Two Members of a Network of Putative Na⁺/H⁺ Antiporters Are Involved in Salt and pH Tolerance of the Freshwater Cyanobacterium *Synechococcus elongatus*^{∇†}

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Synechococcus elongatus strain PCC 7942 is an alkaliphilic cyanobacterium that tolerates a relatively high salt concentration as a freshwater microorganism. Its genome sequence revealed seven genes, *nha1* to *nha7* (syn_pcc79420811, syn_pcc79421264, syn_pcc7942359, syn_pcc79420546, syn_pcc79420307, syn_pcc79422394, and syn_pcc79422186), and the deduced amino acid sequences encoded by these genes are similar to those of Na⁺/H⁺ antiporters. The present work focused on molecular and functional characterization of these *nha* genes encoding Na⁺/H⁺ antiporters. Our results show that of the *nha* genes expressed in *Escherichia coli*, only *nha3* complemented the deficient Na⁺/H⁺ antiporter activity of the Na⁺-sensitive TO114 recipient strain. Moreover, two of the cyanobacterial strains with separate disruptions in the *nha* genes (Δ *nha1*, Δ *nha2*, Δ *nha3*, Δ *nha4*, Δ *nha5*, and Δ *nha7*) had a phenotype different from that of the wild type. In particular, Δ *nha3* cells showed a high-salt- and alkaline-pH-sensitive phenotype, while Δ *nha2* cells showed low salt and alkaline pH sensitivity. Finally, the transcriptional profile of the *nha1* to *nha7* genes, monitored using the real-time PCR technique, revealed that the *nha6* gene is upregulated and the *nha1* gene is downregulated under certain environmental conditions.

Appropriate intracellular concentrations of Na⁺ and H⁺ are crucial for cyanobacterial cell development and survival. Hence, cyanobacteria possess several mechanisms dedicated to maintaining homeostasis of these ions. Na⁺/H⁺ antiporters are ubiquitous transmembrane proteins that mediate the exchange of Na⁺ and H⁺ across the membrane and thus contribute to salt and proton transport (6, 40, 55).

Even though a minimum Na⁺ concentration is essential for the survival of cyanobacteria, mainly when they grow at a high external pH (3, 13), high concentrations can be harmful. In cyanobacterial cells the mechanisms of salt adaptation primarily involve the active export of Na⁺ and accumulation of K⁺ (47, 48). Na⁺/H⁺ antiporters are involved in Na⁺ efflux and consequently prevent the toxic effects of elevated cytoplasmic Na⁺ levels. In the halotolerant cyanobacterium *Synechocystis* sp. strain PCC 6803, NhaS3 Na⁺/H⁺ antiporter activity is necessary for growth since a completely segregated *nhaS3* mutant strain has never been obtained (12, 24, 65). Nevertheless, incompletely segregated *nhaS3* mutant cells showed sensitivity in the presence of high salt concentrations at alkaline pH (65). In addition, two Na⁺/H⁺ antiporters of *Aphanethece halophytica*, ApnhaP and ApNapA1-1, were able to complement the salt-sensitive phenotype of *Escherichia coli* strain TO114, which is deficient in Na⁺/H⁺ antiporter activity (63, 66). Overexpression of ApnhaP in the freshwater cyanobacterium *Synechococcus elongatus* altered the salt tolerance of this organism and

permitted it to grow in seawater (64). Moreover, the NhaA Na⁺/H⁺ antiporter of *E. coli* is a high-capacity Na⁺ extrusion transporter responsible for the salt tolerance of bacterial cells at alkaline pH (39, 41).

Na⁺/H⁺ antiporters not only promote salt tolerance but also enhance bacterial growth under alkaline conditions, due to acidification of the cytoplasm relative to the external milieu (42). In *Bacillus* species a direct correlation between active monovalent cation/H⁺ antiporters and pH homeostasis has been demonstrated (27). Isolation of Na⁺- and alkali-sensitive *Bacillus subtilis* mutants led to the identification of Bs-Tet(L), a multifunctional (tetracycline-metal⁺)(Na⁺)(K⁺)/H⁺ antiporter that contributes to neutral intracellular pH maintenance under alkaline growth conditions (10). In extreme aerobic alkaliphiles, such as *Bacillus pseudofirmus* OF-4 and *Bacillus halodurans* C-125, the Mrp (Sha) Na⁺/H⁺ antiporter is responsible for pH homeostasis that is exclusively coupled to Na⁺ extrusion (20, 58).

Cyanobacterial cells show optimum growth at pH values ranging from 7.5 to 11, and they are practically absent from habitats with pH values below 5 (8). Inactivation of two Na⁺/H⁺ antiporters in *Synechocystis* sp. strain PCC 6803, NhaS2 and NhaS4, resulted in strains sensitive to alkaline and acidic conditions, respectively (65), implying that these antiporters contribute to pH homeostasis.

The unicellular freshwater cyanobacterium *Synechococcus* sp. strain PCC 7942 (*S. elongatus*) tolerates NaCl at concentrations up to 0.4 M and shows optimum growth at physiological pH values ranging from 7.0 to 9.0 (4, 11, 56). Bioenergetic studies revealed that there is Na⁺-coupled secondary ion transport across the membrane of *S. elongatus* at physiological pH values (pH 7.0 to 9.0) (48, 50), which was confirmed by biochemical assays, demonstrating that there is Na⁺/H⁺ antiporter activity (5, 29, 44). Surprisingly, however, high-salt

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stress represses the synthesis and activity of Na⁺/H⁺ antiporters in this organism (1, 2). Thus, the molecular mechanisms involved in adaptation and acclimation of *S. elongatus* to salt and alkaline stress conditions have to be determined.

Analysis of the recently completed genomic sequence of *S. elongatus* (http://genome.jgi-psf.org/finished_microbes/synel/synel.home.html) revealed the presence of seven open reading frames (*nha1* to *nha7*) that encode protein sequences very similar to the sequences of Na⁺/H⁺ antiporters. In the present work we investigated which of the corresponding proteins exhibit Na⁺/H⁺ antiporter activity and therefore contribute to the salt- and pH-responsive mechanisms of *S. elongatus*. Based on our results, the Nha3 protein showed Na⁺/H⁺ antiporter activity in everted membrane vesicles and successfully complemented the salt-sensitive phenotype of recipient TO114 cells. In contrast, the Nha1, Nha4, Nha6, and Nha7 proteins showed low Na⁺/H⁺ antiporter activity and were not able to complement the salt-sensitive phenotype of TO114 cells. Additionally, inactivation of six of the seven *nha* genes in *S. elongatus* revealed the genes that have essential roles in growth at different salt concentrations and pHs. Finally, expression of the *nha* genes was monitored under salt and alkaline stress conditions, using real-time reverse transcription (RT)-PCR. To our knowledge, this is the first report of Na⁺/H⁺ antiporters in the cyanobacterium *S. elongatus* which also provides new insights into understanding the contribution of these proteins to the salt and pH tolerance of a freshwater organism.

