CHARACTERIZATION OF Na^+/H^+ ANTIPORTERS IN THE CYANOBACTERIUM *SYNECHOCYSTIS* sp. PCC 6803

A Thesis Submitted in Fulfillment of the Requirement for the Degree of Doctor of Science

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Contents

Abstract

Na^+/H^+ antiporters are membrane proteins that play a major role in Na^+ and H^+ homeostasis of cells. The cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) has five genes for putative Na^+/H^+ antiporters (designated *nhaS1*, *nhaS2*, *nhaS3*, *nhaS4*, and *nhaS5*). Their products were studied by means of expression in *Escherichia coli*, targeted mutagenesis, sequence analyses, and phylogenetic characterization.

Previous studies on Na^+/H^+ antiporters in various organisms, including bacteria, eukaryotic microorganisms, plants, and animals, are reviewed in Chapter 1.

Chapter 2 describes characterization of *Synechocystis* Na^+/H^+ antiporters by functional expression of the *nhaS* genes in *Escherichia coli*. The results demonstrated that the *nhaS1* and *nhaS3* genes encode low-affinity and high-affinity Na^+/H^+ antiporters, respectively.

Evaluation of effects of targeted inactivation of the *nhaS* genes on phenotypes of *Synechocystis* cells is described in Chapter 3. The results suggested that (i) the function of the *nhaS1* and *nhaS2* genes' products might complement one another, (ii) the *nhaS3* gene is essential for viability of *Synechocystis* cells, and (iii) products of the *nhaS4* and *nhaS5* genes may contribute little to high-salt stress tolerance.

Chapter 4 describes phylogenetic analyses of Na^+/H^+ antiporter homologues from animals, *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, eubacteria, and archaea. The results indicated that (i) the members of the NhaP/NHE and NapA families of Na^+/H^+ antiporters diverged before the divergence of

major lineages of prokaryotes, (ii) Na^+/H^+ antiporter homologues from various cyanobacteria form five distinct groups, namely NhaS1-5 subfamilies; NhaS1 and NhaS2 belong to the NhaP/NHE family, while NhaS3, NhaS4, and NhaS5 belong to the NapA family, (iii) NhaS3 might have a particular importance for cyanobacteria, and (iv) some of the *Arabidopsis* genes for Na^+/H^+ antiporter homologues were acquired from the ancestor of plastids.

Chapter 1

General Introduction

Studies of Na^+/H^+ antiporters

1.1. Introduction

Na^+ and H^+ play primary roles in cell physiology; they are the most important ions in cell bioenergetics and the concentration of these ions within the cell is critical to the functioning of the cell and its proteins. When the concentration of these ions is elevated or de-elevated, they become potent stressors to all cells. Therefore, every cell has an efficient mechanism for the homeostasis of these ions.

Na^+/H^+ antiporters play a major role in the homeostatic mechanism. These are membrane proteins that exchange Na^+ (or Li^+) for H^+. They were discovered by Peter Mitchell and his colleagues (Mitchell and Molyle 1969; West and Mitchell 1974) and, since then, it has been found that they are widely distributed throughout the biological kingdoms. With only one known exception (Speelmans et al. 1993), the presence of Na^+/H^+ antiporters has been described in the cytoplasmic membranes of virtually all types of cells and in the membranes of many organelles (Padan and Schuldiner 1994). Because Na^+/H^+ antiporters are relatively simple in terms of their structural and kinetic properties, they could serve as a model of various ion transporters. On the other hand, their physiological roles largely depend on cell type. The main purpose of this chapter is to review how these universal devises are used by various organisms.

1.2 General properties of Na^+/H^+ antiporters

1.2.1. Function

Na^+/H^+ antiporters are secondary transporters, i.e., the antiport process is driven by an electrochemical gradient of either H^+ ($\Delta\mu H^+$) or Na^+ ($\Delta\mu Na^+$), which is maintained by primary transporters that are linked to either an electron transport, photochemical reaction, or ATP hydrolysis (Fig. 1-1). Under normal physiological conditions, there is a driving force that favors net entry of one of the two ions. In the cytoplasmic membrane of most bacteria, eukaryotic microorganisms, and plant cells, as well as in the inner membrane of mitochondria, a $\Delta\mu H^+$ is maintained by primary H^+ pumps. The $\Delta\mu H^+$ allows the operation of Na^+/H^+ antiporters that couple the downhill movement of protons into the cell or the organelle along the $\Delta\mu H^+$ and the extrusion of Na^+ against its electrochemical gradient (Fig. 1-1A). In the cytoplasmic membrane of animal cells, by contrast, a $\Delta\mu Na^+$ is maintained by the Na^+/K^+-ATPase, and Na^+/H^+ antiporters extrude protons recycling Na^+ ions back down the $\Delta\mu Na^+$ (Fig. 1-1B). However, the cycles of Na^+ and H^+ can alternate even in a single cell, depending on the conditions, and, thus, Na^+/H^+ antiporters can potentially maintain both cycles.

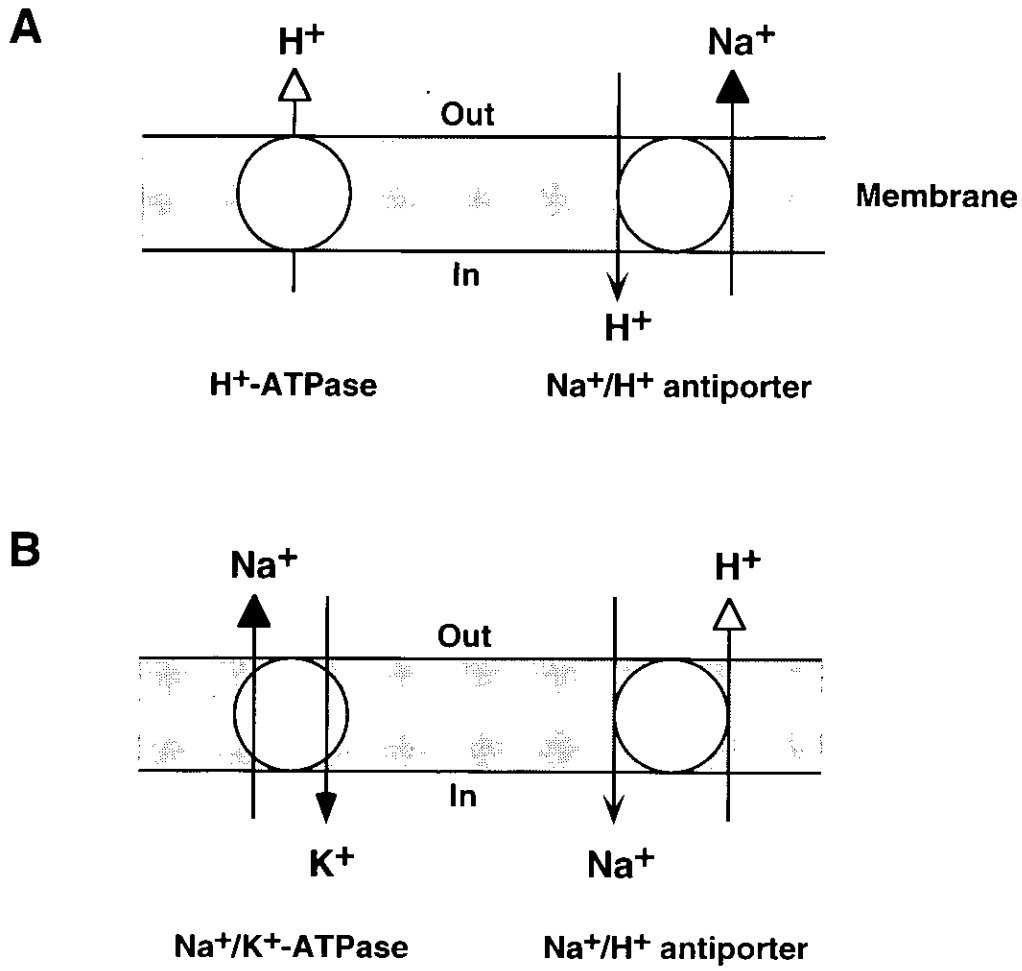

FIG. 1-1. Two modes of Na^+/H^+ antiport. (A) When a $\Delta\mu H^+$ is maintained by a primary H^+ pump, such as the H^+-ATPase, the Na^+/H^+ antiporter utilizes the $\Delta\mu H^+$ to extrude Na^+. (B) When a $\Delta\mu Na^+$ is maintained by a primary Na^+ pump, such as the Na^+/K^+-ATPase, the Na^+/H^+ antiporter utilizes the $\Delta\mu Na^+$ to extrude H^+. Uphill movements of ions are indicated by Δ in the case of H^+ and by ▲ in the case of Na^+, whereas downhill movements of these ions are indicated by ↑.

1.2.2. Structure

Hydropathy plots of amino acid sequences of Na^+/H^+ antiporters, deduced from the nucleotide sequences, suggest that they are similar to many other secondary transporters in being composed of twelve transmembrane α-helices that are connected with hydrophilic loops. Figure 1-2A shows a structure model of the best characterized Na^+/H^+ antiporter, NhaA of *E. coli* (Padan et al. 2001). This model was constructed based on results of various studies, including *phoA* and *lacZ* fusion (Karpel et al. 1991; Rothman et al. 1996), mapping antibody epitope (Padan et al. 1998), measurement of accessibility of the protein to proteases (Rothman et al. 1997), and Cys accessibility scanning (Olami et al. 1997). The structure of NhaA was determined by two-dimensional electron crystallography (Williams et al. 1999; Williams 2000) and the result substantiated the twelve-transmembrane-segment model.

Eukaryotic Na^+/H^+ antiporters, such as the members of the vertebrate Na^+/H^+ exchanger family (NHE isoforms) and Na^+/H^+ antiporters in yeast and plant cells, have a long hydrophilic region that follows the transmembrane segments (Fig. 1-2B; Wakabayashi et al. 2000). Evidence has been presented that the carboxy-terminal hydrophilic region of the NHE isoforms serves as a regulatory domain, whereas their transmembrane region is the catalytic domain that have all the features required to catalyze Na^+/H^+ exchange (Wakabayashi et al. 1997). Some of the Na^+/H^+ antiporter homologues of prokaryotes were predicted to have a similar structure.

Oligomerization is a common feature for many transmembrane proteins, including receptors, channels and transporters. A recent study of the two-dimensional crystals of NhaA of *E. coli* demonstrated that the protein exists as a homooligomer in native membranes (Gerchman et al. 2001). Fafournoux et al. (1994) presented evidence that NHE1 and NHE3 isoforms of the Na^+/H^+ exchanger also form homodimers in the cytoplasmic membrane. However, whether such oligomeric structure is required for the full functioning of these antiporters has not been answered.

