The resultant DNA fragments were labeled with [α-^{32}P]dCTP using a BcaBEST labeling kit (Takara Shuzo).

Northern blotting. Fifteen micrograms of total RNA was fractionated by electrophoresis on a 1.2% agarose gel that contained 6.3% formaldehyde in 3-(*N*-morpholino)propanesulfonic acid buffer, pH 7.0 (39), and bands of RNA were transferred to a nylon membrane (NEN Life Science Products, Boston, Mass.). The membrane was baked at 80°C for 2 h and then incubated for 2 h at 65°C in a solution of 0.5 M sodium phosphate buffer (pH 7.2), 5% SDS, 5× Denhardt's reagent (39), and 100 μg of denatured salmon sperm DNA ml^{-1}. Then the DNA probe was added (2×10^5 cpm ml^{-1}), and hybridization was allowed to proceed for 16 h at 65°C. After a 1-h wash at 55°C in a solution of 0.05 M sodium phosphate buffer (pH 7.2) and 0.5% SDS, the membrane was exposed to an X-ray film (Eastman Kodak Company, Rochester, N.Y.).

Measurement of Na^+/H^+ antiport activities of IMVs. Cells were grown in LBK medium to the middle of the exponential phase of growth (OD_{600}, 1.5). Inverted membrane vesicles (IMVs) were prepared with a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) as described previously (37). The Na^+/H^+ antiport activities of IMVs were estimated from the extent of the collapse of a preformed proton gradient, with acridine orange as the pH indicator, essentially as described previously (14). The assay solution consisted of 140 mM choline chloride, 5 mM $MgCl_2$, 1 μM acridine orange, and 10 mM Tris titrated with 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 8.5). In some cases choline chloride was replaced by 140 mM KCl. An aliquot corresponding to 20 μg of vesicle protein was added to 2 ml of the assay solution that was being stirred in a cuvette. Fluorescence from acridine orange was monitored in a fluorometer (model RF-5000; Shimadzu, Kyoto, Japan). The wavelength of excitation light was 495 nm, and fluorescence was monitored at 530 nm. Addition of Tris-D-lactate to a final concentration of 2 mM energized the IMVs and resulted in quenching of the fluorescence. Subsequent addition of NaCl or LiCl resulted in restoration of fluorescence. The initial rate of this restoration, as measured during the 2-s interval that followed the addition of NaCl or LiCl at various concentrations was taken as the Na^+/H^+ antiport activity, which was expressed in arbitrary units (fluorescence units s^{-1} mg of protein^{-1}). IMVs from pBR322$^+$ cells (negative control) had low Na^+/H^+ antiport activity, which was taken as the background activity. For calculations of kinetic parameters, the Na^+/H^+ antiport activity of IMVs prepared from pBR322$^+$ cells was subtracted from the activity of IMVs prepared from *nhaA*$^+$, *nhaS1*$^+$, and *nhaS3*$^+$ cells.

Evaluation of the sensitivity of cell growth to salt stress. Transformed cells that had been grown in LBK medium were spread on plates prepared with 1.0% tryptone, 0.5% yeast extract, and 1.5% agar (Difco; LBn solid medium) that had been supplemented with various concentrations of NaCl or LiCl, in addition to KCl, for evaluation of the sensitivity of cell growth to high concentrations of Na^+ and Li^+ ions. For evaluation of the sensitivity of cell growth to depletion of K^+ ions, we used plates of LBn solid medium that had been supplemented with various concentrations of KCl. LBn solid medium by itself contained 20 mM Na^+ ions and 5 mM K^+ ions. Formation of colonies was examined after incubation for 24 h at 37°C.

Targeted mutagenesis of the *nhaS* genes in *Synechocystis*. Plasmid pAM1573, which contained a chloramphenicol resistance (Cm^r) gene cartridge, and plasmid pAM1303, which contained a spectinomycin resistance (Sp^r) gene cartridge, were kindly provided by S. S. Golden (Texas A&M University, College Station, Tex.). The *nhaS* genes that had been amplified by PCR, as described above, were subcloned into the TA cloning site of plasmid pT7Blue (Novagen, Madison, Wis.). For construction of a plasmid with a disrupted *nhaS1* gene, the region between the *Bbs*I and *Stu*I sites of the *nhaS1* gene in pT7Blue was removed and the ends of the cleaved plasmid were blunted with the DNA blunting kit. The cleaved and blunted plasmid was ligated with a kanamycin resistance (Km^r) gene cartridge, which had been excised by *Sma*I from plasmid pUC-KIXX (Pharmacia, Uppsala, Sweden). The resultant plasmid was designated p*nhaS1*::Km^r.