MATERIALS AND METHODS

Strains and growth conditions. For routine cultures, wild-type (provided by the Pasteur Culture Collection of Cyanobacteria) and mutant strains of *S. elongatus* were grown photoautotrophically at 31°C in standard BG-11 medium buffered at pH 8.0 with 20 mM HEPES-KOH. The cultures were continuously aerated with 5% (vol/vol) CO₂ in air and illuminated with fluorescent white light (100 microeinsteins · m⁻² · s⁻¹) (57). Media containing defined concentrations of sodium were prepared by adding NaCl to standard BG-11 medium or BG-11 media in which sodium salts (NaNO₃) were replaced by the same concentration of potassium salts (KNO₃) (~18 mM). The pH values of BG-11 media were adjusted with 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-bis-Tris-propane (pH 7.0, 8.0, and 9.0) and remained stable at least until the late log phase of growth. Cyanobacteria were also grown on solid medium by adding Bacto agar (Difco) at a final concentration of 1.5% and 1 mM sodium thiosulfate to standard liquid BG-11 medium.

E. coli TO114 (W3110 *nhaA::Km^r nhaB::Em^r chA::Cm^r*), which was used as the recipient strain for complementation tests with cyanobacterial genes, was generously provided by H. Kobayashi (Chiba University, Chiba, Japan) (38). TO114 cells were grown in LBK medium (pH 7.0) (1% tryptone, 0.5% yeast extract, 100 mM KCl). Growth tests with transformed TO114 cells were performed by adding NaCl as indicated below. LB⁺ medium was prepared like LBK medium except that 300 mM KCl was added instead of 100 mM KCl. Finally, LBn medium contained only the basic components of LB medium (yeast extract, Bacto tryptone) without any further addition of NaCl or KCl (basal levels, 5 mM K⁺ and 20 mM Na⁺). The growth rates of *E. coli* and cyanobacterial cells were monitored by measuring light scattering at *A*₆₀₀ and *A*₇₃₀, respectively.

Plasmid isolation from *E. coli* DH5α [F⁻φ80d *lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR* *recA1* *endA1* *hsd* *RL7*(rK⁻ mK⁺) *phoA* *supE44* λ *thi-1* *gvrA96* *relA*] cells was performed as previously described (54). Transformed *E. coli* TO114 and DH5α cells were selected in the presence of ampicillin (final concentration, 50 μg · ml⁻¹).

S. elongatus transformed cells were initially selected on BG-11 media containing 10 μg · ml⁻¹ of kanamycin, 10 μg · ml⁻¹ of spectinomycin, and 5 μg · ml⁻¹ of chloramphenicol.

Plasmid construction. Seven *S. elongatus* sequences (syn_pcc79420811, syn_pcc79421264, syn_pcc7942359, syn_pcc79420546, syn_pcc79420307, syn_pcc79422394, and syn_pcc79422186) encoding putative Na⁺/H⁺ antiporters, designated *nha1*, *nha2*, *nha3*, *nha4*, *nha5*, *nha6*, and *nha7*, were amplified

using appropriate oligonucleotides (oligonucleotides 1 to 7 [see Table S1 in the supplemental material]) from cyanobacterial genomic DNA with *Pfu* polymerase (Fermentas). The amplified sequences were inserted into the BamHI/XbaI sites of pBluescript KS (+/-), resulting in plasmids designated pBnha1, pBnha2, pBnha3, pBnha4, pBnha5, pBnha6, and pBnha7. The inserted DNA fragment in each plasmid was verified by sequence analysis (MWG Biotech). In order to be expressed in *E. coli* cells, all seven *nha* sequences were amplified from cyanobacterial genomic DNA using *Pfx* polymerase (Invitrogen) and appropriate oligonucleotides (oligonucleotides 8 to 14 [see Table S1 in the supplemental material]) and inserted into the BamHI/EcoRI (*nha1*, *nha2*, *nha3*, *nha5*, *nha6*, and *nha7*) or BamHI/HindIII (*nha4*) sites of the pTrcHis2A vector (Invitrogen) as in-frame C-terminal His-tagged translational fusions. The inserted DNA fragment in each plasmid was verified by sequence analysis (MWG Biotech). The resulting plasmids, designated pTnha1, pTnha2, pTnha3, pTnha4, pTnha5, pTnha6, and pTnha7, were used to transform *E. coli* DH5α and TO114 cells.

For inactivation of *nha* genes, all coding sequences were isolated as BamHI/HindIII fragments from pTnha vectors and inserted into the BamHI/HindIII sites of the pUC19 vector (Fermentas), producing the constructs pUnha1 to pUnha7. The HincII restriction fragment containing the gene encoding resistance to kanamycin (Km^r) (1.1 kb) was isolated from the pUC4K vector (kindly provided by C. Mullineaux, Queen Mary University of London) and inserted into the HincII sites of pUnha1, pUnha3, pUnha4, and pUnha5 to produce the pΔ*nha1::Km*, pΔ*nha3::Km*, pΔ*nha4::Km*, and pΔ*nha5::Km* plasmids, respectively. In plasmids pUnha2 and pUnha6, a SalI restriction site and a HincII restriction site were generated in the middle of the *nha2* and *nha6* coding sequences, respectively, by in vitro directed mutagenesis using the *Pfx* polymerase (Invitrogen) and appropriate oligonucleotides (primers 15 and 16 [see Table S1 in the supplemental material]). Following this, a SalI fragment containing the Km^r cassette was inserted into the SalI site of plasmid pUnha2, producing the pΔ*nha2::Km* plasmid. On the other hand, two different HincII fragments were inserted into the unique HincII site of plasmid pUnha6; one of these fragments contained the Km^r cassette, and the other contained the chloramphenicol resistance (Cm^r) cassette (0.8 kb) derived from plasmid pUC4C (kindly provided by C. Mullineaux). The resulting plasmids were designated pΔ*nha6::Km* and pΔ*nha6::Cm*, respectively. Additionally, the 2.0-kb Sp sequence containing the gene encoding resistance to streptomycin/spectinomycin was PCR amplified from plasmid pHP45Ω (kindly provided by S. S. Golden, Texas A&M University) using appropriate oligonucleotides having HincII sites at their ends and was inserted into the HincII site of pUnha6 to produce plasmid pΔ*nha6::Sp* (data not shown). Finally, digestion of plasmid pUnha7 with PpuMI and subsequent insertion of a Km^r fragment bearing PpuMI sites into its ends resulted in plasmid pΔ*nha7::Km*.