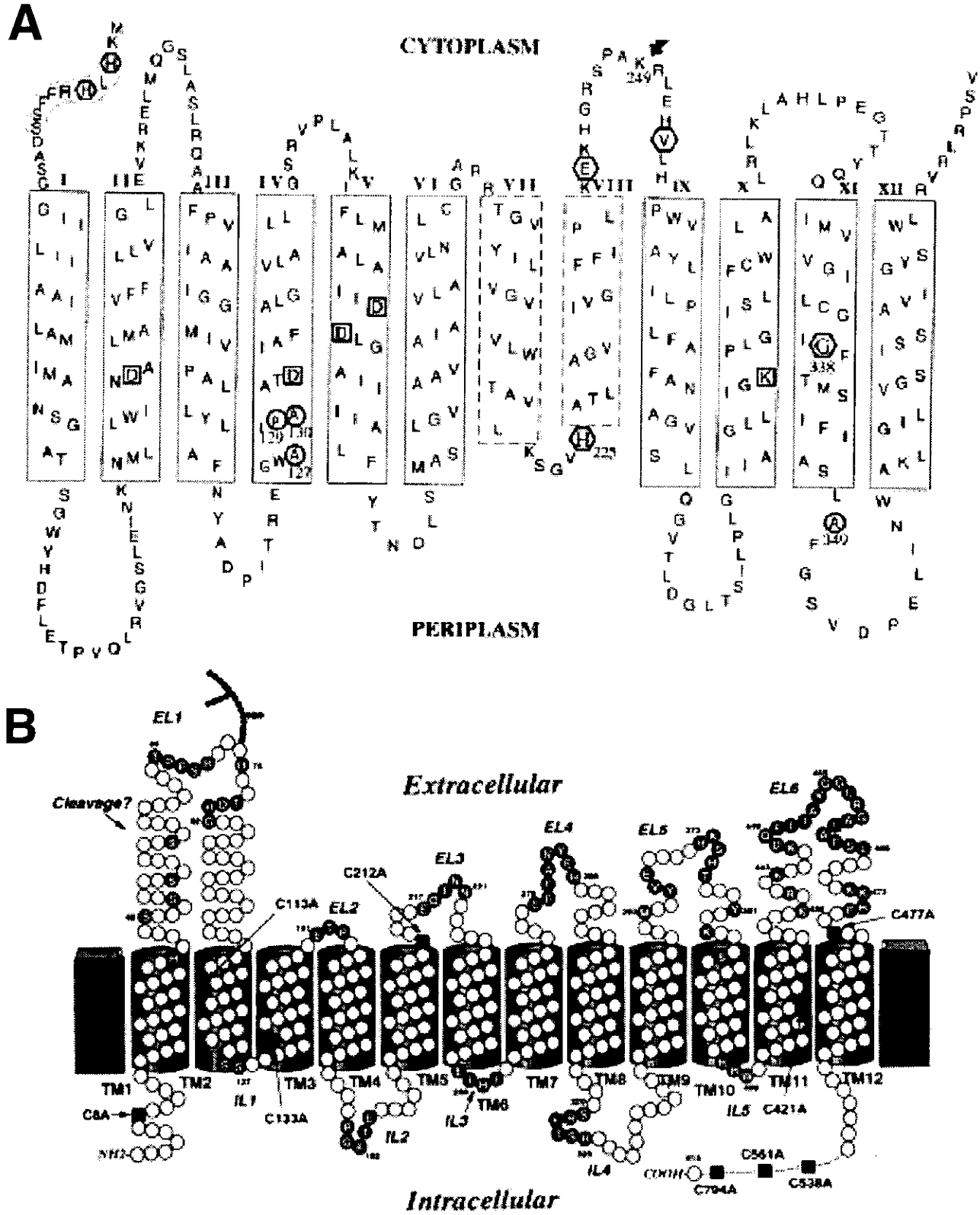

FIG. 1-2. Contemporary models for the secondary structure of Na^+/H^+ antiporters. (A) NhaA of *Escherichia coli* (Rothman et al. 1997). (B) NHE1 isoform of human (Wakabayashi et al. 2000).

1.3. The families of Na^+/H^+ antiporters

1.3.1. The prokaryotic families of Na^+/H^+ antiporters

Several families of Na^+/H^+ antiporters can be recognized in prokaryotes. These include NhaA, NapA, NhaP, NhaC, NhaD, NhaB, Mrp and TetA(L) (Table 1-1). NapA is similar to the putative K^+/H^+ antiporter of *E. coli*, KefC (Reizer et al. 1992). NhaP shows sequence similarity to Na^+/H^+ antiporters in eukaryotes. Many now available DNA sequences belong to one of these families, based upon the similarity of their putative open reading frame. There is little or no sequence similarity between members of different families. In other words, there are several groups of polypeptides with Na^+/H^+ antiport activity. Their diversity suggests that the residues essential for the activity might be very few or the activity might be fulfilled by various different combinations.

1.3.2. The eukaryotic families of Na^+/H^+ antiporters

NHE isoforms exist in the cytoplasmic membrane and the inner membrane of mitochondria of vertebrate cells (Wakabayashi et al. 1997). Yeast has Na^+/H^+ antiporters that show distinct homology to NHE isoforms. These include NhxI, an Na^+/H^+ antiporter in an endosomal compartment (Nass and Rao 1998), and SOD2, a plasma membrane Na^+/H^+ antiporter (Prior et al. 1996; Bañuelos et al. 1998). An NHX1 homologue of *Arabidopsis thaliana*, AtNHX1, is a tonoplast Na^+/H^+ antiporter that functions in compartmentation of Na^+ ions into the vacuole

(Gaxiola et al. 1999; Apse et al. 1999). In addition, *Arabidopsis thaliana* has the *SOS1* gene for a putative Na^+/H^+ antiporter that is essential for Na^+ and K^+ homeostasis (Shi et al. 2000). SOS1 shows significant similarity to NHE isoforms and bacterial NhaP.

TABLE 1-1. Characteristics of the Na^+/H^+ antiporter families

(A) Prokaryotic Na^+/H^+ antiporter families

	Characteristics	Organism	Reference
NhaA	Electrogenic High affinity for Na^+ Activated at alkaline pH	*Escherichia coli* *Salmonella enteritidis* *Vibrio parahaemolyticus* *Vibrio alginolyticus* *Helicobacter pylori*	Goldberg et al. 1987 Pinner et al. 1992 Kuroda et al. 1994 Nakamura et al. 1994 Inoue et al. 1999
NapA	High affinity for Na^+ Homologous to K^+/H^+ antiporters	*Enterococcus hirae* *Bacillus megaterium* (GrmA) *Bacillus cereus* (GerN) *Synechocystis* (NhaS3)	Waser et al. 1992 Tani et al. 1996 Thackray et al. 2001 Inaba et al. 2001
NhaP	Homologous to eukaryotic Na^+/H^+ antiporters	*Pseudomonas aeruginosa* *Synechocystis* (NhaS1)	Utsugi et al. 1998 Inaba et al. 2001
NhaC	Electrogenic High affinity for Na^+	*Bacillus firmus OF4* *Bacillus subtilis* (YheL)	Ivey et al. 1991 Wang et al. 2000
NhaD	Active at alkaline pH	*Vibrio parahaemolyticus*	Nozaki et al. 1998
NhaB	Electrogenic Low affinity for Na^+ Active at pH <8	*Escherichia coli* *Vibrio parahaemolyticus* *Vibrio alginolyticus*	Pinner et al. 1992 Nozaki et al. 1996 Nakamura et al. 1996
Mrp	Na^+(or K^+)/H^+ antiporter Electrogenic Encoded by a multigene locus (multisubunit?)	*Staphylococcus aureus* *Bacillus subtilis* *Bacillus* sp. C-125	Hiramatsu et al. 1998 Ito et al. 1999 Hamamoto et al. 1994
TetA	Tc-Co^{2+}/H^+ and Na^+(K^+,Li^+)/H^+ antiporter Electrogenic Low affinity for Na^+	*Bacillus subtilis*	Cheng et al. 1994

(B) Plant and yeast Na^+/H^+ antiporter families

	Characteristics	Organism	Reference
SOD2	$Na^+(K^+)/H^+$ antiporter	*Schizosaccharomyces pombe*	Jia et al. 1992
	Cytoplasmic membrane	*Zygosaccharomyces rouxii*	Watanabe et al. 1995
	Activated at alkaline pH	(zSOD2)	
		Saccharomyces cerevisiae	Prior et al. 1996
		(NHA1)	
		Zygosaccharomyces rouxii	Iwaki et al. 1998
		(zSOD22)	
NHX1	$Na^+(K^+)/H^+$ antiporter	*Saccharomyces cerevisiae*	Nass et al. 1997
	Vacuole	*Arabidopsis thaliana*	Gaxiola et al. 1999
	Electroneutral	(AtNHX1)	
		Oryza sativa (OsNHX1)	Fukuda et al. 1999
SOS1	$Na^+(K^+)/H^+$ antiporter?	*Arabidopsis thaliana*	Shi et al. 2000
	Cytoplasmic membrane?		

(C) Animal Na^+/H^+ antiporter families

	Localization	Other characteristics	Organism	Reference
NHE1	Cytoplasmic membrane Ubiquitous	Sensitive to amiloride Calmodulin binding Phosphorylation K^+ inhibitable *N*- and *O*-glycosylation	Human Rabbit Pig Rat Hamster	Sadat et al. 1989 Tse et al. 1991 Reilly et al. 1991 Orlowski et al. 1992 Counillon et al. 1993
NHE2	Cytoplasmic membrane Kidney, intestine, stomach, etc.	Sensitive to amiloride Calmodulin binding *O*-glycosylation	Rat Rabbit Human	Collins et al. 1993 Tse et al. 1993 Ghishan et al. 1995
NHE3	Cytoplasmic membrane Kidney, intestine, stomach	Resistant to amiloride Calmodulin binding Phosphorylation No glycosylation	Rabbit Rat Human Pig	Tse et al. 1992 Orlowski et al. 1992 Brant et al. 1995 Shugrue et al. 1999
NHE4	Cytoplasmic membrane Stomach, intestine, kidney, brain, etc.	Calmodulin binding	Rat	Orlowski et al. 1992
NHE5	Cytoplasmic membrane Brain	Resistant to amiloride Homologous to NHE3	Human Rat	Baird et al. 1999 Attaphitaya et al. 1999
NHE6	Mitochondrion	Homologous to NHX1	Human	Numata et al. 1998
βNHE	Red cell	Activated by cAMP, protein kinase A, protein kinase C	Trout	Borgese et al. 1992
XLNHE	Heart, brain skeletal muscle, reticulocytes, oocytes	Sensitive to amiloride	*Xenopus laevis*	Busch et al. 1997

1.4. Na^+/H^+ antiporters in various organisms

1.4.1. Neutrophilic bacteria

In neutrophilic bacteria (Fig. 1-3), such as *Escherichia coli* and *Bacillus subtilis*, there is a primary H^+ cycle, by which a $\Delta\mu H^+$, positive and acidic outside relative to the cytoplasm, is maintained during respiration. Some can generate a $\Delta\mu H^+$ either by an anaerobic electron transport or by the H^+-ATPase. The $\Delta\mu H^+$ is the driving force for cation/H^+ antiporters, H^+-coupled solute symporters, H^+-translocating ATP synthase, and flagellar motor. Na^+/H^+ antiporters are often electrogenic and serve as the main system for extrusion of Na^+ ions and pH regulation. In some instances, a primary Na^+-translocating system becomes active at low $\Delta\mu H^+$ (Avetisyan et al. 1992; Cheng et al. 1997).

Escherichia coli

E. coli has two Na^+- and Li^+-specific Na^+/H^+ antiporters, NhaA and NhaB (Fig. 1-3A). NhaA is the major system responsible for acclimation to the upper limit concentration of Na^+ (0.9 M, pH 7.0) and to the upper limit of pH in the presence of Na^+ (0.7 M, pH 8.5). NhaA is activated by pH shifts towards alkaline (Taglicht et al. 1991). By contrast, NhaB is active below pH 8.0 and becomes essential only when cells are devoid of functional NhaA (Pinner et al. 1993). In addition, *E. coli* has an $Na^+,Ca^{2+}/H^+$ antiporter, ChaA, that plays a role in circulation of Na^+ and Ca^{2+} ions, mainly at alkaline pH (Ohyama

et al. 1994). The presence of Na^+/H^+ antiporters with different dependence on pH appears to allow cells to tolerate high concentrations of Na^+ in a wide range of external pH.

NhaA has been the most extensively studied. The *nhaA* gene was cloned by characterizing a mutant with increased activity of Na^+/H^+ antiport (Goldberg et al. 1987; Karpel et al. 1988). The mutant was selected for the ability to grow at toxic levels of Li^+ ions (Niiya et al. 1982). On a concentration basis, Li^+ is more than ten times as toxic as Na^+ to the wild-type of *E. coli*. Therefore, Li^+ ions provided a screen for cells that were capable of maintaining low levels of intracellular Na^+ and Li^+ ions without selecting for tolerance to high osmolarity. A similar approach was taken for cloning of the *SOD2* gene that codes for an Na^+/H^+ antiporter of the fission yeast *Schizosaccharomyces pombe* (Jia et al. 1992; see *1.4.6*).

Transcription of the *nhaA* gene is dependent on NhaR, a LysR-type *trans*-acting positive regulator (Rahav-Manor et al. 1992), and regulated by intracellular Na^+ and Li^+ ions (Karpel et al. 1991). Na^+ ions affect the *nhaA*-NhaR interaction directly in a pH-dependent fashion (Carmel et al. 1997). The expression of the *nhaA* gene is also under the control of H-NS, a global regulator in enteric bacteria (Dover et al. 1996).

NhaA was purified and reconstituted in proteoliposomes (Taglicht et al. 1991; Olami et al. 1997) and the stoichiometry of the antiport reaction was determined as $Na^+/2H^+$ (Taglicht et al. 1993). The K_m value for Na^+ ions of the reconstituted NhaA was 0.11 mM at pH 8.6 (Taglicht et al. 1991).