For construction of a plasmid with a disrupted *nhaS2* gene, the region between the *Bst*EII and *Hpa*I sites of the *nhaS2* gene in pT7Blue was removed and the ends of the cleaved plasmid were blunted with the DNA blunting kit. The cleaved and blunted plasmid was ligated with a Cm^r gene cartridge, which had been excised by *Bst*EII and *Hin*dIII from pAM1573 and blunted with the DNA blunting kit. The resultant plasmid was designated p*nhaS2*::Cm^r. A plasmid with a disrupted *nhaS3* gene was constructed by inserting the Km^r gene cartridge, which had been excised from pUC-KIXX with *Sma*I, into the *Eco*RV site of the *nhaS3* gene in pT7Blue. The resultant plasmid was designated p*nhaS3*::Km^r. For construction of a plasmid with a disrupted *nhaS4* gene, the region between the *Bst*EII and *Bbs*I sites of the *nhaS4* gene in pT7Blue was removed and the ends of the cleaved plasmid were blunted with the DNA blunting kit. The cleaved and blunted plasmid was ligated with the Cm^r gene cartridge, which had been excised by *Bst*EII and *Hin*dIII from pAM1573 and blunted with the DNA blunting kit. The resultant plasmid was designated p*nhaS4*::Cm^r. A disrupted *nhaS5* gene was constructed by replacing the region between the two *Bal*I sites in the *nhaS5* gene in pT7Blue by the Sp^r gene cartridge, which had been excised by *Eco*RV and *Sma*I from pAM1303. The resultant plasmid was designated p*nhaS5*::Sp^r.

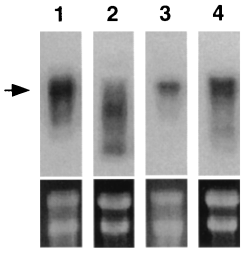

FIG. 2. Northern blotting analysis of the expression of *nhaS* genes in transformed *E. coli* TO114 cells. Total RNA was extracted from cells that had been grown in LBK medium. Results are shown for *nhaS1* transcripts in *nhaS1*$^+$ cells (lane 1), *nhaS2* transcripts in *nhaS2*$^+$ cells (lane 2), *nhaS3* transcripts in *nhaS3*$^+$ cells (lane 3), and *nhaS4* transcripts in *nhaS4*$^+$ cells (lane 4). The positions of the expected transcripts are indicated (arrow). The lower panels show bands that correspond to 16S and 23S rRNAs on each gel, as revealed after staining with ethidium bromide prior to blotting. Three independent experiments yielded essentially the same results.

Wild-type cells of *Synechocystis* were transformed with the individual plasmids to generate Δ*nhaS* cells, as described previously (44). For the construction of the double mutants Δ*nhaS1*Δ*nhaS2* and Δ*nhaS4*Δ*nhaS5*, we transformed Δ*nhaS1* cells with p*nhaS2*::Cm^r and Δ*nhaS4* cells with p*nhaS5*::Sp^r, respectively. For selection of mutant cells, kanamycin, spectinomycin, and chloramphenicol were included in the medium at 25, 15, and 15 μg/ml, respectively. Disruption with the antibiotic resistance cartridges of the *nhaS* genes on all copies of the chromosome was examined by PCR (44).

Concentrations of proteins in IMVs. The concentrations of proteins in IMVs were determined as described elsewhere (9).

RESULTS

Expression of *nhaS* genes in *E. coli* TO114. We transformed *E. coli* TO114 cells with plasmids that contained individual *nhaS* genes (Fig. 1B). The resultant transformed cells were grown on solid LBK medium supplemented with 50 μg of ampicillin ml^{-1}. We obtained colonies only when cells had been transformed with p*nhaS1*, p*nhaS2*, pR*nhaS3*, or p*nhaS4*. Transformation with plasmids pR*nhaS1*, pR*nhaS2*, pR*nhaS4*, pR*nhaS5*, and p*nhaS5* failed to yield colonies under our selection conditions. Thus, transformation of cells with the *nhaS5* gene was unsuccessful. We also obtained TO114 cells that harbored pBR322, pR*nhaA*, or p*nhaA*.

We attempted to determine the levels of products of *nhaS* genes in membrane fractions of transformed *E. coli* cells by SDS-polyacrylamide gel electrophoresis and silver staining. However, we failed to detect bands that corresponded unequivocally to the *Synechocystis* proteins either before or after induction by NaCl. Thus, to evaluate whether *nhaS* genes were at least transcribed in *E. coli* under control of the *nhaAp* promoter, we performed Northern blotting of total RNA extracted from p*nhaS1*/TO114 (*nhaS1*$^+$), p*nhaS2*/TO114 (*nhaS2*$^+$), pR*nhaS3*/TO114 (*nhaS3*$^+$), and p*nhaS4*/TO114 (*nhaS4*$^+$) cells that had been grown in LBK medium, using probes derived from each *nhaS* gene (Fig. 2). Transcripts of the *nhaS1*, *nhaS3*, and *nhaS4* genes accumulated in *nhaS1*$^+$, *nhaS3*$^+$, and *nhaS4*$^+$ cells, respectively. In contrast, most transcripts of the *nhaS2* gene in *nhaS2*$^+$ cells were shorter than the expected length of *nhaS2* mRNA. These transcripts might be degradation products of the *nhaS2* mRNA.