DNA isolation and Southern blot analysis. *S. elongatus* genomic DNA was isolated by using a standard protocol for gram-negative bacteria. Briefly, cells from a 10-ml culture in the stationary phase were harvested and washed twice with 5 ml of Tris-EDTA (TE). These cells were centrifuged at 5,000 × *g* for 10 min, resuspended in a buffer containing 10 mM Tris and 50 mM EDTA, and treated with lysozyme (3 mg/ml) for 90 min at 37°C. After centrifugation the cells were resuspended in a buffer containing TE, sodium dodecyl sulfate (SDS) (0.5%), and NaCl (125 mM) and treated with proteinase K (10 mg/ml) for 30 min at 65°C. Standard phenol, phenol-chloroform, and chloroform-isoamyl alcohol extractions and ethanol precipitation were then performed. Approximately 10 μg of genomic DNA was digested with appropriate restriction enzymes for 4 h at 37°C and resolved in a 1% agarose gel in Tris-acetate-EDTA. The gel was developed at a rate of 2.5 cm/min for 16 h. It was denatured in 1.5 M NaCl-0.5 M NaOH, neutralized in 1.5 M NaCl-0.5 M Tris-HCl (pH 7.5), and plotted onto a Hybond-N membrane (Amersham Biosciences). Hybridization was performed using ³²P-labeled probes (the *nha1* to *nha7* opening reading frames) according to the instructions provided with a Megaprime labeling system kit (Amersham Biosciences).

RNA isolation and real-time RT-PCR. For RNA isolation, 50-ml cultures of *S. elongatus* cells grown to the early exponential phase (optical density at 730 nm [OD₇₃₀], 1.5) under various conditions were harvested at 4°C, immediately frozen in liquid nitrogen, and stored at -80°C. Each sample was thawed in 1 ml Trizol reagent (Invitrogen) supplemented with sand and vigorously shaken twice in a mini Bead-Beater (Biospec Products) at 42 × 10² rpm for 2 min. After 10 min of incubation in Trizol reagent, each mixture was centrifuged at 2,000 × *g* for 10 min at 4°C. The supernatant was collected, and 0.2 ml of chloroform was added. Following vortexing for 1 min, each mixture was incubated on ice for 15 min and centrifuged at 2,000 × *g* for 15 min at 4°C. The aqueous phase was carefully collected and was precipitated by adding an equal volume of isopropanol. The pellet was washed with 75% ethanol, dried, and redissolved in RNase-free water (Ambion).

RNAs collected by the Trizol reagent method were further purified using an RNeasy mini kit (Qiagen) according to the instructions of the manufacturer. Moreover, to avoid contamination with genomic DNA, about 10 µg of each RNA sample was treated as described in the instructions of a TURBO DNA-free kit (Ambion). The absence of DNA contamination was verified by a conventional PCR (approximately 45 cycles) using as the template at least 2 µg of each RNA sample. The quality of isolated RNAs was checked by conventional gel electrophoresis using a 2% agarose gel stained with ethidium bromide (1 mg/ml). Finally, the concentration of each RNA sample was calculated using Nanodrop equipment (ND-1000 spectrophotometer) according to the instructions of the manufacturer.

Approximately 1 µg of each RNA sample was used for RT with the SuperScript II RNase H reverse transcriptase (Invitrogen) by following the instructions of the manufacturer. Briefly, ~1 µg of RNA template with 250 ng of random hexamer primers (Sigma) was heated at 70°C for 10 min and chilled immediately on ice for 2 min. RT buffer and dithiothreitol were added at appropriate concentrations, and the mixture was annealed at 25°C for 5 min. Reverse transcriptase was added, and then the mixture was incubated at 25°C for 10 min. Finally, an extension step consisting of 42°C for 1 h and a heat inactivation step consisting of 70°C for 15 min were performed.

Real-time PCR was carried out using the LightCycler system (Roche Molecular Biochemicals). Oligonucleotides used for amplification of the *nha1* to *nha7* genes and the 16S rRNA reference gene were designed using the Primer Premier software and yielded PCR products that were between ~100 and 200 bp long (primers 17 to 23 [see Table S1 in the supplemental material]). The appropriate template, primer, and Mg²⁺ concentrations, as well as the annealing temperature that provided the optimum experimental efficiency and specificity, were determined. The fluorescence signal due to SYBR green intercalation was monitored to quantify the double-stranded DNA product formed in each PCR cycle. For LightCycler PCRs, a master mixture containing 4 mM MgCl₂, forward and reverse primers (5 pmol each), and LightCycler-FastStart DNA Master SYBR green I (2 µl per reaction mixture; Roche Molecular Biochemicals) was prepared. Aliquots of the master mixture (19 µl) were dispensed into LightCycler glass capillaries, and then 1 µl of 10⁻¹-diluted cDNA was added as a PCR template to obtain a final volume of 20 µl. The PCR amplification program used for each pair of primers with various cDNAs as the templates consisted of the following steps: (i) initial denaturation at 95°C for 10 min, (ii) an amplification and quantification program consisting of 40 cycles (95°C for 10 s, 60°C for 10 s, and 72°C for 9 s with a single fluorescence measurement and a temperature transition rate of 20°C/s), (iii) a melting curve program (55 to 95°C with a heating rate of 0.1°C/s with continuous fluorescence measurement), and (iv) final cooling to 4°C. Negative controls without a cDNA template were run with every assay.

The PCR conditions described above were initially used for each pair of primers, using as the templates a series of dilutions (10⁻¹, 10⁻², 10⁻³, and 10⁻⁴) of a total cDNA mixture prepared from an RNA sample corresponding to an *S. elongatus* untreated culture at an OD₇₃₀ of 1.5. LightCycler 4.05 software was used to plot the crossing points of dilutions against the logarithm of input amounts and to generate a standard curve for each set of primers with a slope calculated by the program. The slopes for the seven genes under the PCR conditions described above ranged from -3.2 to -3.6 (indicating PCR efficiencies of 1.92 to 2.08). The efficiency values obtained correspond to comparable PCR efficiencies that can be used for relative quantification according to the instructions for "critical factors for successful real-time PCR" (Qiagen). The melting curves for each set of primers verified the absence of a primer dimer or other nonspecific products under the experimental conditions used. The real-time PCR assays were performed using as the templates total cDNA mixtures prepared from RNAs of *S. elongatus* cultures treated as indicated above. Differences in the transcript levels of the *nha1*, *nha2*, *nha3*, *nha4*, *nha5*, *nha6*, and *nha7* genes between normal (reference) growth conditions and the growth conditions examined were calculated by using the $\Delta\Delta C_T$ method (user bulletin no. 2, comparative C_T method; Applied Biosystems), using as reference gene (or calibrator) the 16S rRNA housekeeping gene, which is constitutively expressed (data not shown). The whole procedure was repeated independently at least three times in order to correctly evaluate the significance of calculated transcription changes, and the final values reported below are the averages of at least three independent experiments.