Importance of acidic residues in the transmembrane helices, namely Asp-133, Asp-163, and Asp-164, for the activity

of Na^+/H^+ antiport was demonstrated (Inoue et al. 1995). The antiport activity of NhaA is extremely dependent on pH; its V_{max} value for Na^+ ions was increased by three orders of magnitude upon a pH shift from 7.0 to 8.5 (Taglicht et al. 1991; Gerchman et al. 1993). It was demonstrated that His-225 and Gly-338 of NhaA are involved in the pH response (Gerchman et al. 1993; Rimon et al. 1995; Rimon et al. 1998).

NhaB plays a role in tolerance to high concentrations of Na^+ and Li^+ ions at acidic and neutral pH (Pinner et al. 1993; Ohyama et al. 1994). The *nhaB* gene was cloned by functional complementation of a Δ*nhaA* strain of *E. coli* (Pinner et al. 1992). NhaB was reconstituted in proteoliposomes and the stoichiometry of the antiport reaction was determined as $2Na^+/3H^+$ (Pinner et al. 1994). The antiport activity of NhaB has some pH dependence; the K_m value for Na^+ ions of the reconstituted protein changed by tenfold, from 16.66 mM at pH 7.2 to 1.55 mM at pH 8.5, while its V_{max} value remained constant (Pinner et al. 1994). The antiport activity of NhaB is inhibited by amiloride and its derivatives, specific inhibitors of the NHE isoforms, and many other Na^+-translocating proteins (Pinner et al. 1995). In contrast, amiloride had no effect on the activity of purified NhaA (Taglicht et al. 1991).

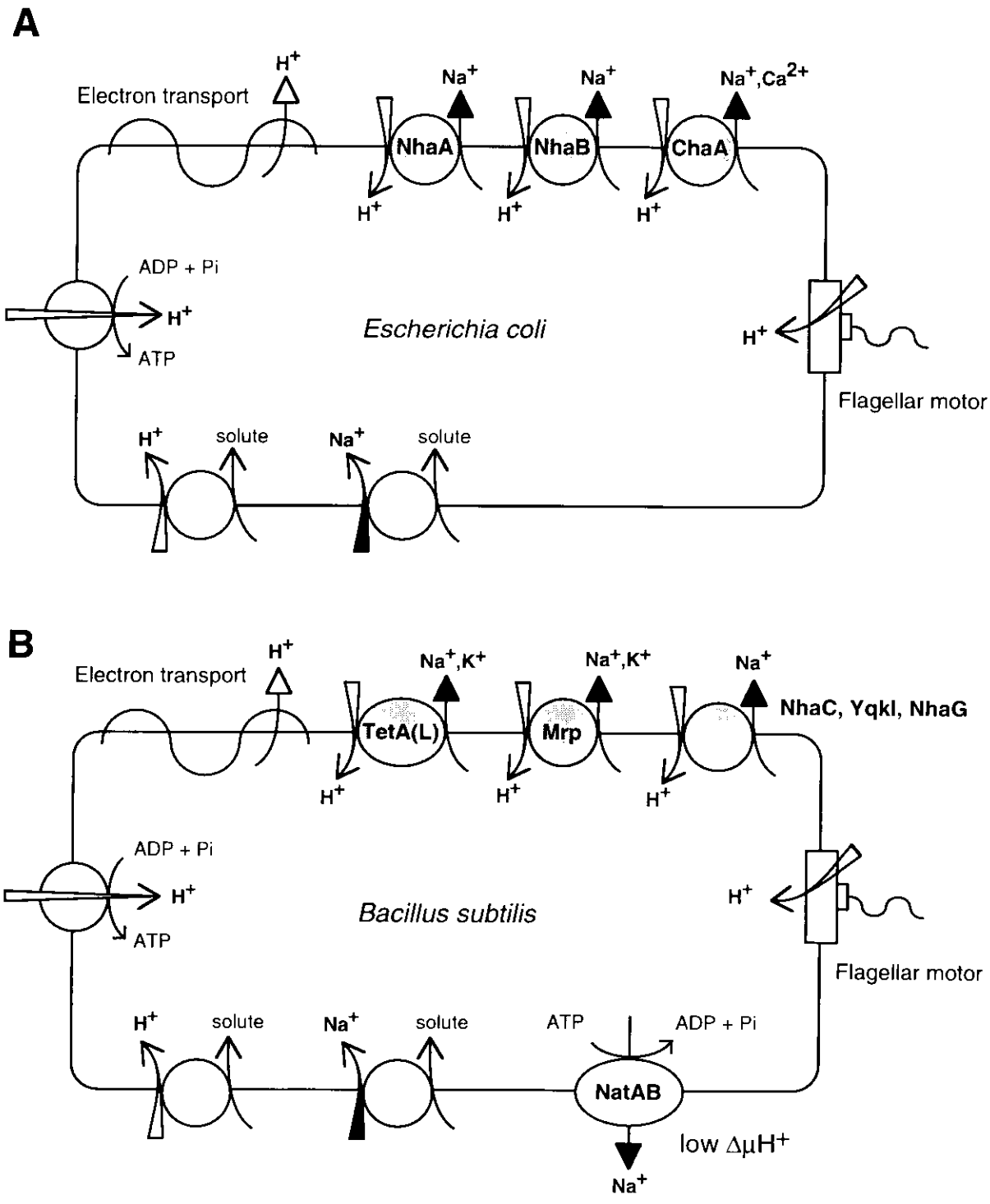

FIG. 1-3. Patterns of H^+ and Na^+ cycles in neutrophilic bacteria, such as *Escherichia coli* (A) and *Bacillus subtilis* (B). Na^+/H^+ antiporters are indicated by shading.

Bacillus subtilis

Bacillus subtilis is an endospore-forming Gram-positive bacterium, which is similar to *E. coli* with respect to optimal conditions for growth, tolerance to Na^+ ions, and dependence on pH. However, homologues of neither NhaA nor NhaB have been found in this organism. *Bacillus subtilis* has another set of transporters that are responsible for the activity of Na^+/H^+ antiport, namely, TetA(L), MrpA, YheL, and YqkI (Fig. 1-3B).

TetA(L) is a multifunctional transporter that catalyzes electrogenic antiport of tetracycline-Co^{2+}/H^+ and $Na^+(K^+,Li^+)/H^+$ (Cheng et al. 1996). The deduced amino acid sequence of TetA(L) exhibits 58-81% identity with those of class L Tet proteins, Tet(L), a major category of tetracycline efflux proteins that accounts for resistance to this antibiotic. *tet*(L) genes have been found exclusively in plasmids of Gram-positive bacteria (Salyers et al. 1990). However, the *tetA*(L) gene is localized on the chromosome of *Bacillus subtilis* strains that are nonetheless tetracycline-sensitive (Sakaguchi et al. 1988; Amano et al. 1991). Involvement of TetA(L) in Na^+/H^+ antiport was discovered by characterizing a mutant that was sensitive to high concentrations of Na^+ ions and to alkaline pH (Cheng et al. 1994). Guffanti and Krulwich (1995) expressed the *tetA(L)* gene in an Na^+/H^+ antiporter-deficient mutant of *E. coli* and demonstrated that TetA(L) catalyzes both high-affinity tetracycline/H^+ antiport, in the presence of Co^{2+} ions, and low-affinity Na^+/H^+ antiport. Upon a pH shift from 7.5 to 8.5, the apparent K_m value for tetracycline was increased from 14 μM to 40 μM, while the same value for Na^+ ions decreased from 69 mM

to 29 mM. Guffanti et al. (1998) proposed that the activity of TetA(L) includes an $Na^+(K^+)/K^+$ mode that mediates net uptake of K^+ ions. TetA(L) plays a major role in the Na^+- and K^+-dependent pH regulation, extrusion of Na^+ ions, and acquisition of K^+ ions (Cheng et al. 1994; Guffanti and Krulwich 1995; Cheng et al. 1996; Wang et al. 2000). This protein also confers the resistance to low concentrations of tetracycline.

MrpA is the product of the first gene of the *mrp* locus that contains a cluster of seven genes designated *mrpA* to *mrpG* (Oudega et al. 1997). Ito et al. (1999) demonstrated that the *mrp* genes are co-transcribed as a single transcript. However, from analyses of several mutants with mutations in the *mrp* genes, the authors proposed that the *mrpA* gene encodes an Na^+/H^+ antiporter that can function independently of a fixed complex with products of the other *mrp* genes, whereas products of the other *mrp* genes might be required for stability, assembly, or functionality of MrpA. They also presented evidence for the role of MrpA in tolerance to Na^+ ions and in Na^+-dependent regulation of intracellular pH.

Homologues of the *mrp* locus were found in other bacteria. The *pha* locus from the Gram-negative soil bacterium *Rhizobium meliloti* includes seven genes and is responsible for K^+/H^+ antiport and K^+-dependent regulation of intracellular pH (Putnoky et al. 1998). The alkalophilic *Bacillus* sp. strain C-125 has a locus, encompassing only the first three genes, which is responsible for Na^+/H^+ antiport (Hamamoto et al. 1994). The *mnh* locus of the Gram-positive bacterium *Staphylococcus aureus* contains seven genes. Hiramatsu et al. (1998) demonstrated that the whole *mnh* locus is required for an activity of Na^+/H^+

antiport when expressed in an *E. coli* mutant. However, it remains controversial whether this and the other multigene loci do or do not encode multisubunit Na^+/H^+ antiporters.

YheL and YqkI are homologues of NhaC, an Na^+/H^+ antiporter of the alkalophile *Bacillus firmus* OF4 (Section *1.4.2*; Ito et al. 1997). Wei et al. (2000) demonstrated that, when expressed in *E. coli*, YheL catalyzed an electrogenic Na^+/H^+ antiport. Evidence that the *yheL* encodes an Na^+/H^+ antiporter was first presented by Wang et al. (2000). The authors examined expression of several genes in the Δ*tetA*(L) mutants that might compensate for different functions of TetA(L). These included genes for homologues of TetA(L), the *mrp* locus and three loci for homologues of Na^+/H^+ antiporters, and genes for putative K^+ uptake systems. The mRNA level of *yheL* was much higher in the Δ*tetA*(L) mutants than in the wild-type. Phenotypes, resulting from deletion of the *yheL* gene, revealed that YheL plays a minor role in Na^+-dependent pH regulation; however no contribution to Na^+ resistance is detected. These properties are similar to those of NhaC of *Bacillus firmus* OF4 (Ito et al. 1997).

YqkI catalyzes malate- and lactate-dependent electroneutral exchange of Na^+ and H^+ (Wei et al. 2000). This protein complemented an *E. coli* mutant deficient in malate transport, only when Na^+ and lactate were present. When the *yqkI* gene was expressed in an Na^+/H^+ antiporter-deficient mutant of *E. coli*, inverted membrane vesicles, prepared from transformed cells, gained Na^+ uptake capacity, which depended on intravesicular malate and extravesicular lactate. Based upon

these results, Wei et al. (2000) proposed that YqkI is a malic^{-2}-2H$^+$/Na$^+$-lactate^{-1} antiporter.

Bacillus subtilis has an Na$^+$-translocating ABC-type transporter, NatAB (Cheng et al. 1997). This transporter is encoded by two genes, one of which (*natA*) encodes the ATP-binding hydrophilic subunit, while the other (*natB*) encodes an integral membrane protein. These genes are expressed as an operon, and the expression is induced at low $\Delta\mu H^+$. The phenotype of mutants with disruption of the *natAB* genes indicates that this locus plays no role in pH regulation *per se*.

1.4.2. Alkalophilic bacteria

Alkalophilic bacteria grow optimally at alkaline pH and can survive at or above pH 11.0. They exhibit a variation of the pattern of Na$^+$ and H$^+$ cycles in neutrophilic bacteria (Fig. 1-4A). Respiration and ATP synthesis are coupled entirely with protons. Electrogenic Na$^+$/H$^+$ antiporters have a crucial role in the regulation of intracellular pH. In contrast to neutrophilic bacteria, all solute symporters and motility are Na$^+$-coupled. They provide re-entry routes for Na$^+$ ions, allowing the activity of Na$^+$/H$^+$ antiporters to be sustained.