We also examined changes in the levels of transcripts upon an increase in the concentration of NaCl in the medium to 200 mM (data not shown). During exposure to 200 mM NaCl, the level of *nhaS3* transcripts in *nhaS3*$^+$ cells increased gradually over the course of 40 min, while the levels of transcripts of *nhaS1*, *nhaS2*, and *nhaS4* in *nhaS1*$^+$, *nhaS2*$^+$, and *nhaS4*$^+$ cells, respectively, did not change significantly. This result was probably due to the presence of the Na^+-dependent regulatory gene *nhaR* in the construct for expression of the *nhaS3* gene (Fig. 1B), which might have promoted transcription of the *nhaS3* gene under high-salt conditions (11).

Na^+/H^+ antiport activities of IMVs. We measured the Na^+/H^+ antiport activity of IMVs prepared from transformed cells as the Na^+-mediated and Li^+-mediated net efflux of protons, which we monitored by observing changes in the fluorescence of acridine orange. Since IMVs from p*nhaA*/TO114 and pR*nhaA*/TO114 cells had almost the same Na^+/H^+ antiport activity (data not shown), we used pR*nhaA*/TO114 cells in further experiments as the positive control, referring to them as *nhaA*$^+$ cells. We used pBR322/TO114 (pBR322$^+$) cells as the negative control.

Figure 3 shows profiles of Na^+/H^+ antiport activity after addition of 5 mM NaCl in the presence of 140 mM choline chloride under K^+-free conditions (Fig. 3A) or in the presence of 140 mM KCl (Fig. 3B; K^+-rich conditions). Under K^+-free conditions, IMVs from pBR322$^+$ cells had low Na^+/H^+ antiport activity (Fig. 3A). Such activity might have been due to a nonspecific monovalent cation/H^+ antiport system (35) that did not transport Na^+ ions under K^+-rich conditions (Fig. 3B). IMVs from *nhaS1*$^+$ and *nhaS3*$^+$ cells had significant Na^+/H^+ antiport activity under K^+-rich conditions, as did the IMVs from *nhaA*$^+$ cells (Fig. 3B). These results clearly demonstrated that the Na^+/H^+ antiport activity had been transferred to the host *E. coli* cells by transformation with the *nhaS1* and *nhaS3* genes. The IMVs prepared from *nhaS2*$^+$ and *nhaS4*$^+$ cells did not have Na^+/H^+ antiport activity under K^+-rich conditions.

Figure 4 shows profiles of Li^+/H^+ antiport activity, as determined upon addition of 5 mM LiCl under K^+-free conditions (Fig. 4A) and under K^+-rich conditions (Fig. 4B). The

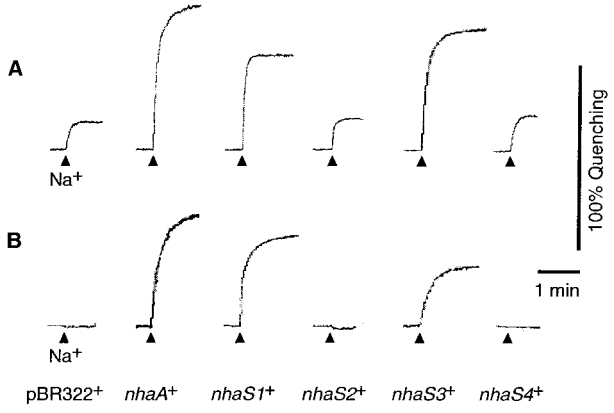

FIG. 3. Activities of the Na^+/H^+ antiport system in IMVs prepared from transformed cells. IMVs were prepared from cells that had been grown in LBK medium. Activity was assayed in a solution that consisted of 5 mM $MgCl_2$, 1 μM acridine orange, and 10 mM Tris titrated with MES (pH 8.5) and supplemented with 140 mM choline chloride (A) or 140 mM KCl (B). Arrowheads, time at which 5 mM NaCl was added to the assay solution.