Measurement of Na⁺/H⁺ antiporter activity in *E. coli*. Na⁺/H⁺ antiporter activity was examined using everted membrane vesicles prepared from transformed *E. coli* TO114 cells that were grown to the mid-exponential phase (OD₆₀₀, 1.0) as previously described (52). Briefly, *E. coli* cells were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C and then washed with TCDS suspension buffer (10 mM Tris-HCl [pH 7.5], 0.14 M choline chloride, 0.5 mM dithiothreitol, 0.25 M sucrose). The pellet was suspended in an appropriate

volume of TCDS suspension buffer and applied to a French pressure cell (4,000 lb/in²). The resulting solution was centrifuged at 12,000 × *g* for 10 min at 4°C to pellet unbroken cells, and the supernatant was recentrifuged at 110,000 × *g* and 4°C to pellet membrane fractions, which were resuspended in approximately 600 µl of TCDS suspension buffer. In everted membrane vesicles, Na⁺/H⁺ antiporter activity was estimated from changes in the vesicular ΔpH after addition of NaCl, using the corresponding changes in the acridine orange fluorescence signal as previously described (18). The fluorescence of acridine orange was monitored with a fluorometer (Perkin Elmer MPF-3L) using an emission wavelength of 530 nm (Δλ = 5 nm) and an excitation wavelength of 495 nm (Δλ = 3 nm). More precisely, ~50 µg of vesicular proteins was added to 2 ml of a solution containing 140 mM choline chloride, 5 mM MgCl₂, 10 mM Tris titrated with MES at the pHs indicated below, and 1 µM acridine orange, which was stirred continuously in a cuvette. Addition of 2 mM DL-lactate resulted in fluorescence quenching (*Q*) due to respiration since lactate energized the vesicles, which in turn accumulated H⁺ in their interiors. Upon addition of 5 mM NaCl the fluorescence signal was increased due to vesicular excretion of H⁺ via the antiporters (Δ*Q* [*Q* = fluorescence quenching]), while addition of 25 mM NH₄Cl caused ΔpH dissipation.

The initial rate of the increase in fluorescence that followed the addition of various concentrations of NaCl was considered the Na⁺/H⁺ antiporter activity. Moreover, the percent increase in fluorescence upon addition of NaCl was calculated as follows: Δ*Q* × 100/*Q* (63). For calculation of half-saturation constant values, the Na⁺/H⁺ antiporter activity of everted membrane vesicles prepared from TO114/pTrc transformed cells was subtracted from the activity of everted membrane vesicles prepared from TO114 *nha3* transformed cells.

Membrane protein extract preparation and Western blotting. Transformed *E. coli* TO114 cells were grown in LBK medium until the mid-exponential phase (OD₆₀₀, 1.5) in the presence of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h. High-pressure membrane fractions were prepared like the everted vesicles, but the pressure used was 20,000 lb/in² (French pressure cell) (17, 59). Approximately 50 µg of membrane proteins was suspended in 2× SDS loading buffer (containing β-mercaptoethanol) and separated by SDS-polyacrylamide gel electrophoresis (Bio-Rad) on a 12.5% gel. Proteins were transferred electrophoretically (Bio-Rad) to a 0.2-µm nitrocellulose membrane (Amersham Hybond ECL), probed with penta-His-tagged horseradish peroxidase-conjugated antibody (Qiagen) that recognized the His epitope, and visualized by ECL (enhanced chemiluminescence; Pierce) technology (46). The protein concentration was determined using a modified Bradford assay (7).

Other methods. Amino acid sequences were aligned with the program CLUSTAL X (60), using the default alignment parameters. Phylogenetic tree construction was performed using the neighbor-joining method as implemented in CLUSTAL X. The statistical significance of nodes was evaluated using 1,000 bootstrap replicates (14). Trees were drawn using Treeview 1.6.6 (43). All similarity searches were performed with the help of the protein-protein BLAST program BLASTP (<http://www.ncbi.nlm.nih.gov/BLAST/>).

RESULTS

In silico analysis and cloning of putative Na⁺/H⁺ antiporters from *S. elongatus*. The majority of the Na⁺/H⁺ antiporters are classified as members of the monovalent cation:proton antiporter (CPA) superfamily, which is divided into the Na⁺-transporting carboxylic acid decarboxylase (NaT-DC) family found only in bacteria and the CPA1 and CPA2 families, whose members are found in several kingdoms of organisms (9). Analysis of the genomic sequence of *S. elongatus* revealed the presence of seven genes, all members of the CPA superfamily, with putative Na⁺/H⁺ antiporter capacity. The locus tags of proteins Nha1 to Nha7 in *S. elongatus*, as well as their individual molecular characteristics, are shown in Table 1.

According to the Transport Classification DataBase (<http://www.tcdb.org/progs/blast.php>), Nha3, Nha4, and Nha5 belong to the CPA2 family and Nha1, Nha2, Nha6, and Nha7 belong to the CPA1 family. As shown in Fig. 1, proteins belonging to the CPA1 and CPA2 families cluster together with characterized Na⁺/H⁺ antiporters belonging to the corresponding families and form distinct monophyletic groups with significant bootstrap support. In addition, Nha6 and Nha7 are

TABLE 1. <i>Synechococcus</i> sp. strain PCC 7942 genes encoding putative Na ⁺ /H ⁺ antiporters used in this study				
Locus tag	Protein	Gene size (bp)	Protein size (amino acids)	Protein mol wt (10 ³)
syn_pcc79420811	Nha1	1,584	527	57.340
syn_pcc79421264	Nha2	1,644	547	58.503
syn_pcc79422359	Nha3	1,383	460	47.391
syn_pcc79420546	Nha4	2,148	715	77.963
syn_pcc79420307	Nha5	1,227	408	43.885
syn_pcc79422394	Nha6	1,782	593	63.325
syn_pcc79422186	Nha7	1,638	545	58.067

derived from the same node and form a discrete group that appears to cluster with the CPA1 family, although not with a very high level of support (66%).

Nha4 and Nha7 share an extended hydrophilic C terminus with a universal stress protein A (UspA)-like domain (37). This type of C-terminal tail is found in Na⁺/H⁺ antiporters of some cyanobacterial species and is believed to be involved in stress response mechanisms (28). Computational analysis revealed

strong amino acid sequence similarity of the seven Nha proteins of *S. elongatus* to the six Na⁺/H⁺ antiporters of *Synechocystis* sp. strain PCC 6803 (19, 24, 65) (encoded by slr1727, slI0273, slI0689, slr1595, slr0415, and slI0556 and designated NhaS1, NhaS2, NhaS3, NhaS4, NhaS5, and NhaS6, respectively), as well as to the two characterized antiporters of *A. halophytica*; both *Synechocystis* sp. strain PCC 6803 and *A. halophytica* are distantly related organisms. In particular, Nha1 shows significant similarity to NhaS1 (52% identity and 72% similarity) and ApnhaP (56% identity and 74% similarity) from *A. halophytica* (63), Nha2 shows significant similarity to NhaS2 (52% identity and 68% similarity), Nha3 shows significant similarity to NhaS3 (64% identity and 80% similarity) and ApNapA1-1 (62% identity and 74% similarity) of *A. halophytica* (66), Nha4 shows significant similarity to NhaS4 (52% identity and 73% similarity), and Nha5 shows significant similarity to NhaS5 (51% identity and 69% similarity). Additionally, Nha6 shows significant similarity to the putative Na⁺/H⁺ antiporter NhaS6 (slI0556) (46% identity and 63% similarity). In contrast, there is no significant similarity between Nha7