Bacillus firmus OF4

Bacillus firmus OF4 share a similar set of Na$^+$/H$^+$ antiporters with *Bacillus subtilis* (Fig. 1-4A). However, *Bacillus firmus* OF4, but not *Bacillus subtilis*, can acidify its

cytoplasm relative to the exterior upon a shift of pH from 8.5 to 10.5. Upon such a shift, the alkalophile maintains a substantial level of $\Delta\mu H^+$, the driving force for the Na^+/H^+ antiporters, whereas there is a pronounced reduction of $\Delta\mu H^+$ in the neutrophile. The aggregate activity of Na^+/H^+ antiport in the membrane of *Bacillus firmus* OF4 was at least ten times higher than that of *Bacillus subtilis* at pH 9. Interestingly, growth in the upper limit of pH for *Bacillus firmus* OF4 exclusively depends on Na^+ ions, whereas that for *Bacillus subtilis* depends on either Na^+ or K^+ ions (Krulwich et al. 1994).

NhaC of *Bacillus firmus* OF4 was cloned using a Δ*nhaA* strain of *E. coli* by functional complementation (Ivey et al. 1991). This is a relatively high-affinity electrogenic Na^+/H^+ antiporter. Disruption of the *nhaC* gene in *Bacillus firmus* OF4 did not affect the capacity of cells to grow at pH 10.5, indicating that NhaC is not required for alkalophily (Ito et al. 1997). The *nhaC* gene is a part of an operon that consists of *nhaC* and a small gene designated *nhaS*. NhaS is an Na^+-binding regulatory protein. Expression of the *nhaS* gene alone in an Na^+/H^+ antiporter-deficient mutant of *E. coli* increased tolerance of cells to Na^+ ions without enhancing the activity of Na^+/H^+ antiport. It has been suggested that NhaS might associate with the membrane under some conditions, where it could have a role in Na^+ sensing (Ito et al. 1997).

A primary Na^+-translocating system exists in *Bacillus firmus* OF4 as does *Bacillus subtilis* (Wei et al. 1999). Such a system might be regulated to function only at an inhibitory concentration of Na^+ ions, as loss of intracellular Na^+ ions

through primary extrusion would be detrimental to alkalophilic bacteria.

1.4.3. Halophilic bacteria

Halophilic bacteria specifically require a high concentration of Na^+ ions for growth and can grow in the presence of 3.5 M NaCl or higher. They share most of the general pattern of Na^+ and H^+ cycles for alkalophilic bacteria, although the major biological challenge is quite different (Fig. 1-4B). No Na^+/H^+ antiporter has been cloned so far. However, it has been demonstrated that *Halobacterium halobium* has activity of Na^+/H^+ antiport with unique properties; it requires a gating potential of 100 mV; restricts back-flow of Na^+ ions, regardless of the size of $\Delta\mu H^+$; and is the only bacterial Na^+/H^+ antiporter that is inhibited by N,N'-dicyclohexylcarbodiimide (Murakami and Konishi 1989). Halophilic bacteria seem to tolerate a high intracellular concentration of Na^+ ions (up to 1 M). Therefore, it appears that they compromise with a $\Delta\mu Na^+$ lower than ten, which can be easily produced by Na^+/H^+ antiporters. The presence of Na^+-translocating respiratory chain complexes has been suggested in the moderately halophilic bacterium *Haemophilus influenzae* (Hayashi et al. 1996).

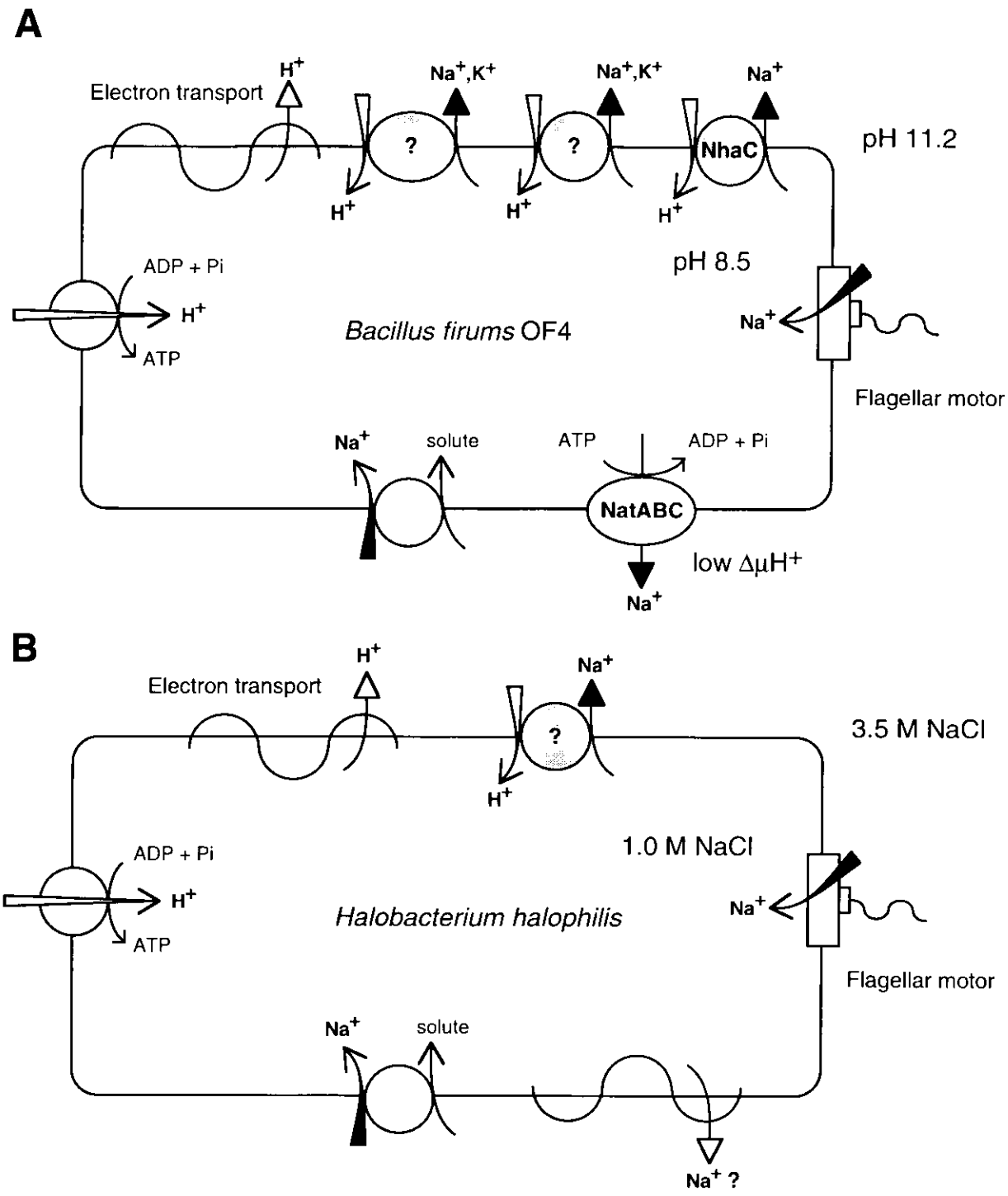

FIG. 1-4. Patterns of H^+ and Na^+ cycles in alkalophilic bacteria, such as *Bacillus firums* OF4 (A), and halophilic bacteria, such as *Halobacterium halophilis* (B). Na^+/H^+ antiporters are indicated by shading.

1.4.4. Anaerobic and marine bacteria

The third pattern of Na^+ and H^+ cycles includes anaerobic and marine bacteria (Fig. 1-5). These bacteria have specialized primary Na^+ pumps that serve as the major systems for extrusion of Na^+ ions. The anaerobe *Enterococcus hirae* has an Na^+-translocating V-type ATPase together with a H^+-translocating P-type ATPase (Takase et al. 1993; Takase et al. 1994). The marine bacterium *Priopionigenium modestum* and homoacetogenic *Acetobacterium woodii* have Na^+-translocating P-type ATPases (Dimroth 1990; Heise et al. 1992). In *Vibrio alginolyticus* and other marine bacteria there are respiratory chain complexes that include Na^+-translocating NADH:ubiquinone oxidoreductase, in addition to H^+-translocating respiratory chain complexes (Unemoto and Hayashi 1993). Activity of membrane-bound Na^+-translocating decarboxylases have been described in many anaerobes (Dimroth 1997). In methylotrophic archaea, the N5-methyltetrahydromethanopterin coenzyme M methyltransferase functions as a reversible Na^+ pump (Lienard and Gottschalk 1998).

These bacteria also have Na^+/H^+ antiporters. An exceptional case is *Clostridium fervidus*, a Gram-positive thermophilic anaerobe. It has an Na^+-ATPase and lacks Na^+/H^+ antiporter (Speelmans et al. 1993). The absence of Na^+/H^+ antiporter might be because the cytoplasmic membrane of this bacterium is much more permeable for protons than for Na^+ ions at the optimal temperature of growth.

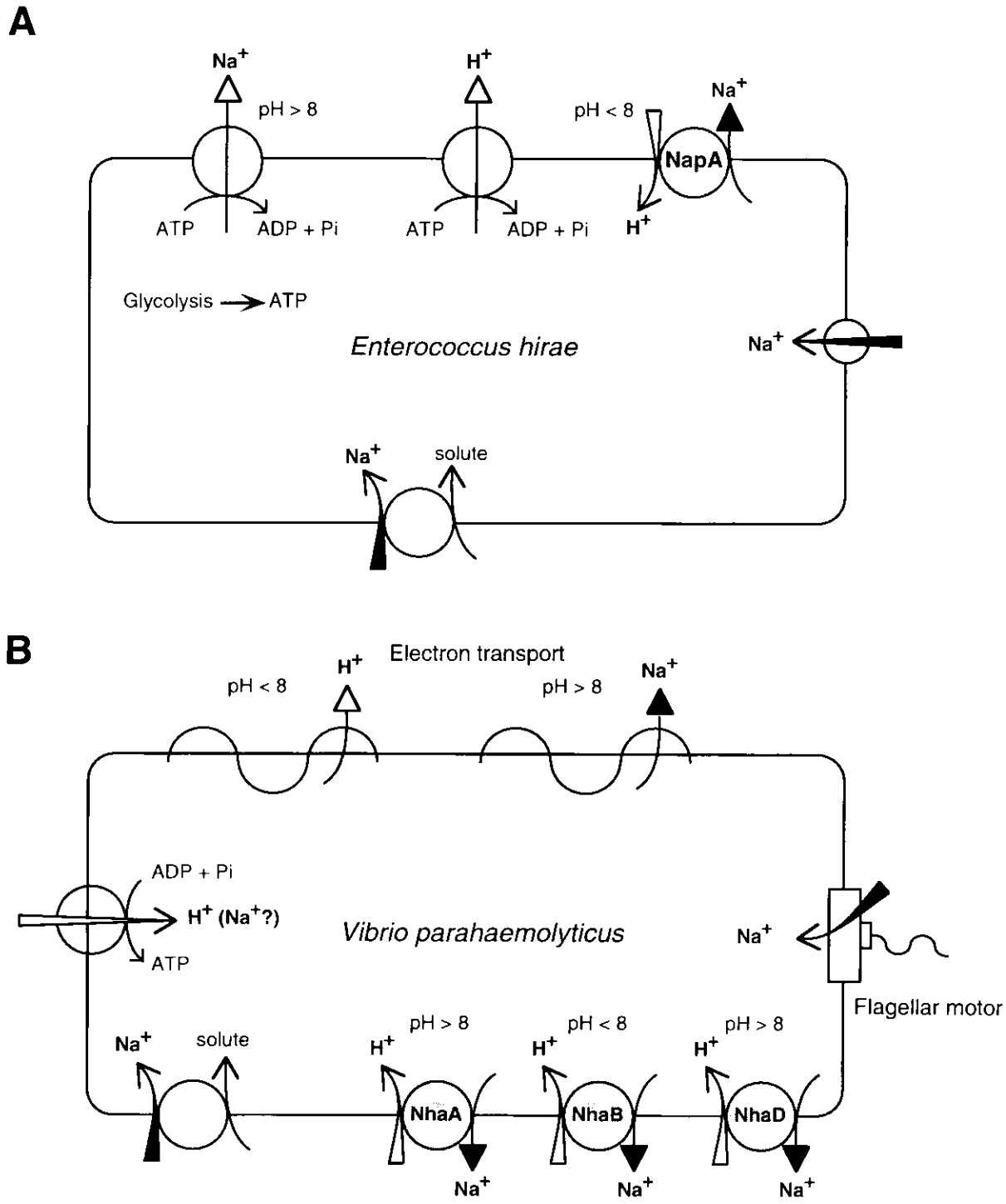

FIG. 1-6. Patterns of H^+ and Na^+ cycles in anaerobic bacteria, such as *Enterococcus hirae* (A), and marine bacteria, such as *Vibrio parahaemolyticus* (B). Na^+/H^+ antiporters are indicated by shading.