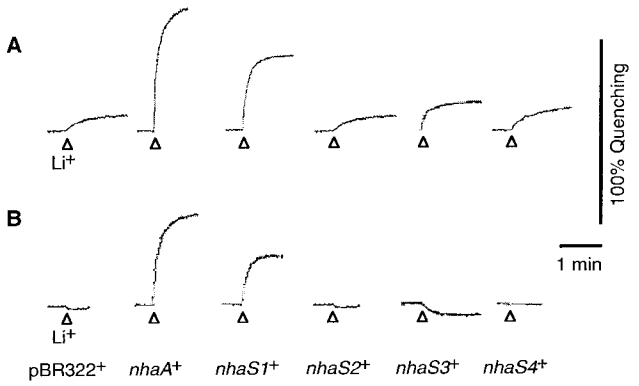

FIG. 4. Activities of the Li^+/H^+ antiport system in IMVs prepared from transformed cells. Experiments were carried out as described in the legend to Fig. 3 except that 5 mM LiCl was added to the assay solution instead of 5 mM NaCl.

IMVs prepared from *nhaS1*$^+$ cells had high Li^+/H^+ antiport activity under K^+-rich conditions (Fig. 4B), demonstrating that Li^+/H^+-antiport activity had also been transferred to the host *E. coli* cells by transformation with the *nhaS1* gene. IMVs prepared from *nhaS3*$^+$ cells did not have Li^+/H^+ antiport activity under K^+-rich conditions (Fig. 4B), but they had considerably higher Li^+/H^+ antiport activity under K^+-free conditions than IMVs prepared from pBR322$^+$ cells (Fig. 4A). These results indicated that Li^+/H^+ antiport activity had been transferred to the host *E. coli* cells upon transformation with the *nhaS3* gene and that the activity was strongly inhibited by the presence of K^+ ions in the assay solution.

Table 1 shows the kinetic parameters of the Na^+/H^+ antiport activity of IMVs prepared from *nhaA*$^+$, *nhaS1*$^+$, and *nhaS3*$^+$ cells and assayed under K^+-free conditions. For both Na^+ and Li^+ ions, the activity of IMVs from *nhaS1*$^+$ cells gave larger values of K_m than the activity of IMVs from *nhaS3*$^+$ cells. The activity of IMVs from *nhaS3*$^+$ cells revealed a strikingly high affinity for Li^+ ions. The K_m of the activity of IMVs from *nhaA*$^+$ cells for Na^+ ions was of the same magnitude as the value reported previously for purified NhaA (i.e., 0.1 mM at pH 8.6) (43).

Sensitivity of cell growth to high concentrations of Na^+ and Li^+ ions. Table 2 shows the maximum concentrations of Na^+ and Li^+ ions that allowed growth of transformed cells on solid LBn medium prepared with 5, 25, 105, or 305 mM K^+ ions. In the presence of 105 mM K^+ ions, growth of pBR322$^+$ cells was inhibited at 120 mM Na^+ ions and at 3 mM Li^+ ions, and it was completely arrested at 170 mM Na^+ ions and at 5 mM Li^+ ions. In contrast, *nhaS1*$^+$ and *nhaS3*$^+$ cells were able to grow at 570 mM Na^+ ions and 10 mM Li^+ ions and at 420 mM Na^+ ions and 70 mM Li^+ ions, respectively, in the presence of 105 mM K^+ ions. These results were consistent with the restored Na^+/H^+ antiport activity in the membranes isolated from the respective cell lines. *nhaS1*$^+$ and *nhaS3*$^+$ cells retained their high tolerance to Na^+ and Li^+ ions when the concentration of K^+ ions was decreased to 5 mM.

Both *nhaS2*$^+$ and *nhaS4*$^+$ cells were as sensitive as pBR322$^+$ cells to Na^+ and Li^+ ions, as expected from the absence under K^+-rich conditions of Na^+/H^+ antiport activity of the IMVs prepared from such cells. This sensitivity of pBR322$^+$, *nhaS2*$^+$, and *nhaS4*$^+$ cells to Na^+ and Li^+ ions decreased as the concentration of K^+ ions in the medium was increased from 5 to 105 mM. As described below, this dependence on K^+ ions seemed to reflect the absence of Na^+/H^+ antiport activity in the membranes.

Sensitivity of cell growth to depletion of K^+ ions. pBR322$^+$ cells did not grow in the presence of 5 mM K^+ ions, the

TABLE 1. Kinetic parameters under K^+-free conditions, of Na^+/H^+ antiport activities of IMVs from transformed cells[a]

Cells	Na^+		Li^+	
	V_{max}[b]	K_m (mM)	V_{max}[b]	K_m (mM)
nhaA$^+$	42	0.2	42	0.3
nhaS1$^+$	120	7.7	40	2.5
nhaS3$^+$	22	0.7	5	0.01

[a] The activities of IMVs from pBR322$^+$ cells (K_m = 1.5 mM and V_{max} = 12 U s^{-1} mg of protein^{-1} for Na^+ ions; K_m = 0.7 mM and V_{max} = 1.5 U s^{-1} mg of protein^{-1} for Li^+ ions) were taken as background activities, as described in Materials and Methods. Two independent experiments yielded essentially the same results.