FIG. 1. Neighbor-joining phylogenetic dendrogram of functionally characterized Na⁺/H⁺ antiporter proteins (members of the CPA superfamily) from different organisms. Open circles indicate nodes with bootstrap support of >70%, while filled circles indicate nodes with bootstrap support of >90%, based on 1,000 replicates. The areas surrounded by dashed lines indicate monophyletic groups corresponding to protein families CPA1 and CPA2. Note that Nha6 and Nha7 form a distinct group that appears to be more closely associated (66%) with the CPA1 family. Even though CPA2 appears to be an entirely separate family with a high statistical score (>90%), the CPA1 family appears to be more diverged (statistical score, 75%). The following proteins were used to construct the dendrogram: KefC (*E. coli*) (accession number NP_414589), NhaP (*Pseudomonas aeruginosa* PAO1) (accession number NP_252576), NHX1 (*Saccharomyces cerevisiae*) (accession number NP_010744), NapA (*Enterococcus hirae*) (accession number CAD22163), SOS1 (*Arabidopsis thaliana*) (accession number NP_178307), NhaS1 (*Synechocystis* sp. strain PCC 6803) (accession number NP_441245), NhaS2 (*Synechocystis* sp. strain PCC 6803) (accession number NP_441812), NhaS3 (*Synechocystis* sp. strain PCC 6803) (accession number NP_442262), NhaS4 (*Synechocystis* sp. strain PCC 6803) (accession number NP_440311), NhaS5 (*Synechocystis* sp. strain PCC 6803) (accession number NP_442308), ApnhaP (*A. halophytica*) (accession number BAB69459), ApNapA1-1 (*A. halophytica*) (accession number BAD97367), NhaG (*B. subtilis*) (accession number BAA89487), ATNHX8 (*A. thaliana*) (accession number NP_172918), NhaA (*E. coli*) (accession number NP_414560), GerN (*Bacillus cereus*) (accession number AAF91326), VP2867 (*Vibrio parahaemolyticus*) (accession number NP_799246), KefB (*E. coli*) (accession number YP_312276), SLC9A7 (*Homo sapiens*) (accession number NP_115980), YvgP (*B. subtilis*) (accession number NP_391222), and ATCHX17 (*A. thaliana*) (accession number NP_194101).

FIG. 2. Complementation tests and growth rates of *E. coli nha*⁺ transformed strains and the control TO114 strain transformed with the pTrcHis2A vector alone in LBK medium (105 mM K⁺) (pH 7.0). *nha*⁺ transformants and the recipient strain were grown in (A) solid LBK medium with different concentrations of NaCl (20, 220, 320, and 420 mM Na⁺) and (B) liquid LBK medium (pH 7.0) supplemented with 200 and 300 mM NaCl. The results shown panel B are mean values of three independent experiments.

and any characterized cyanobacterial Na⁺/H⁺ antiporter, based on data from NCBI BLAST and protein BLAST analyses (see Materials and Methods).

Functional characterization of Nha Na⁺/H⁺ antiporters in *E. coli*. In *E. coli* cells inactivation of the three native Na⁺/H⁺ antiporters, NhaA, NhaB, and ChA, resulted in an inability of the derived TO114 strain (38) to grow in the presence of elevated concentrations of Na⁺. We initially assessed the abilities of the seven Nha proteins of *S. elongatus* to function as Na⁺/H⁺ antiporters by examining whether they could complement the salt-sensitive phenotype of TO114 cells. To do this, each of the *nha* genes was inserted under control of the 5' and 3' regulated regions of the TrcHis2A plasmid (see Materials and Methods), and the resulting constructs were introduced by transformation into TO114 cells. Several transformants were isolated in each case, except when the plasmid carrying the *nha5* sequence was introduced into TO114 cells, when no transformants were obtained on media containing the appropriate concentration of ampicillin. This is in accordance with previous data showing that efforts to isolate TO114 cells carrying the *nhaS5* gene, which is the *nha5* homologue in *Synechocystis* sp. strain PCC 6803, were unsuccessful (24).

Recipient TO114 cells are able to grow in the presence of less than 0.2 M NaCl at neutral pH (53). Thus, transformants carrying plasmids containing each of the *nha1* to *nha7* sequences were analyzed for the ability to grow with various concentrations of Na⁺ in LBK medium (105 mM K⁺). As shown in Fig. 2A, only cells carrying the *nha3* sequence were able to overcome the threshold concentration of NaCl, 0.2 M, and to grow at NaCl concentrations up to 0.42 M. These results were confirmed by monitoring the growth of the transformed

cells in liquid cultures, as shown in Fig. 2B. Additionally, we investigated whether *nha1* to *nha7* can complement the alkaline sensitivity of TO114 cells in the presence of Na⁺ (16, 39) by examining the effect of both acidic and alkaline pHs on growth. Our results showed that there was no difference in the growth phenotypes of the TO114 recipient and cells transformed with *nha*⁺ sequences at pH values of 5.5, 8.0, and 9.0 (data not shown).

Since the TO114 strain is sensitive to the absence of K⁺ (21, 62), the effect of K⁺ depletion on the growth of TO114 cells transformed with *nha*⁺ sequences was examined. Our results revealed that only TO114 cells expressing *nha3* were able to grow in the absence of K⁺ at pH 7.0, when the concentration of Na⁺ was less than 0.2 M (data not shown). Moreover, at a high K⁺ concentration that does not restrict growth, the salt tolerance of both transformed *nha*⁺ and recipient cells is slightly enhanced (21, 46). Under these conditions *nha3*⁺ cells again had an advantageous phenotype compared with the other *nha*⁺ transformed cells, as they were the only cells able to grow at an Na⁺ concentration of 420 mM (data not shown).

Immunodetection of Nha proteins and measurement of Na⁺/H⁺ antiporter activities in *E. coli* cells. To investigate whether the *nha1* to *nha7* genes are expressed in *E. coli* TO114 cells, the His-tagged proteins of each transformed strain were analyzed by Western blotting. As shown in Fig. 3A, the Nha1, Nha3, Nha4, Nha6, and Nha7 proteins were specifically detected in cell membrane protein fractions extracted from the corresponding *E. coli* TO114 transformed cells and blotted against a His-specific antibody (see Materials and Methods). Each Nha protein detected had a mobility consistent with the estimated molecular weight shown in Table 1. No His-tagged

FIG. 3. (A) Western blot analysis of His-tagged Nha proteins. The Nha1, Nha3, Nha4, Nha6, and Nha7 proteins were detected using the penta-His-tagged horseradish peroxidase-conjugated antibody. The bands of molecular mass markers are indicated on the left. (B and C) Na⁺/H⁺ antiporter activity of the Nha3 protein in everted membrane vesicles as revealed by measuring the fluorescence of acridine orange (see Materials and Methods). (B) Increase in acridine orange fluorescence in Nha3-expressing vesicles compared to pTrcHis2A (control) vesicles upon addition of 5 mM NaCl, after the vesicles were energized by addition of DL-lactate (decrease in the fluorescence signal). (C) pH dependence of Na⁺/H⁺ antiporter activity in Nha3⁺ everted membrane vesicles. Each bar indicates the average of three independent measurements.