Enterococcus hirae

Enterococcus hirae is an obligatory anaerobic Gram-positive bacterium. It has an Na^+/H^+ antiporter, NapA (Fig. 1-5A). The *napA* gene was cloned by complementation of an *Enterococcus hirae* mutant that was deficient in both ATP-driven Na^+ transport and Na^+/H^+ antiport with a DNA library derived from this bacterium on the basis of the growth sensitivity to Na^+ ions (Waser et al. 1992). Expression of the *napA* gene enabled cells of the mutant to grow in the presence of high concentrations of Na^+ ions at neutral pH. Disruption of the *napA* gene in another mutant that was deficient in the ATP-driven Na^+ transport, but not in the wild-type, resulted in hypersensitivity to Na^+ ions. When the *napA* gene was expressed in a *ΔnhaAΔnhaB* strain of *E. coli*, the transformed cells gained an activity of Na^+/H^+ antiport with a K_m value of 1 mM for Na^+ ions at pH 7.5, which is similar to the value obtained for reconstituted NhaA of *E. coli* (Taglicht et al. 1991). Unlike NhaA, however, the activity decreased as the external pH was raised to 8.5, with little change in the K_m value, and became zero at pH 9.0 (Strausak et al. 1993). These results altogether suggested that NapA functions mainly at pH below 8.0 and becomes essential for growth at high concentrations of Na^+ ions when the Na^+-ATPase is not operative.

Vibrio parahaemolyticus

Vibrio parahaemolyticus is a slightly halophilic marine bacterium that requires 30 mM of Na^+ ions for growth. Three Na^+/H^+ antiporters, Vp-NhaA, Vp-NhaB and NhaD, have been identified (Fig. 1-5B). Genes for these Na^+/H^+ antiporters were cloned using a Δ*nhaA*Δ*nhaB* mutant of *E. coli* by functional complementation (Kuroda et al. 1994; Nozaki et al. 1996; Nozaki et al. 1998). The deduced amino acid sequences of Vp-NhaA and Vp-NhaB show 59% and 72% identity to those of NhaA and NhaB of *E. coli*, respectively. The Na^+/H^+ antiport activity of Vp-NhaA increases as the external pH is raised from 7.0 to 9.0. By contrast, the activity of Vp-NhaB is independent on pH. Hence, they are also functionally similar to the *E. coli* antiporters. The third gene, *nhaD*, was cloned on the basis of sensitivity of the Δ*nhaA*Δ*nhaB* mutant of *E. coli* to Li^+ ions. NhaD shows the highest activity of Na^+/H^+ antiport between pH 8.5 and 9.0 and zero activity between pH 7.0 and 7.5.

1.4.5. Cyanobacteria

Cyanobacteria are, by definition, prokaryotes that perform oxygenic photosynthesis. They are assumed to have risen nearly 3.5 billion years ago and, today, constitute the largest and perhaps most diverse group of eubacteria. According to the traditional classification of prokaryotes, cyanobacteria are classified as Gram-negative bacteria; their cell envelope is composed of the outer membrane and cytoplasmic (inner) membrane separated by a peptidoglycan layer. Their cell envelope, however, shows a unique combination of Gram-negative and Gram-positive features (Hoiczyk and Hansel 2000). In addition to the cell envelope, cyanobacteria have intracellular membranes, i. e., thylakoid membranes, which provide the site of both photosynthetic and respiratory electron transport (Omata and Murata 1985). At least in some cyanobacteria, complete respiratory chain exists also in the cytoplasmic membrane (Schmetterer 1995).

The pattern of H^+ and Na^+ cycles in non-marine cyanobacteria is shown in Figure 1-6. They have a primary H^+ cycle at the cytoplasmic membrane, by which a $\Delta\mu H^+$ is generated by the H^+-ATPase or by a respiratory electron transport. The $\Delta\mu H^+$ is the driving force for cation/H^+ antiporters and H^+-coupled solute symporters. Na^+/H^+ antiporters appear to serve as the main system for extrusion of Na^+ ions and pH regulation. In some instances, a primary Na^+-translocating system may function to extrude Na^+ ions. The status of Na^+/H^+ antiporters or other Na^+ transport systems in the thylakoid membranes is unclear. In

the cytoplasmic membrane of marine cyanobacteria, a primary Na^+ cycle is maintained by Na^+-ATPases (Brown et al. 1990).

Sodium ions are specifically required for photosynthesis and uptake of HCO_3^- and CO_2 (Kaplan et al. 1984; Reinhold et al. 1984). Previously it was considered that HCO_3^- uptake might be directly coupled with Na^+ transport by an Na^+/HCO_3^- symporter (Kaplan et al. 1984; Reinhold et al. 1984). This possibility was excluded, at least in *Synechocystis*, by the finding that the genome of this cyanobacterium does not contain genes encoding such transporters. Moreover, an ATP-binding cassette protein involved in high affinity uptake of inorganic carbon was identified in *Synechococcus* sp. PCC 7942 (Omata et al. 1999). The Na^+ dependence of HCO_3^- uptake appears to be more likely due to the requirement of Na^+ ions for the activity of photosystem II (Zhao and Brand 1988).

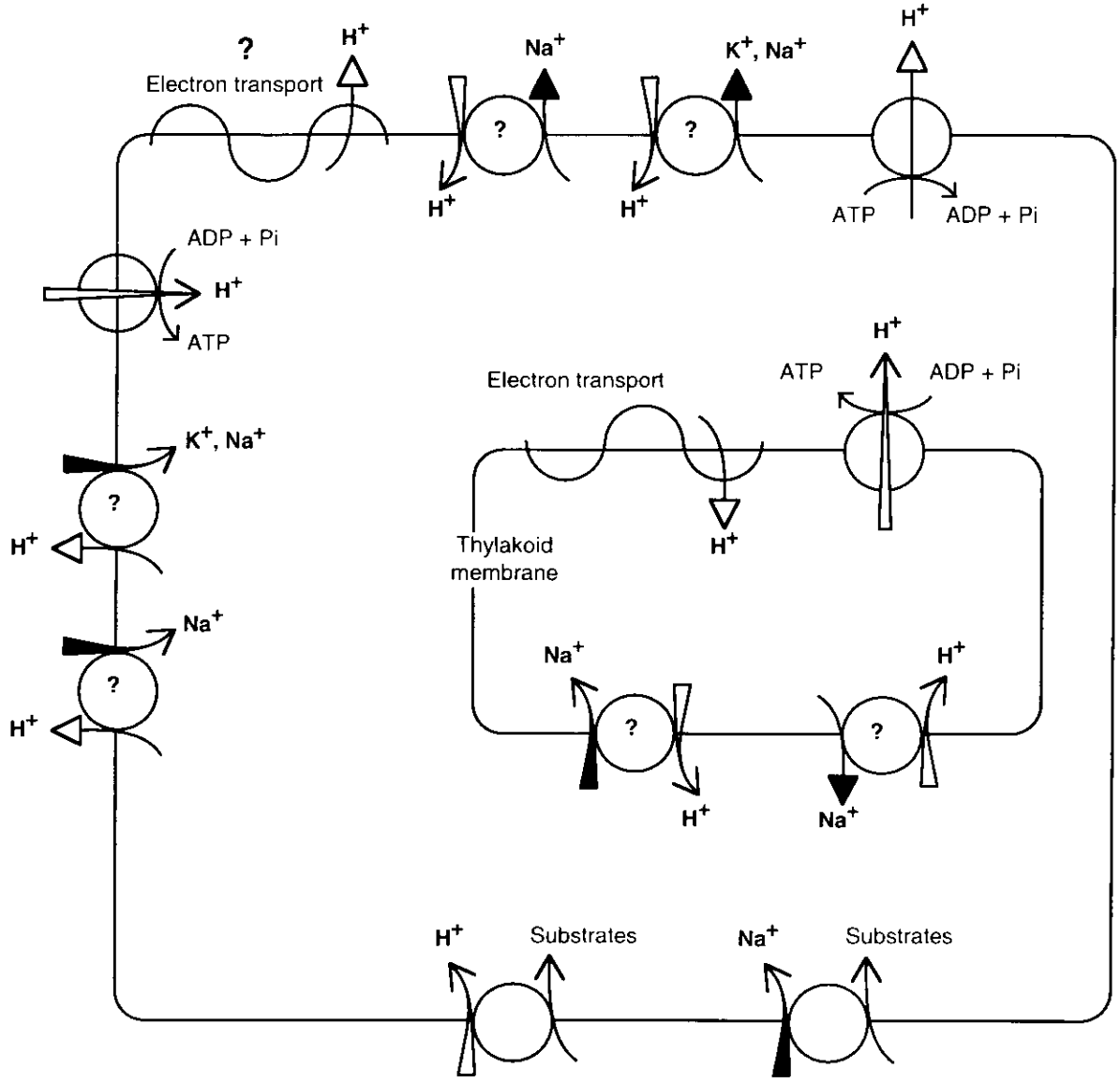

FIG. 1-6. Patterns of H^+ and Na^+ cycles in cyanobacterial cells. Status of Na^+ transport in the thylakoid membranes is unclear.

1.4.6. Eukaryotic microorganisms and plants

Cells of eukaryotic microorganisms and plants share basically the same pattern of Na^+ and H^+ cycles (Fig. 1-7; Blumwald et al. 2000). The cytoplasmic membrane maintains a primary H^+ cycle initiated by the H^+-ATPase. Solute symporters are coupled to protons. Na^+/H^+ antiporters are widely distributed in the cytoplasmic membrane of eukaryotic microorganisms and plants. Tonoplast Na^+/H^+ antiporters mediate the transport of Na^+ ions into the compartment. The driving force for the tonoplast Na^+/H^+ antiporters is generated by the vacuolar H^+-translocating enzymes, the V-type H^+-ATPase and H^+-pyrophosphatase. *Saccharomyces cerevisiae* has Na^+-ATPases, while the existence of Na^+-ATPases has been suggested in marine algae *Tetraselmis* (*Platymonas*) *viridis* and *Heterosigma akashiwo* (Wada et al. 1992; Gimmler 2000). By contrast, no primary Na^+-translocating system has been found in higher plants, even halophytes. Therefore, plant Na^+/H^+ antiporters play important roles both in extrusion and compartmentation of Na^+, the two main mechanisms that function cooperatively to prevent the accumulation of Na^+ in the cytosol and to maintain a high cytosolic K^+/Na^+ concentration ratio.

Na^+ ions can be transported into the cell through K^+ carriers. This is due to the similarity between the hydrated ionic radii of sodium and potassium, which makes difficult the discrimination between the two ions by the carriers. This discrimination problem is also the basis for Na^+ toxicity. Na^+ ions have adverse effects on K^+ nutrition, cytosolic enzyme activities, photosynthesis, and metabolism. Yeast and plant

cells utilize a variety of low- and high-affinity transporters to take up K^+ ions from the extracellular medium. Low-affinity K^+ carriers, such as AKT1 of *Arabidopsis thaliana*, are inwardly rectifying channels that activate K^+ influx upon hyper-polarization of the cytoplasmic membrane (Sentenac et al. 1992). They display a high K^+/Na^+ selectivity ratio at physiological external concentrations of these ions. K^+ uptake by high-affinity K^+ carriers, such as the wheat K^+/Na^+ symporter, HKT1, is activated by micromolar concentrations of Na^+ ions in the external medium (Rubio et al. 1995). In *Saccharomyces cerevisiae*, it has been proposed that a K^+ transporter, Trk1, limits Na^+ entry by increasing discrimination between K^+ and Na^+ ions under conditions of high-salt stress or K^+ depletion (Goméz et al. 1996). Nevertheless, such K^+ carriers could mediate a significant entry of Na^+ ions at toxic levels of Na^+ ions in the external medium (Rubio et al. 1995). Outwardly rectifying K^+ channels, which open during depolarization of the cytoplasmic membrane, could also mediate the efflux of K^+ and the influx of Na^+ (Wegner et al. 1994). Therefore, it is crucial to remove Na^+ ions actively from the cytosol under conditions of high-salt stress.