[b] In fluorescence units s^{-1} mg of protein^{-1}.

TABLE 2. Effects of K^+ ions on the maximum concentrations of Na^+ and Li^+ ions that allowed growth of transformed cells on solid LBn medium

Concn of K^+ in the medium (mM)	Maximum concn for growth (mM)[a]	
	Na^+	Li^+
pBR322$^+$		
5	<20	0
25	60	2
105	120	3
305	120	3
nhaA$^+$		
5	1,070	400
25	1,070	400
105	970	350
305	770	300
nhaS1$^+$		
5	570	15
25	570	15
105	570	10
305	420	5
nhaS2$^+$		
5	<20	0
25	60	2
105	120	3
305	120	3
nhaS3$^+$		
5	370	40
25	370	40
105	420	70
305	320	90
nhaS4$^+$		
5	<20	0
25	60	2
105	120	3
305	120	3

[a] Three independent experiments yielded essentially similar results.

background level, even in the absence of additional Na^+ and Li^+ ions (Table 2), an observation that was consistent with previous reports on a Δ*nhaA*Δ*nhaB* strain of *E. coli* (16, 47) and was probably due to the inability of these cells to maintain intracellular concentrations of Na^+ ions at an appropriate level when the ratio of K^+ ions to Na^+ ions in the medium was low (16). To elucidate the effect of transformation on the sensitivity to depletion of K^+ ions, we examined the growth of transformed cells at various concentrations of K^+ ions. pBR322$^+$ cells required at least 20 mM K^+ ions; *nhaS1*$^+$ and *nhaS3*$^+$ cells grew at 5 mM K^+ ions, the background level, as did *nhaA*$^+$ cells. *nhaS4*$^+$ cells also exhibited a lower requirement for K^+ ions (6 mM) than that of pBR322$^+$ cells. In contrast, the requirement of *nhaS2*$^+$ cells for K^+ ions did not differ significantly from that of pBR322$^+$ cells.

Disruption of *nhaS* genes in *Synechocystis*. We created single and double mutants of *Synechocystis* in which individual *nhaS* genes were disrupted by insertion of an antibiotic resistance gene cartridge. We verified the disruption of the *nhaS1*, *nhaS2*, *nhaS4*, and *nhaS5* genes on all copies of the chromosomal DNA by PCR. We failed to disrupt the *nhaS3* gene under any conditions tested. In our efforts to disrupt the *nhaS3* gene we used the following media: BG11 medium that contained 18 mM Na^+ ions (pH 7.5), a low-sodium medium in which all the sodium salts of BG11 medium had been replaced by potassium salts (this medium was estimated to contain 50 μM Na^+ ions from the extent of contamination by Na^+ ions of the potassium salts [Wako Pure Chemical Industries, Ltd., Oaska, Japan] that we used), and media prepared by adding different concentrations of NaCl (100 μM to 100 mM) to the low-salt medium. The single mutants that we did obtain did not show any phenotypic changes in terms of sensitivity to high concentrations of NaCl (data not shown). Δ*nhaS1*Δ*nhaS2* cells grew more slowly than wild-type cells both in BG11 medium and in a high-salt medium prepared by adding NaCl to 0.5 M to BG11 medium (Fig. 5A). The retardation of growth of Δ*nhaS1*Δ*nhaS2* cells, compared to the growth of wild-type cells, appeared to be greater in the presence of 0.5 M NaCl than in its absence. In contrast, Δ*nhaS4*Δ*nhaS5* cells grew as rapidly as wild-type cells regardless of the presence or absence of 0.5 M NaCl (Fig. 5B).

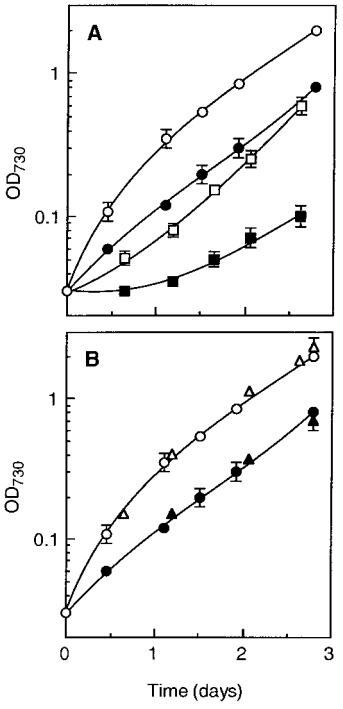

FIG. 5. Growth curves for *Synechocystis* in BG11 medium that contained 18 mM Na^+ ions (open symbols) or in high-salt medium prepared by increasing the concentration of NaCl in BG11 medium to 0.5 M (closed symbols). (A) Wild-type cells (circles) and mutant cells with disrupted *nhaS1* and *nhaS2* genes (Δ*nhaS1*Δ*nhaS2*) (squares). (B) Wild-type cells (circles) and mutant cells with disrupted *nhaS4* and *nhaS5* genes (Δ*nhaS4*Δ*nhaS5*) (triangles). The results were obtained from three independent determinations for each line of cells.