Nha protein was detected in membrane protein fractions of the TO114 recipient or the strain transformed with the *nha2* gene. The latter result was due to *nha2* RNA degradation in *nha2*⁺ cells, as revealed by Northern blot analysis (data not shown).

The Na⁺/H⁺ activity of *nha1*⁺, *nha3*⁺, *nha4*⁺, *nha6*⁺, and *nha7*⁺ cells was monitored by measuring the increase in the acridine orange fluorescence signal (due to changes in the vesicular ΔpH) upon addition of NaCl at pHs ranging from 6.5

to 9.5 in everted membrane vesicles prepared from *nha*⁺ and control TO114/pTrc cells (see Materials and Methods). In vesicles that were energized with lactate, addition of 5 mM NaCl at pH 8.0 caused an increase in the fluorescence yield, which was minor in Nha1-, Nha4-, Nha6-, Nha7-expressing vesicles (data not shown) and apparent in Nha3⁺ vesicles compared to the control TO114/pTrc vesicles. Subsequent addition of 25 mM NH₄Cl resulted in a further increase in the acridine or-

FIG. 4. Southern blot analysis of $\Delta nha1$, $\Delta snha2$, $\Delta nha3$, $\Delta nha4$, $\Delta nha5$, $\Delta nha7$, and wild-type strains. Genomic DNA ($\sim 10 \mu\text{g}$) from wild-type and mutant strains were digested with HindIII and resolved on 1% agarose gels. (A) Blots of the fully segregated $\Delta nha1$, $\Delta snha2$, $\Delta nha4$, $\Delta nha5$, and $\Delta nha7$ strains. (B) Blot of the incompletely segregated $\Delta nha3^*$ strain with the kanamycin resistance cartridge. Decreased amounts of the wild-type alleles are apparent in the $\Delta nha3^*$ strain compared to the wild-type strain. For each blot the sizes of the wild-type and corresponding disrupted *nha* alleles are indicated. WT, wild type.

ange fluorescence signal due to ΔpH collapse (Fig. 3B). The results described above indicate that $nha3^+$ cells exhibit an apparent Na^+/H^+ antiporter activity. Moreover, the results shown in Fig. 3C revealed that $nha3^+$ vesicles have significant Na^+/H^+ exchange activity at pH values ranging from 6.5 to 9.0 and minor activity at pH 9.5.

Finally, the Nha3 antiporter had an half-saturation constant for Na^+ of 4 mM in *E. coli* cells, as calculated using $nha3^+$ everted membrane vesicles assayed with a wide range of NaCl concentrations (0.1 to 60 mM NaCl) and measuring the corresponding increase in the acridine orange fluorescence yield upon addition of distinct NaCl concentrations (data not shown).

Disruption of *nha* genes in *S. elongatus*. In order to examine the possible contributions of all Nha proteins in the salt and pH response mechanisms of *S. elongatus*, we disrupted each of the corresponding *nha* loci in the genome of the organism using the kanamycin resistance gene cassette, as described in Materials and Methods.

Six of seven Δnha mutant strains, each having one of the *nha* genes disrupted, were isolated and verified by Southern blot analysis. Figure 4 shows genetically homologous strains isolated for the *nha1*, *nha2*, *nha4*, *nha5*, and *nha7* genes as a result of a double-crossover event that disrupted all the corresponding wild-type alleles. For disruption of the *nha3* gene, both kanamycin and chloramphenicol selectable markers were used to enforce complete segregation. Even though high concentrations of antibiotics ($100 \mu\text{g ml}^{-1}$ of kanamycin and $30 \mu\text{g ml}^{-1}$ of chloramphenicol) were added to the selective media, only incompletely segregated *nha3* cells were obtained. These results suggest that the *nha3* gene is essential for the survival of cyanobacterial cells under the experimental conditions used. One of the merodiploid $\Delta nha3^*$ strains isolated in the presence of kanamycin was used for further analysis.

For disruption of the *nha6* gene three different selectable markers, kanamycin, chloramphenicol, and streptomycin/spectinomycin, were used. Transformed cells were initially selected with a wide range of antibiotic concentrations and different concentrations of Na^+ and K^+ , as well as different pH values. Despite the fact that several antibiotic-resistant *nha6* mutant strains were randomly isolated, these strains either lost their

antibiotic resistance after several rounds of culturing or resulted from an out-of-locus antibiotic cassette insertion, as revealed by Southern blot analyses (data not shown).

The unsuccessful inactivation of the *nha6* gene could imply that this gene is vital for growth under the experimental conditions used. To our knowledge, no homologue of the *nha6* gene has been inactivated in any cyanobacterial species. Overall, polar effects of insertional mutagenesis on a broader gene area might be the reason that only incompletely segregated *nha3* cells were obtained and no *nha6* mutant cells were obtained. To examine this, a detailed genome map of the seven *nha* genes is shown in Fig. S1 in the supplemental material.

Functional characterization of the $\Delta nha4$ mutant cells. To assess the effect of inactivation of the *nha1* to *nha7* genes on the salt and pH tolerance of *S. elongatus*, the growth of Δnha mutant strains was examined using a multiwell plate assay and liquid BG-11 medium buffered at three different pH values (pH 7.0, 8.0, and 9.0) and supplemented with various concentrations of NaCl (50, 100, 200, 300, and 400 mM). Homozygous $\Delta nha1$, $\Delta nha4$, $\Delta nha5$, and $\Delta nha7$ strains showed a growth phenotype similar to that of the wild-type strain with respect to high-salt and alkaline-pH sensitivity. However, $\Delta nha3^*$ merodiploid cells exhibited an apparent growth deficiency in the presence of 100 mM NaCl at pH 7.0, 8.0, and 9.0 (Fig. 5A, C, and D).

Interestingly, growth of *nha3* mutant cells was significantly impaired without NaCl addition, indicating the general growth sensitivity. The greatest growth reduction was observed at pH 9.0, suggesting that there was alkaline sensitivity as well. To examine whether the internal concentration of Na^+ in BG-11 medium ($\sim 18 \text{ mM Na}^+$) was the cause of the reduced growth of *nha3* mutant cells, we also tested $\Delta nha3^*$ merodiploid cells in modified BG-11 medium in which the Na^+ was replaced by the same concentration of K^+ . As shown in Fig. 5B, mutant cells maintained their growth sensitivity, suggesting that the $\Delta nha3^*$ strain exhibited an overall reduction in growth, which was radical (more severe) in the presence of elevated Na^+ concentrations and alkaline pH values. The reduced tolerance of $\Delta nha3^*$ merodiploid cells to both a high salt concentration and elevated pH values might be the result of low levels of the

FIG. 5. Multiwell plate assay of the $\Delta nha3^*$ strain in standard BG-11 medium buffered at pH 7.0 (A) and pH 8.0 (C) and supplemented with different concentrations of NaCl (50, 100, 200, 300, and 400 mM), in modified BG-11 medium in which Na⁺ was replaced by the same amount of K⁺ (~18 mM) and which was buffered at pH 7.0 and supplemented with different concentrations of NaCl (50, 100, 200, 300, and 400 mM) (B), and in standard BG-11 medium buffered at pH 9.0 and supplemented with various concentrations of NaCl (D). Overall, $\Delta nha3^*$ cells showed not only reduced salt tolerance but also reduced growth even in the absence of NaCl.