Schizosaccharomyces pombe

The fission yeast *Schizosaccharomyces pombe* grow over a wide range of external pH from 3.5 to 7.0, with optimum growth at pH 6.0 in low-salt medium. The growth is arrested by 15 mM LiCl or 600 mM NaCl. The yeast has an electroneutral Na^+/H^+ antiporter, SOD2. The *SOD2* gene was cloned by characterizing

mutants with enhanced activity of Na^+/H^+ antiport, which were selected for tolerance of cell growth to Li^+ ions (Jia et al. 1992). The tolerance to Li^+ ions was pH dependent; these mutants grew up to 60 mM LiCl at pH 5.0, while they were as sensitive to Li^+ ions as wild-type at pH 7.0. Amplification of the *SOD2* locus had occurred on the chromosome of the mutants, and the degree of such amplification correlated with the level of tolerance to Na^+ and Li^+ ions. On the other hand, haploid cells that contained only disrupted *SOD2* genes were deficient in activity of Na^+/H^+ antiport (Jia et al. 1992). They grew much slower than wild-type cells in both rich and minimal media and were unable to grow in the presence of 125 mM NaCl. Moreover, the mutant cells could not grow at or above pH 6.5, even without the addition of Na^+ ions. These results indicated that SOD2 is responsible for tolerance to Na^+ and Li^+ ions and to high values of external pH.

Saccharomyces cerevisiae

Saccharomyces cerevisiae has P-type Na^+-ATPases that play a predominant role in extrusion of Na^+ ions and are essential for tolerance to Na^+ ions at high values of external pH. An Na^+-ATPase was cloned based on its ability to increase the tolerance to Li^+ ions in a Li^+-sensitive strain of *Saccharomyces cerevisiae* (Haro et al. 1991). The gene is the first unit of the *ENA/PMR2* locus that consists of a tandem array of nearly identical four (*ENA1* to *ENA4*) or five (*PMR2a* to *PMR2e*) genes, depending on the strain. The expression of the *ENA1* gene is induced by Na^+ and Li^+ ions and high pH values (Garciadeblas et

al. 1993) and modulated by calcineurin (Mendoza et al. 1994). By contrast, the *ENA2*, *ENA3*, and *ENA4* genes are expressed constitutively at low levels (Garciadeblas et al. 1993).

Besides these Na^+-ATPases, *Saccharomyces cerevisiae* has two Na^+/H^+ antiporters, NHA1 and NHX1. NHA1 is a plasma membrane $Na^+(K^+)/H^+$ antiporter. It plays a role in regulation of cytosolic pH, being activated by increased cytosolic pH (Prior et al. 1996; Bañuelos et al. 1998). Bañuelos et al. (1998) proposed that NHA1 functions to eliminate a toxic amount of Na^+ ions or an excess of K^+ ions from cells at acidic external pH, while it might contribute to the buffering of cytosolic pH when the cytosolic pH raises, using the outward gradient of K^+ (Na^+) ions. NHA1 exhibits high homology to SOD2 of *Schizosaccharomyces pombe* (40.1% identity) and to Z-SOD2 of *Zygosaccharomyces rouxii* (71.7% identity). The *NHA1* gene was cloned by complementation of a strain that was sensitive to Na^+ ions with a genomic DNA library of *Saccharomyces cerevisiae* (Prior et al. 1996). The sensitivity of the strain to Na^+ ions is probably due to the fact that the strain contains only a single *ENA1/PMR2* gene. Overexpression of the *NHA1* gene in a mutant in which all *ENA* genes were deleted increased the tolerance to Na^+ and Li^+ ions at acidic pH. Disruption of the *NHA1* gene in this mutant brought about hypersensitivity to Na^+ ions. By contrast, the high tolerance to Na^+ ions of a strain that contained four *ENA* genes was not increased by overexpression of the *NHA1* gene.

NHX1 is an electroneutral Na^+/H^+ antiporter that mediates intracellular sequestration of Na^+ ions. Deduced amino acid sequence of NHX1 shows significant similarity to NHE isoforms

of the vertebrate Na^+/H^+ exchanger. The process of identification of the *NHX1* gene was unique. Na^+-sensitive mutants, disrupted in the gene for calcineurin B, which lacks the ability to increase the expression of the *ENA1* gene and modulate the K^+ transport to high affinity (Mendoza et al. 1994), were used in screening for genetic suppressers (Nass et al. 1997). Mutations that conferred tolerance to Na^+ ions were mapped to the *PMA1* gene for the P-type H^+-ATPase. Cells of such a mutant, *pma1-α4*, showed increased levels of intracellular Na^+ ions in addition to a reduced influx of Na^+ ions. These results suggested the existence of a mechanism for intracellular sequestration of Na^+ ions. The authors identified the *NHX1* gene by analysis of the genome of *Saccharomyces cerevisiae* and examined its role in tolerance to Na^+ ions. The *NHX1* gene encodes a protein which shares homology (about 30% identity) with the NHE isoforms. Disruption of the *NHX1* gene in wild-type strains led to a significant decrease in tolerance to Na^+ ions at acidic pH, but not at neutral pH. By contrast, disruption of the *NHX1* gene in the *pma1-α4* mutant nullified its Na^+-tolerant phenotype both at acidic and neutral pH. These results indicate that the cytosolic pH may be a controlling factor in tolerance to Na^+ ions. It was proposed that reduced proton pumping in the *pma1-α4* mutant leads to a reduction in the cytosolic pH, which in turn facilitates the sequestration of Na^+ ions by NHX1.

Nass and Rao (1998) proposed the localization of NHX1 in a late endosomal compartment. The authors tagged NHX1 with either the triple hemagglutinin (HA) epitope or the green fluorescent protein (GFP) at the carboxy-terminus. Cells of a mutant that was disrupted for the *NHX1* gene, *Δnhx1*, expressing either the

HA- or GFP-tagged NHX1 under the control of the endogenous promoter of the *NHX1* gene did not exhibit the pH-dependent Na^+-sensitive phenotype, which suggested that the chimera proteins were fully functional. The expression of the tagged genes were induced by addition of NaCl to the medium, suggesting a role for NHX1 in tolerance to Na^+ ions. Subcellular fractionation experiments revealed that both HA- and GFP-tagged NHX1 co-localized with makers for the vacuole and prevacuolar compartments. Confocal laser microscopy of GFP-tagged NHX1 showed the localization of the protein to the membrane of the prevacuolar compartment with a bipolar distribution.

On the other hand, Numata et al. (1998) proposed that NHX1 is localized in mitochondria. The authors tagged the *NHX1* gene with the gene encoding GFP at its carboxy-terminus, and expressed the GFP-tagged NHX1 chimera under control of the constitutive, methionine-repressible MET25 promoter in wild-type cells. GFP fluorescence co-localized with a DNA-binding dye for mitochondrial DNA staining. In addition, the amiloride analogue benzamil, which inhibits activity of Na^+/H^+ antiport in mammalian mitochondria, inhibited $^{22}Na^+$ influx into isolated mitochondria. In *Δnhx1* cells, the benzamil-sensitive $^{22}Na^+$ influx was absent. Moreover, when *Δnhx1* cells were grown on non-fermentative carbon sources, retardation of growth was observed compared to wild-type cells. These results altogether suggested a role for NHX1 in mitochondrial function.

The conflicting results regarding the subcellular localization of NHX1 may be due in part to the different promoters they used to express the chimera proteins. The pH-dependent Na^+-sensitive phenotype of *Δnhx1* cells seems to favor

the model that NHX1 mediates sequestration of Na^+ ions into the prevacuolar compartment. This model is supported by the recent findings that NHX1 and the Cl^- channel, Gef1, are required for the sequestration of Na^+ ions and that NHX1 and Gef1 are co-localized to the prevacuolar compartment (Gaxiola et al. 1999).

Arabidopsis thaliana

The genome sequencing of *Arabidopsis thaliana* led to identification of genes for putative Na^+/H^+ antiporters with similarity to NHX1 of *Saccharomyces cerevisiae*. On the basis of this fact, Gaxiola et al. (1999) cloned a sequence from *Arabidopsis thaliana* with high homology to the *NHX1* gene and designated *AtNHX1*. Expression of the *AtNHX1* gene in *Δnhx1* cells of *Saccharomyces cerevisiae* suppressed the Na^+-sensitive phenotype of the host cells only under conditions in which the K^+ availability was reduced. When *Arabidopsis* plants were exposed to either 250 mM NaCl or 250 mM KCl for 6 h, transcripts of the *AtNHX1* gene increased by 4.2-fold and 2.8-fold, respectively. These results altogether might indicate that the *AtNHX1* gene encodes an $Na^+(K^+)/H^+$ antiporter. Apse et al. (1999) presented evidence that the *AtNHX1* gene encodes a vacuolar Na^+/H^+ antiporter. To assess the function of AtNHX1 in Na^+/H^+ antiport, the authors measured Na^+-dependent H^+ movement in intact vacuoles isolated from wild-type plants and plants overexpressing the *AtNHX1* gene. The rate of Na^+/H^+ antiport in vacuoles isolated from transgenic plants was much higher than that in vacuoles isolated from wild-type plants. Moreover, the overexpression of the *AtNHX1* gene promoted growth and

development of transgenic plants in soil watered with up to 200 mM NaCl. This result is consistent with the role of the vacuolar Na^+/H^+ antiport mediated by AtNHX1 in tolerance to Na^+ ions. Apparent K_m value of the Na^+/H^+ antiport for Na^+ obtained with transgenic plants was 7 mM.

SOS1 is a putative Na^+/H^+ antiporter that is essential for Na^+ and K^+ homeostasis. The *SOS1* gene is predicted to encode a protein that consists of 12 transmembrane segments followed by a long hydrophilic domain. The transmembrane region of SOS1 shows significant sequence similarity to cytoplasmic membrane Na^+/H^+ antiporters in animals (NHE isoforms), bacteria (NhaP), and yeast (SOD2 and NHA1). The *SOS1* locus was identified by characterizing *sos* (salt overly sensitive) mutants defective in tolerance to Na^+ ions (Wu et al. 1996; Liu and Zhu 1997; Zhu et al. 1998). The *sos* mutants were hypersensitive to Na^+ and Li^+ ions, as well as K^+ depletion (Zhu et al. 1998). Allelic tests indicated that the *sos* mutants define three loci, i.e., *SOS1*, *SOS2*, and *SOS3* (Zhu et al. 1998). The *SOS3* gene encodes a homologue of the yeast calcineurin B (Zhu et al. 1998), and the *SOS2* gene encodes a serine/threonine type protein kinase (Liu et al. 2000). SOS2 and SOS3 constitute for a regulatory pathway for Na^+ and K^+ homeostasis (Halfter et al. 2000). The *SOS1* locus has been isolated through positional cloning (Shi et al. 2000). Expression of the *SOS1* gene is induced by salt stress and is regulated by the SOS2/SOS3 regulatory pathway (Shi et al. 2000).

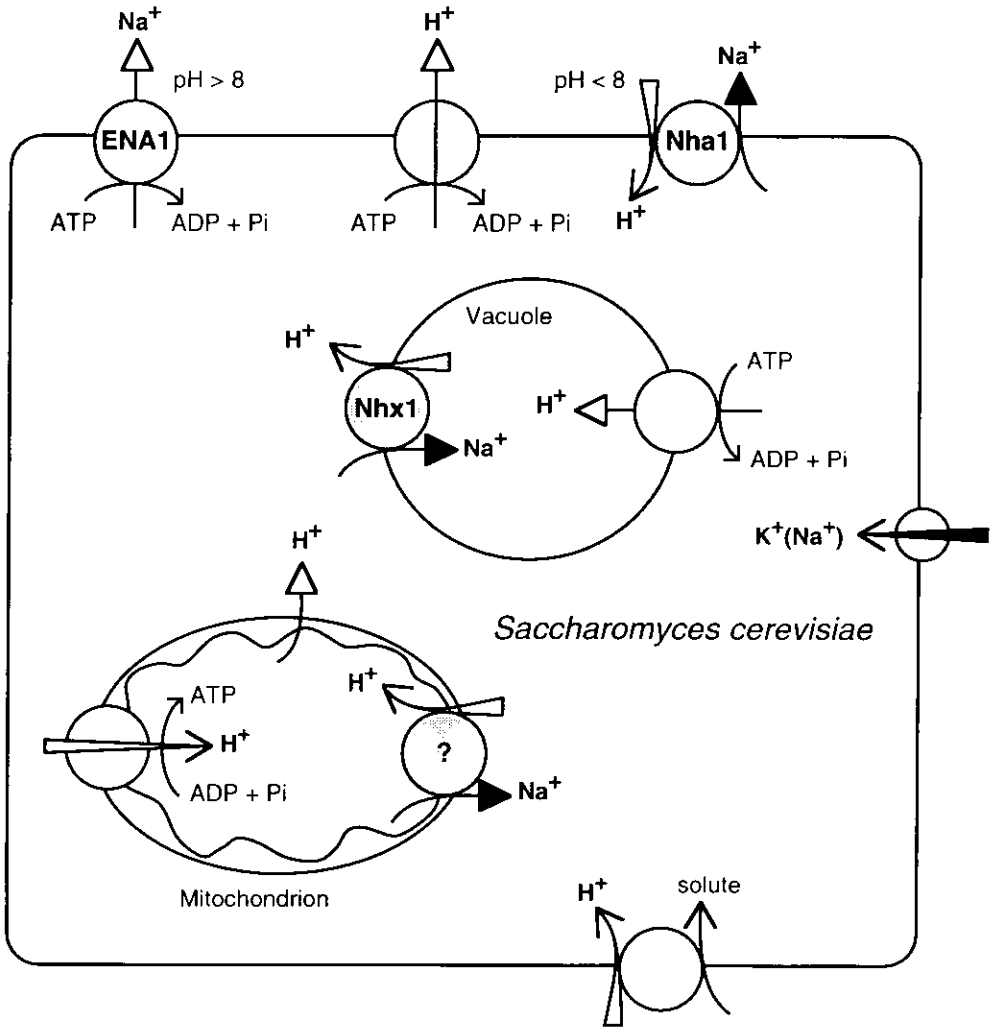

FIG. 1-7. Patterns of H^+ and Na^+ cycles in eukaryotic microorganisms, such as *Saccharomyces cerevisiae*. Na^+/H^+ antiporters are indicated by shading. Plant cells exhibit essentially similar patterns of H^+ and Na^+ cycles. Status of ion transport in chloroplasts is unclear.