DISCUSSION

Homologs of eukaryotic and prokaryotic Na^+/H^+ antiporters in *Synechocystis*. Phylogenetic analysis (Fig. 6A) revealed that NhaS1 and NhaS2 are related to isoforms of NHE found in vertebrates (NHE1 to -6 and βNHE) and to NHE-like Na^+/H^+ antiporters in plant, fungal, and bacterial cells. NhaS1 and NhaS2 appeared to be most similar to NhaP, an Na^+/H^+ antiporter of *Pseudomonas aeruginosa* (46), and to SOS1, a putative Na^+/H^+ antiporter in *A. thaliana* (40). NhaS3, NhaS4, and NhaS5 resembled NapA, an Na^+/H^+ antiporter in *Enterococcus hirae* (42, 51), as well as KefC, a putative K^+/H^+ antiporter in *E. coli* (27). Genes for homologs of both NHE-like ("eukaryotic") and NapA-like ("prokaryotic") Na^+/H^+ antiporters have been found in many eubacteria, archaea, and eukaryotes, suggesting that the two types of Na^+/H^+ antiporter might have been selected early in evolution. NHE-like and NapA-like Na^+/H^+ antiporters appear to have distinct properties. The isoforms of NHE catalyze the electroneutral exchange of Na^+ ions for protons, being activated by internal protons (50). It has been proposed that Nhx1 of *S. cerevisiae*, an NHE-like Na^+/H^+ antiporter, might be activated by decreases in cytoplasmic pH (28). The isoforms of NHE have rather high K_m values for Na^+ ions, which range from 4.7 to 59 mM (32). A high K_m (7 mM) for Na^+ ions was also reported for vacuoles of *A. thaliana* that overexpressed the *AtNHX1* gene (1). In contrast, it was reported that NapA has a relatively low K_m (1.0 mM) for Na^+ ions (42). The present study of the expression in *E. coli* of cyanobacterial genes from *Synechocystis* provides the first example, to our knowledge, of the functional identification of the two types of Na^+/H^+ antiporter in a single organism. *A. thaliana* has a number of genes for putative NHE-like and NapA-like Na^+/H^+ antiporters. They might be localized in different tissues and membranes.

Each of the NhaS proteins appears to contain 11 transmembrane segments (Fig. 6B). NhaS1, NhaS2, and NhaS5 include a large hydrophilic extension at the carboxyl terminus, as do the NHE isoforms and the NHE-like Na^+/H^+ antiporters in eukaryotic cells. In the various isoforms of NHE, the carboxy-terminal extension mediates the response of the antiporter to various stimuli (50). Therefore, the carboxy-terminal extensions of NhaS1, NhaS2, and NhaS5 might each also play a role in the regulation of the activity.

The strongest homology was found within the putative fifth and sixth transmembrane segments of the NhaS proteins and the corresponding regions of the Na^+/H^+ antiporters from