Nha3 protein due to reduced numbers of copies of the *nha3* wild-type alleles.

These observations were further examined by measuring the growth rates of both wild-type and mutant cells. As shown in Fig. 6, $\Delta nha3^*$ cells had a reduced growth rate compared to the wild-type cells even in the absence of NaCl at both pH 8.0 and pH 9.0. Addition of 100 mM NaCl, although it did not affect

the growth rate of wild-type cells, resulted in strongly reduced growth of $\Delta nha3^*$ cells compared to the profile in the absence of NaCl. Moreover, the reduced growth of $\Delta nha3^*$ cells both in the presence and in the absence of NaCl was more severe at pH 9.0 than at pH 8.0 and 7.0 (data not shown), implying that the growth of these cells was significantly affected at elevated pH values.

To test growth sensitivity under low-salt conditions, *nha1* to *nha7* mutant cells were cultured in a modified BG-11 medium in which the Na⁺ (~18 mM) was replaced by the same amount of K⁺, using a multiwell plate assay. Growth was tested at pH 7.0, 8.0, and 9.0 with different concentrations of Na⁺ (0, 0.1, 10, 50, 100, and 200 mM NaCl). Under these conditions, only $\Delta nha2$ mutant cells showed a requirement for a low concentration of Na⁺ (0 and 0.1 mM NaCl), which increased from pH 7.0 to pH 8.0 and was essential at pH 9.0 (Fig. 7A, B, and C). Growth rate measurements confirmed the results described above showing that there was reduced growth of $\Delta nha2$ cells compared to wild-type cells under Na⁺-depleted conditions at pH 8.0 and no growth at pH 9.0. These results indicated that an absence of the Nha2 protein causes sensitivity to low Na⁺ concentrations in *S. elongatus* cells, which becomes severe at pH 9.0 (Fig. 7D). This pH-dependent growth sensitivity of $\Delta nha2$ cells indicates that they have a preference for low pH, possibly because they have lost the capacity for Na⁺-dependent pH homeostasis at low Na⁺ concentrations (25).

Transcriptional profile of *nha* genes. Na⁺/H⁺ antiporters are considered proteins that are involved in the first line of defense under salt stress conditions (36). Thus, we examined whether the expression of the *nha1* to *nha7* genes is subject to Na⁺- and pH-dependent regulation at the transcriptional level with respect to acute response and acclimation.

Using real-time RT-PCR, the expression of the *nha1* to *nha7* genes in wild-type cells in the early exponential stage of growth (OD₇₃₀, ~1.5) was monitored after short- or long-term accli-

FIG. 6. Growth curves for wild-type (squares) and incompletely segregated $\Delta nha3^*$ (Km^r) (circles) cells grown in BG-11 medium containing ~20 mM Na⁺ (filled symbols) or in high-salt BG-11 medium prepared by adding 100 mM NaCl (open symbols). BG-11 media were buffered with (A) 20 mM bis-Tris-propane/HEPES (pH 8.0) or (B) 20 mM bis-Tris-propane/MES (pH 9.0). In $\Delta nha3^*$ (Km^r) cultures, 25 μ g ml⁻¹ of kanamycin was added. The symbols indicate the means of three independent culture measurements.

FIG. 7. Multiwell plate assay of $\Delta nha2$ cells in BG-11 media in which Na^+ was replaced by the same amount of K^+ (~ 18 mM) and which were supplemented with different concentrations of NaCl (0.1, 10, 50, 100, 200 mM) and buffered at (A) pH 7.0, (B) pH 8.0, and (C) pH 9.0. (D) Growth curves for wild-type (squares) and $\Delta nha2$ (triangles) cells in modified BG-11 media. BG-11 media were buffered with 20 mM bis-Tris-propane/HEPES at pH 8.0 (open symbols) or 20 mM bis-Tris-propane/MES at pH 9.0 (filled symbols). The symbols indicate the means of three independent culture measurements.

mation to salt stress and alkaline-pH conditions. More precisely, changes in *nha1* to *nha7* RNA transcript levels were examined in wild-type cells in the early exponential stage after addition of different concentrations of NaCl (50, 100, and 180 mM) at pH 8.0, as well as after addition of 100 mM NaCl and a simultaneous shift to pH 9.0 for 4 h (30, 32) (short-term acclimation). Moreover, changes in *nha1* to *nha7* RNA transcript levels were also monitored when cyanobacterial cells were exposed until the early exponential stage of growth (long-term acclimation) in medium buffered at pH 9.0 with subsequent addition of 100 mM NaCl for 4 h and in medium supplemented with 100 mM NaCl (pH 8.0).

As shown in Fig. 8, in the presence of salt and alkali stimuli, the expression of the *nha2*, *nha3*, *nha4*, *nha5*, and *nha7* genes remained constitutive. The results presented here were quan-

tified using the corresponding *nha* transcript levels in standard BG-11 medium (see below and Materials and Methods). Nevertheless, the transcript levels of *nha6* appeared to be slightly increased under all short-term acclimation conditions examined and to be radically increased under the long-term acclimation conditions used (2.15 ± 0.6 -fold change for long-term acclimation with salt and 2.23 ± 0.63 -fold change for long-term acclimation with an alkaline pH with short-term induction with 100 mM NaCl). These results suggested that transcription of *nha6* is upregulated when cyanobacterial cells are exposed for an extended period to a high salt concentration or for a short period to a high salt concentration at an alkaline pH.

The transcript levels of *nha1* are slightly decreased under short-term exposure conditions, a change which can be either

FIG. 8. Transcript accumulation for the *nha1* to *nha7* genes as determined by real-time PCR, with the values normalized to the value for the constitutively expressed 16S rRNA gene. The bars indicate the relative changes in RNA levels compared with the average expression of each gene in cells grown in standard BG-11 medium buffered at pH 8.0 (control conditions). Calculations were performed using the relative quantitative $2^{-\Delta\Delta C_T}$ method, and the results are the means of at least three independent experiments. The error bars indicate the standard errors of the means. All RNAs were derived from cultures grown until the early exponential phase ($OD_{730} = 1.5$). The gray bars indicate expression in standard BG-11 medium calculated as previously described (31), the purple bars indicate the results for short-term acclimation with 50 mM NaCl, the yellow bars indicate the results for short-term acclimation with 100 mM NaCl, the blue bars indicate the results for short-term acclimation with 180 mM NaCl, the orange bars indicate the results for short-term acclimation to pH 9.0 with simultaneous addition of 100 mM NaCl, the green bars indicate the results for long-term acclimation to 100 mM NaCl, and the dark red bars indicate the results for long-term acclimation to pH 9.0 with subsequent short-term acclimation to 100 mM NaCl.

transient or stable (32). Interestingly, with long-term acclimation under high-salt conditions, the levels of the *nha1* transcripts remained basal, indicating that the slight downregulation observed with short-term exposure to 100 mM NaCl was a result of a transient response. However, a combination of long-term alkaline pH acclimation with short-term high-salt induction resulted in significant downregulation (0.45 ± 0.26 -fold change) of *nha1* gene expression. These results indicated either that high-salt and alkaline-pH conditions act cooperatively or that long-term exposure at an alkaline pH is responsible for the negative regulation of *nha1* expression.