1.4.7. Animals

In the cytoplasmic membrane of animal cells, a high concentration gradient of Na^+ is maintained by the Na^+/K^+-ATPase (Fig. 1-8). By contrast, intracellular pH is maintained at a neutral range (~7.2) under physiological conditions, which is much higher than that calculated by assuming that extracellular and intracellular distribution of protons follows purely its electrochemical gradient (~6.2). Such high intracellular pH is maintained by several H^+ extrusion systems, including Na^+/H^+ exchangers, Cl^-/HCO_3^- exchangers, and the H^+-translocating ATPase. The Na^+/H^+ exchangers constitute extremely efficient systems for protecting cells from internal acidification.

The members of the Na^+/H^+ exchanger family (NHE isoforms) catalyze an electroneutral exchange of Na^+ and H^+. The Na^+/H^+ exchange can be inhibited by amiloride and its analogues. Intracellular pH is the most important regulator of the Na^+/H^+ exchange under physiological conditions. Internal protons dramatically stimulate the Na^+/H^+ exchange. The Na^+/H^+ exchangers are also activated in response to a variety of stimuli, including growth factors, hormones, and mechanical signals, such as hyperosmotic stress and cell spreading.

To date, six isoforms (NHE1-6) have been identified in mammalian cells. These isoforms differ in tissue localization, inhibitor sensitivity, kinetics, and regulation mode by various stimuli (Table 1-2C). The NHE1 isoform is expressed in virtually all cells and tissues, whereas the expression pattern of the other NHE isoforms exhibits variation among different tissues. A cDNA clone that encodes the NHE1 isoform was first

isolated using an expression strategy based on the ability of Na^+/H^+ exchangers to protect antiporter-deficient cells from otherwise lethal intracellular acidification (Sardet et al. 1989). NHE2, NHE3, and NHE4 isoforms were then identified by low-stringency screening of cDNA libraries with the NHE1 cDNA as a probe (Tse et al. 1992; Tse et al. 1993; Wang et al. 1993; Orlowski et al. 1992). In addition, non-epithelial isoforms, such as NHE5 (Klanke et al. 1995; Baird et al. 1999), and NHE6 (Numata et al. 1998) were cloned. By contrast to the other Na^+/H^+ exchangers, NHE6 is expressed not in the cytoplasmic membrane but in mitochondria.

Each NHE isoform is composed of two domains, namely, the amino-terminal catalytic domain, which is predicted to span the membrane twelve times, and the carboxy-terminal hydrophilic domain that is responsible for hormonal regulation of the exchanger (Fig. 1-2B). It has been shown that the hydrophilic domain is located in the cytoplasm, at least for the NHE1 isoform (Shrode et al. 1998). Sequence comparison between NHE isoforms revealed that the transmembrane domain exhibits strong homology (47-74% identity), whereas the carboxy-terminal hydrophilic domain is poorly conserved, but clearly exhibits similarity (28-49% identity). Detailed analysis of the sequence homology reveals the presence of two subfamilies of isoforms: NHE2 and NHE4, and NHE3 and NHE5. The central part of the transmembrane domain (putative transmembrane segments M5a and M5b) is nearly identical in all the NHE isoforms and seems to constitute the catalytic core of the Na^+/H^+ exchangers. By contrast, the first putative transmembrane segment is not conserved even in the same isoforms cloned from various

organisms. This segment has the features of a signal peptide (von Heijne 1990).

Amiloride and its analogues are potent inhibitors of the Na^+/H^+ exchange. It has been proposed that these drugs compete with external sodium at the Na^+-translocating site of the Na^+/H^+ exchangers (Grinstain and Smith 1987). The region between putative fourth and ninth transmembrane segments contain several residues that are crucial for the interaction (Counillon et al. 1993; Counillon et al. 1997; Orlowski and Kandasamy 1996; Wang et al. 1995).

The activities of NHE1, NHE2, and NHE3 isoforms are extremely sensitive to intracellular pH (pH_i). It has been demonstrated that the pH_i dependence of NHE1 exhibits a Hill coefficient of 1.5-2, suggesting that an additional proton binding site can serve as a pH sensor that modulates the affinity of the transport site for intracellular protons (Aronson et al. 1982). The pH_i sensitivity is greatly affected by the presence of the carboxy-terminal domain (Wakabayashi et al. 1992). The activation of NHE1 by various stimuli results from an increase in affinity of the exchanger for intracellular protons. It has been proposed that the carboxy-terminal domain interacts with the pH sensor site to modulate the pH threshold value of NHE1 (Wakabayashi et al. 1992). By contrast, changes in the activity of the NHE3 isoform are caused by modifications of the V_{max} value of the exchanger.

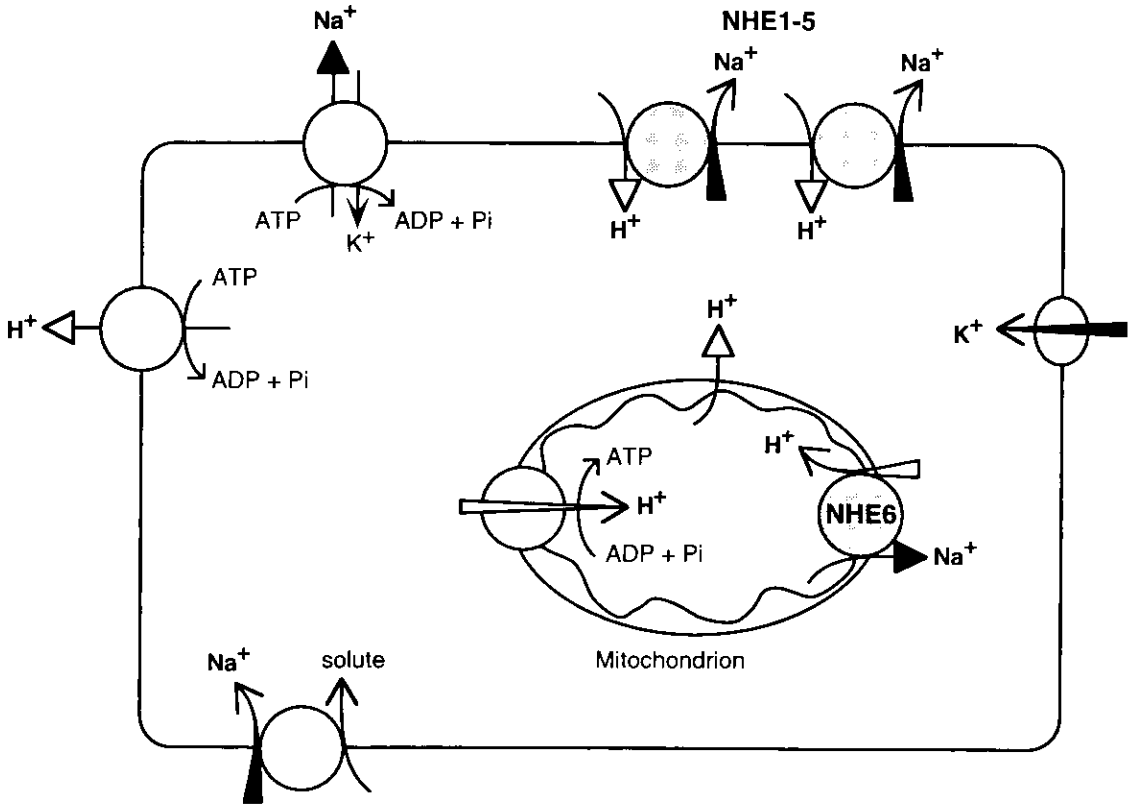

FIG. 1-8. Patterns of H^+ and Na^+ cycles in vertebrate cells. NHE isoforms of the Na^+/H^+ exchanger are indicated by shading. The NHE1 isoform is expressed in virtually all cells and tissues, whereas the expression pattern of the other NHE isoforms depends on cell type and tissue. By contrast to the localization of the NHE1-5 isoforms in the cytoplasmic membrane, NHE6 is expressed in the inner membrane of mitochondria.

1.5. Aim of this study

Salinity is the major environmental factor that limits growth and productivity of plants. I 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 found in plant chloroplasts. (ii) The structure and the lipid compositions of cyanobacterial membranes resemble those of chloroplasts of higher plants and algae (Wada and Murata 1998). (iii) Cyanobacterial cells exhibit more obvious responses to salt stress than plant cells do, 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* and *Synechococcus* sp. PCC 7942, are naturally transformable and can easily be modified by transformation and gene targeting (Golden et al. 1987). (v) The entire nucleotide sequence of the *Synechocystis* genome has been determined (Kaneko et al. 1996). 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 (Reed et al. 1985). Therefore, they must have transport systems that discriminate between K^+ and Na^+ ions. When cyanobacterial cells are grown under high-salt conditions, the pH gradient-dependent (ΔpH-dependent) transport of Na^+ ions across the cytoplasmic membrane is enhanced (Blumwald et al. 1984; Nitschmann and Packer 1992). Respiratory and cytochrome *c* oxidase activities are also enhanced under high-salt conditions (Fry et al. 1986; Jeanjean et al. 1993; Molitor et al. 1986). 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 (Apte et al. 1989; Bhagwat and Apte 1989) and of salt-regulated genes (Apte and Haselkorn 1990; Vinnemeier and Hagemann 1999). However, Na^+/H^+ antiporters and other transporters involved in the efflux of Na^+ ions have not yet been identified in cyanobacteria.

The present study aims to characterize Na^+/H^+ antiporters of *Synechocystis*, a moderately halotolerant fresh-water cyanobacterium (Joset et al. 1996). *Synechocystis* has two genes (*nhaS1* and *nhaS2*) for homologues of NhaP and Na^+/H^+ antiporters of eukaryotes, and three genes (*nhaS3*, *nhaS4*, and *nhaS5*) for proteins with sequence similarity to NapA. The following are scopes included in this research. (i) Functional expression in *Escherichia coli* of the *Synechocystis* genes for putative Na^+/H^+ antiporters. (ii) Evaluation of effects of targeted

inactivation of these genes on phenotypes of *Synechocystis* cells. (iii) Sequence analyses and phylogenetic characterization of Na^+/H^+ antiporter homologues in cyanobacteria and other organisms.