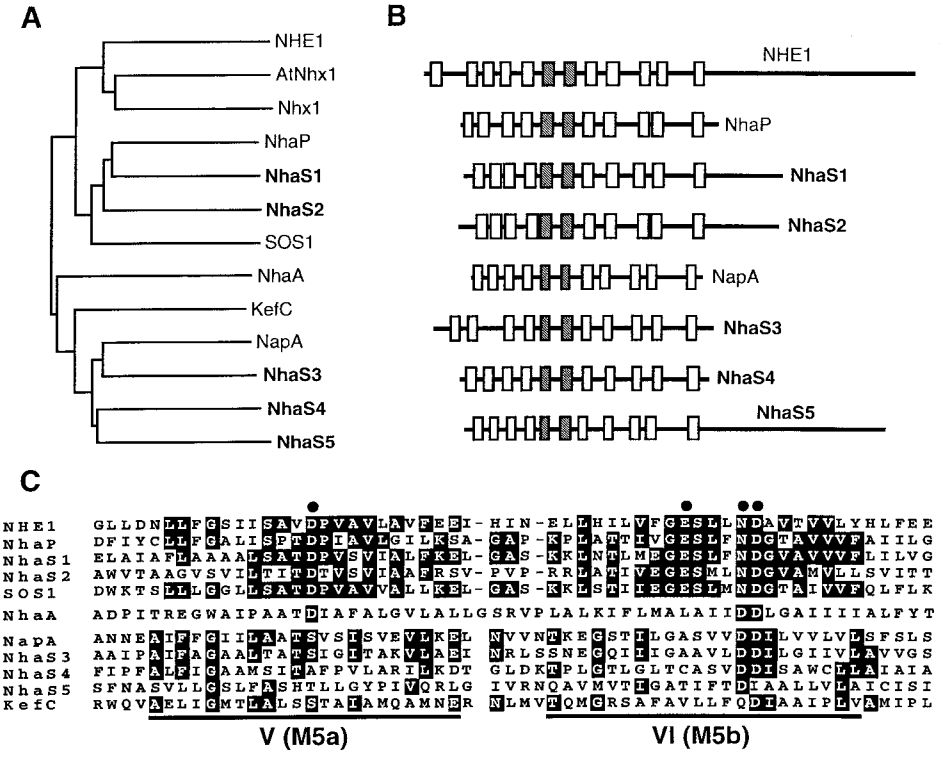

FIG. 6. Relationships of NhaS proteins to other Na^+/H^+ antiporters and related proteins. (A) Phylogenetic relationships as determined with the CLUSTAL W multiple sequence alignment algorithm (45). (B) Secondary structures predicted from hydropathy profiles, as determined by the algorithm of Kyte and Doolittle (24). Putative transmembrane segments are boxed, and segments that exhibit the strongest homology are shaded. (C) Alignment of amino acid sequences of two strongly homologous segments—the putative fifth (V) and sixth (VI) segments—of NhaS proteins, which correspond, respectively, to the sixth (M5a) and seventh (M5b) segments of NHE1. Identical residues are shaded, and conserved Glu and Asp residues are indicated by dots. NHE1, human P19634; AtNhx1, *A. thaliana* AAD16946; Nhx1, *S. cerevisiae* NP 010744; NhaP, *P. aeruginosa* BAA31695; SOS1, *A. thaliana* AAF76139; NhaA, NhaB, and KefC, *E. coli* C64722, G64864, and QQECRD, respectively; NapA, *Enterococcus hirae* A42111.

other organisms (Fig. 6C). This region is the most strongly conserved among the NHE isoforms and includes several acidic residues, the importance of which has been demonstrated both for human NHE1 (12) and for NhaA of *E. coli* (18). Some of these residues are also conserved in the NhaS proteins.

Functional expression of the *nhaS1*, *nhaS3*, and *nhaS4* genes in *E. coli* under the control of the *nhaAp* promoter. The *nhaS* genes were expressed at very low levels in wild-type *Synechocystis* (unpublished results). This observation suggested that expression of each *nhaS* gene in *E. coli* from its own promoter would not result in a sufficient level of product. However, overproduction of proteins that contain several transmembrane segments might be expected to have detrimental effects on host cells. In wild-type *E. coli*, NhaA is a membrane-bound protein that is present at a low level (less than 0.2% of the total membrane proteins [43]). When this protein was overexpressed under the control of the strongly inducible *tac* promoter, cell growth ceased (43). Therefore, we chose to use the *nhaAp* promoter for expression of the various *nhaS* genes in *E. coli* at appropriate levels.

Expression in *E. coli* TO114 of the *nhaS1* and *nhaS3* genes under control of the *nhaAp* promoter resulted in production of functional Na^+/H^+ antiporters. In contrast, the expression of the *nhaS4* gene did not result in expression of detectable Na^+/H^+ antiport activity in the transformed host cells. This failure might have been due to an insufficient level of the expressed protein, which, in turn, would have resulted in the inability of *nhaS4*$^+$ cells to acquire Na^+/H^+ antiport activity. Alternatively, NhaS4 might not function as an efficient system for extrusion of Na^+ ions.

Transcripts of the *nhaS2* gene appeared to be degraded in the absence of NaCl. The instability of the heterologous transcripts might have been related to inefficient translation, due in turn to the presence of codons that are used at low frequencies in *E. coli* (21). Inefficient translation can increase the susceptibility of transcripts to RNases (10). However, this situation does not appear to have been operative in the present case because the proportion of such unusual codons in *nhaS2* transcripts was not much higher than that in the transcripts of the other *nhaS* genes (unpublished data). It has been suggested that NhaS2 might be required for the uptake of Na^+ ions in *Synechocystis* (25). The instability of *nhaS2* transcripts might have been a consequence of the disturbed balance of ions in the transformed *E. coli* cells.

Our failure to introduce the *nhaS5* gene into TO114 cells suggests that the introduction of this gene under the control of the *nhaAp* promoter might have had a detrimental effect on the host cells, even when expression was not induced by high concentrations of Na^+ ions.