For real-time RT-PCR analysis, a series of control experiments were performed to ensure the specificity and efficiency of PCRs (see Materials and Methods). Our results revealed that in standard BG-11 medium all seven *nha* genes are expressed, although at rather low levels. The relative expression of the *nha* genes decreased in the following order: *nha3* > *nha1* > *nha4*, *nha6*, and *nha7* > *nha5* > *nha2*. The *nha3* gene was expressed at moderately elevated levels compared to the other six *nha* genes (see Table S2 in the supplemental material).

DISCUSSION

In this study we cloned seven genes encoding proteins that are members of the CPA superfamily and strongly resemble previously characterized Na⁺/H⁺ antiporters. A series of experimental approaches was used to investigate whether the products of these genes exhibit Na⁺/H⁺ antiporter activity and/or participate in the salt- and pH-responsive mechanisms of the freshwater cyanobacterium *S. elongatus*.

Heterologous expression of the Nha proteins in *E. coli* TO114 cells demonstrated that only the Nha3 protein can complement the salt-sensitive phenotype of the recipient strain. Additionally, an Nha3 orthologue from *Synechocystis* sp. strain PCC 6803 (NhaS3) also complements the salt-sensitive phenotype of TO114 cells, suggesting a common functional origin for these two transporters (24). One possible explanation for this finding is that the Nha3 protein possesses the flux capacity to compensate for the missing native Na⁺/H⁺ antiporters of TO114 cells, while the other Nha proteins do not have this capacity. This argument was supported by the results of Na⁺/H⁺ antiporter activity assays of Nha proteins expressed in everted vesicles, which showed that the Nha3 protein has activity that is at least fourfold higher than the activities of the other Nha proteins. The low Na⁺/H⁺ antiporter activity of the remaining Nha proteins might be due to nonproper function and/or different ion specificities (Na⁺ [K⁺]/H⁺, K⁺/H⁺, or Ca²⁺/H⁺).

Hence, strong evidence for the fundamental Na⁺/H⁺ antiporter activity of the Nha3 protein in *S. elongatus* was obtained by its incomplete inactivation and the high-salt- as well as low-salt- and alkaline-pH-sensitive phenotypes of the corresponding $\Delta nha3$ merodiploid strain. In accordance, the inability to completely inactivate the orthologue *nhaS3* in *Synechocystis* sp. strain PCC 6803 (12, 24, 65) also resulted in a salt-sensitive phenotype in the corresponding mutant strain, which was, however, observed only under alkaline environmental conditions (65). Moreover, the higher transcript levels of *nha3* than of the other *nha* genes indicate the significance of *nha3* under the experimental conditions used. Conclusively, Nha3 exhibited a high Na⁺/H⁺ flux capacity in *E. coli*

cells, which was shown to be necessary for growth of cyanobacterial cells not only under high- Na^+ conditions but also under low- Na^+ conditions, probably by controlling the Na^+/H^+ ratio.

Additionally, disruption of the *nha2* gene resulted in a low- Na^+ requirement that was essential at alkaline pH. A threshold concentration of Na^+ in growth media is essential for cyanobacteria to establish the necessary Na^+ gradient across their membranes in order to take up nutrients (34, 49, 51, 61) and to survive under alkaline conditions (3, 13). Although the Nha2 protein is unable to complement the salt-sensitive phenotype of TO114 cells and despite the low transcript levels of the *nha2* gene, our results suggest that Nha2 is involved in low- Na^+ -dependent pH regulation of cyanobacterial cells under alkaline conditions. A low- Na^+ requirement and high-pH lethality were also reported for a $\Delta nhaS2$ (orthologue of *nha2*) mutant of *Synechocystis* sp. strain PCC 6803 (33, 65), reflecting conserved characteristics of these orthologues beyond amino acid sequence similarities.

In contrast, inactivation of the Nha1, Nha4, Nha5, and Nha7 proteins resulted in strains whose growth was indistinguishable from that of the wild type. One possible explanation for this is that these proteins may make minor and/or overlapping contributions to the mechanisms of salt and pH tolerance of cyanobacterial cells. In this case combinatorial inactivation of more than one *nha* gene might be necessary to demonstrate the actual function of the genes. Inaba et al. (24) showed that only double NhaS mutants of *Synechocystis* sp. strain PCC 6803 can exhibit a salt-sensitive phenotype. Moreover, it has been shown that in bacteria mutational loss of a single Na^+/H^+ antiporter can be compensated for by upregulation of the other antiporters (45). In conclusion, among the seven *nha* genes, *nha2* and *nha3* seem to be the key elements contributing to the salt and pH tolerance of *S. elongatus*, while the other *nha* genes have supporting roles.

Furthermore, expression of all seven *nha* genes under the experimental conditions tested implies that their products make synergistic contributions to the salt and pH tolerance of *S. elongatus* cells. In particular, the steady presence of *nha2*, *nha3*, *nha4*, *nha5*, and *nha7* gene transcripts under stress conditions may reflect an attentive, ready-to-act mechanism. Moreover, our results demonstrated that expression of the *nha6* and *nha1* genes was slightly altered under distinct stress conditions. Similar studies with *Synechocystis* sp. strain PCC 6803 did not detect any changes in the expression of Na^+/H^+ antiporter genes under high-salt and alkaline conditions (12, 15, 22, 23, 26, 33, 35). However, a microarray analysis in which *Synechocystis* sp. strain PCC 6803 cells were exposed to an elevated salt concentration for 24 h revealed slight upregulation of *nhaS6* (32), the homologue of *nha6*. This is in agreement with our results indicating that *nha6* is the only putative Na^+/H^+ antiporter gene showing increased transcript levels with long-term acclimation (OD_{730} , ~1.5; approximately 2.5 days) with 100 mM NaCl, as well as at pH 9.0 with subsequent addition of 100 mM NaCl. Many other aspects must be investigated in order to clarify whether transcriptional activation/regulation is significant for the activity of the Na^+/H^+ antiporters.

Overall, our results indicate that there was a common functional origin for members of the CPA superfamily of *Synechocystis* sp. strain PCC 6803 and *S. elongatus*, which might occur

in other cyanobacterial species as well. The fact that an alkaliphilic cyanobacterium with a restricted salt tolerance range possesses seven genes with putative Na^+/H^+ antiporter activity raises questions about the distinct function of each protein. Given that the Nha3 and Nha2 proteins contribute to salt and alkaline pH response mechanisms of *S. elongatus*, further studies should elucidate the possible roles of the remaining Nha proteins.

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