NhaS1 and NhaS3 are low-affinity and high-affinity Na^+/H^+ antiporters, respectively. The kinetic properties of the Na^+/H^+ antiport system in IMVs prepared from *nhaS1*$^+$ cells (Table 1) indicated that the expressed protein, NhaS1, had low affinity for Na^+ ions (K_m, 7.7 mM) and for Li^+ ions (K_m, 2.5 mM). The K_m for Na^+ ions is close to that reported for AtNhx1 of *A. thaliana* (1). The lower K_m of NhaS1 for Li^+ ions than for Na^+ ions suggests that Li^+ ions might be a better substrate than Na^+ ions. However, transformation with the *nhaS1* gene had only a minimal effect on the tolerance of the host cells to Li^+ ions, while it dramatically increased the tolerance of host cells to Na^+ ions (Table 2). This result suggests that the Li^+/H^+ antiport activity of NhaS1 might not have any physiological relevance.

The Na^+/H^+ antiport system in IMVs prepared from *nhaS3*$^+$ cells had high affinity for Na^+ ions (K_m, 0.7 mM) and extremely high affinity for Li^+ ions (K_m, 0.01 mM). These results suggest that Li^+ ions might be a better substrate of NhaS3 than Na^+ ions. The K_m of NhaS3 for Na^+ ions was similar to the value obtained for NapA of *Enterococcus hirae* that was expressed in *E. coli* (i.e., 1.0 mM [42]). However, the K_m of NhaS3 for Li^+ ions was much smaller than that reported for NapA (i.e., 0.1 mM [42]). K^+ ions in the assay solution significantly inhibited the Li^+/H^+ antiport activity (Fig. 4). However, the tolerance of *nhaS3*$^+$ cells to Li^+ ions increased as the concentration of K^+ ions in the medium was increased (Table 2), suggesting that K^+ ions in the medium might have had a positive rather than a negative effect on the extrusion in vivo of Li^+ ions by NhaS3. There might be a direct interaction between K^+ ions and NhaS3. For example, extracellular K^+ ions might activate the extrusion of Li^+ ions by NhaS3.

Possible roles of NhaS1 and NhaS3 in *Synechocystis*. The existence of high-affinity and low-affinity Na^+/H^+ antiporters in *Synechocystis* is consistent with the ability of this organism to acclimate to a wide range of extracellular concentrations of Na^+ ions. The low affinity of NhaS1 for Na^+ ions suggests that this Na^+/H^+ antiporter might be able to function at relatively high concentrations of Na^+ ions. However, disruption of the *nhaS1* gene did not cause any phenotypic changes in the tolerance to high salt, suggesting that other Na^+/H^+ antiporters might complement the function of NhaS1. Disruption of both the *nhaS1* and the *nhaS2* genes resulted in retardation of growth in the standard BG11 medium. Moreover, retardation of the growth of Δ*nhaS1*Δ*nhaS2* cells appeared to be enhanced by high salt. These results suggest that the functions of NhaS1 and NhaS2, homologs of eukaryotic Na^+/H^+ antiporters, might complement one another and that both might be involved in the tolerance of *Synechocystis* to high-salt stress.

Synechocystis requires a very low concentration of Na^+ ions for optimal growth. Wild-type cells grow more slowly in low-sodium medium (50 μM Na^+) than in the standard BG11 medium (18 mM Na^+). The *nhaS3* gene is essential for the viability of *Synechocystis* even in the low-sodium medium at close to neutral pH. This requirement for the *nhaS3* gene is very specific: all other Na^+/H^+ antiporters characterized to date in heterotrophic bacteria have been shown to be dispensable under such conditions. In contrast, disruption of both the *nhaS4* and the *nhaS5* genes had no effects on phenotypes in terms of high-salt tolerance, an observation that suggests that NhaS4 and NhaS5 might make little contribution to tolerance to high-salt stress. The high affinity of NhaS3 for Na^+ ions and for Li^+ ions indicates that NhaS3 is able to transport Na^+ and Li^+ ions at low concentrations. Therefore, NhaS3 might function in monitoring changes in intracellular concentrations of ions and might be involved in the appropriate adjustment of such concentrations.

It remains to be determined whether the various Na^+/H^+ antiporters are localized on the plasma membrane, on the thylakoid membrane, or on both. Their locations should help us to clarify their physiological roles in *Synechocystis*. Furthermore, we cannot exclude the possibility that NhaS2, NhaS4, and NhaS5 are also Na^+/H^+ antiporters. The *nhaS4* gene reversed the inability of TO114 cells to grow under K^+-depleted conditions, as did the *nhaS1* and *nhaS3* genes, an observation that suggests that the *nhaS4* gene might encode a membrane-bound protein that transports K^+ and/or Na^+ ions.

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