Chapter 2

Functional characterization of Na^+/H^+ antiporters of *Synechocystis* by expression in *Escherichia coli*

2.1. Summary

Products of the *Synechocystis* genes for putative Na^+/H^+ antiporters (designated *nhaS1*, *nhaS2*, *nhaS3*, *nhaS4*, and *nhaS5*) were analyzed by expression in *E. coli*. The expressions of *nhaS1*, *nhaS3*, and *nhaS4* were successfully induced under control of an Na^+-dependent promoter in *E. coli* TO114, a strain that is deficient in Na^+/H^+-antiport activity. Inverted membrane vesicles prepared from *nhaS1*/TO114 and *nhaS3*/TO114 cells exhibited Na^+/H^+ and Li^+/H^+ antiport activities. Kinetic analysis of these activities revealed that the *nhaS1* gene encoded 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, whereas the *nhaS3* gene encoded 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, and to depletion of K^+ ions. To the best of my knowledge, this is the first functional characterization of Na^+/H^+ antiporters from a cyanobacterium. Inverted membrane vesicles prepared from *nhaS4*/TO114 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 *nhaS4*/TO114 cells were tolerant to depletion of K^+ ions. This result suggested that NhaS4 might be a membrane protein that transports K^+ and/or Na^+ ions.

2.2. Introduction

E. coli has at least three genes for Na^+/H^+ antiporters, namely, *nhaA* (Goldberg et al. 1987; Karpel et al. 1988), *nhaB* (Pinner et al. 1992), and *chaA* (Ivey et al. 1991; Ohyama et al. 1994). Mutants deficient in all three genes have no residual activity of Na^+/H^+ antiport under physiological conditions (Ohyama et al. 1994) and are hypersensitive to Na^+ and Li^+ ions (Ivey et al. 1993; Ohyama et al. 1994). Such mutants are suitable to clone genes that encode Na^+/H^+ antiporters by functional complementation. The majority of bacterial genes for Na^+/H^+ antiporters have been identified with this approach and Δ*nhaA*, Δ*nhaA*Δ*nhaB*, or Δ*nhaA*Δ*nhaB*Δ*chaA* strains of *E. coli*. These include members of all the known families of prokaryotic Na^+/H^+ antiporters: *nhaA* (Pinner et al. 1992; Kuroda et al. 1994; Nakamura et al. 1994; Inoue et al. 1999), *napA* (Strausak et al. 1993), *nhaP* (Utsugi et al. 1998), *nhaC* (Ivey et al. 1991), *nhaD* (Nozaki et al. 1998), *nhaB* (Pinner et al. 1992; Nozaki et al. 1996; Nakamura et al. 1996), *mrp* (Hiramatsu et al. 1998), and *tetA*(L) (Cheng et al. 1994). These genes were expressed from their own promoter in *E. coli*. However, the *nhaS* genes are expressed at very low levels in wild-type *Synechocystis* (our unpublished data). 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. On the other hand, overproduction of proteins that contain several transmembrane segments might have detrimental effects on host cells. NhaA is a membrane-bound protein that is present at a low level (less than 0.2% of total membrane proteins) in

wild-type cells of *E. coli* (Taglicht et al. 1991). Transcription of the *nhaA* gene is controlled by intracellular Na^+ and Li^+ ions (Karpel et al. 1991), through the interaction between the *trans*-acting positive regulator, NhaR, and the promoter of the *nhaA* gene, *nhaAp* (Rahav-Manor et al. 1992; Carmel et al. 1997). Once this protein was overexpressed under the control of the strongly inducible *tac* promoter, growth of the cells was arrested (Taglicht et al. 1991). Thus, I chose to use the *nhaAp* promoter to obtain expression of *nhaS* genes in *E. coli* at appropriate levels. Expression in *E. coli* of the *nhaS1* and *nhaS3* genes under control of the *nhaAp* promoter resulted in production of functional Na^+/H^+ antiporters.

2.3. Materials and Methods

Nomenclature of genes

I refer to the putative genes for Na^+/H^+ antiporters in *Synechocystis* as *nhaS1* (slr1727; this nomenclature refers to the designations of open reading frames by Kaneko et al. 1996), *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; Ohyama et al. 1994) was generously provided by Dr. H. Kobayashi (Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan). It was used as the host for complementation tests with cyanobacterial genes. Cells were grown in modified Luria-Bertani medium (Sambrook et al. 1989) that consisted of 1.0% tryptone (Difco; Detroit, MI), 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 $\mu g\ ml^{-1}$.

Construction of plasmids

Plasmid pGM42 (Goldberg et al. 1987) was kindly provided by Dr. E. Padan (Institute of Life Sciences, 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*, containing the *nhaA* gene under control of the Na$^+$-

inducible promoter *nhaAp* and the *nhaR* gene for the positive *trans*-acting regulator of the *nhaA* gene (Rahav-Manor et al. 1992; Carmel et al. 1997). Plasmids for expression of each of the five *nhaS* genes in *E. coli* were constructed from pGM42 as shown in Figure 2-1. (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 the polymerase chain reaction (PCR) with pGM42A as template, the forward primer P1 (CGTCCATCAGTTTGAgAGctCGGTTTACCG, which corresponded to nucleotides +1153 to +1182, counted from the site of initiation of translation of the *nhaA* gene that was taken as +1) and the reverse primer P2 (GATGCAGATGTTgCAtgcTTTATTTCTCTTTCAGG, which was complementary to nucleotides +13 to -19). Underlined sequences represent restriction sites for *Sph*I (GCATGC) and *Sac*I (GAGCTC). Lowercase letters indicate nucleotides that differ from the ones in the template. The *nhaA* gene was also amplified with pGM42A as template, the forward primer A1 (CCTGAAAGAGAAATAAAgcaTGcAACATCTGCATC, which corresponded to nucleotides -19 to +13, counted from the site of initiation of translation of the *nhaA* gene that was taken as +1) and the reverse primer A2 (CGGTAAACCGagCTcTCAAACTGATGGACG, which was 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 p*RnhaA*, 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 CAgCaTGcATACAGCGGTCAACGA (corresponding to nucleotides -4 to +20, counted from the site of initiation of translation of the *nhaS1* gene, which was taken as +1) and reverse primer aagagctcCTAGGATGGTTCGGCCACAT (complementary to nucleotides +1584 to +1565) for the *nhaS1* gene; forward primer CTgCATGcCTTAAGCTCCCTGTGC (corresponding to nucleotides -4 to +19, counted from the site of initiation of translation of the *nhaS2* gene, which was taken as +1) and reverse primer TTgAGcTCGTCAGTCATCCTGCAGG (complementary to nucleotides +1632 to +1608) for the *nhaS2* gene; forward primer ttgcATGcTTATGAACCCATTGCTCCCTC (corresponding to nucleotides +1 to +25, counted from the site of initiation of translation of the *nhaS3* gene, which was taken as +1) and reverse primer ttgagctcCTAATCTGGGGTGGGAACTG (complementary to nucleotides +1386 to +1367) for the *nhaS3* gene; forward primer AAgcATGcACACCAATACTTTACTGCTAATT (corresponding to nucleotides -4 to +27, counted from the site of initiation of translation of the *nhaS4* gene, which was taken as +1) and reverse primer ttgaGcTcTTAATGGGCTGGGGCAGGAT (complementary to nucleotides +1237 to +1214) for the *nhaS4* gene; and forward primer ttgcATGcATGGCCTATTCGCACCAATTC (corresponding to nucleotides +1 to +25, counted from the site of initiation of translation of the *nhaS5* gene, which was taken as +1) and reverse primer

aagagctcCTAGGCGTAGGGATCGCCA (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* (Fig. 2-2) 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* (Fig. 2-2). I failed to generate p*nhaS3*. All the amplified fragments and the ligated junctions were verified by determination of nucleotide sequences.

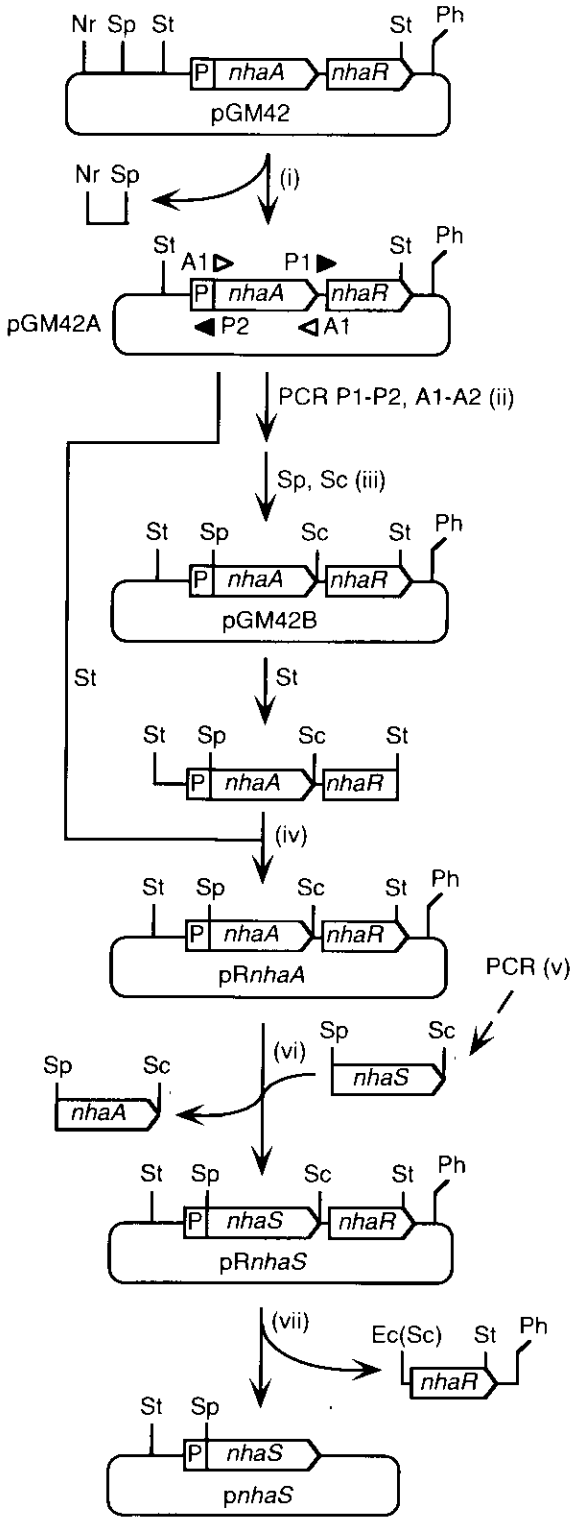

FIG. 2-1. Strategy for the construction of plasmids used for the expression of *nhaS* genes in *E. coli* TO114. Solid lines represent the pBR322 backbone and the flanking regions of the *nhaA* and *nhaR* genes of *E. coli*. **P**, The Na^+-inducible promoter of the *nhaA* gene (*nhaAp*). Restriction sites are abbreviated as follows: Nr, *Nru*I; Sp, *Sph*I; St, *Stu*I; Sc, *Sac*I; Ph, *Psh*AI; and Ec, *Ecl*136II.

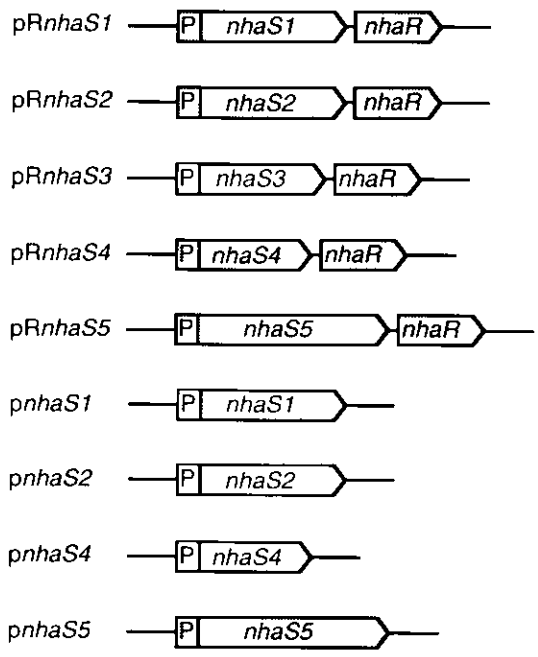

FIG. 2-2. Features of the various plasmids. Plasmids *pnhaS1*, *pnhaS2*, *pRnhaS3*, and *pnhaS4* were successfully introduced into TO114 cells (indicated by the absence of shading).

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% (w/v) 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, and 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 (v/v), buffered with an equal volume of 50 mM sodium acetate, pH 5.2]. This 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 *Hinc*II, 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 analysis

Fifteen μg of total RNA were fractionated by electrophoresis on a 1.2% agarose gel that contained 6.3% formaldehyde in 3-(*N*-morpholino)propanesulfonic acid buffer, pH 7.0 (Sambrook et al. 1989), and bands of RNA were transferred to a nylon membrane (NEN™ Life Science Products, Boston, MA). 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 (Sambrook et al. 1989) and 100 μg ml^{-1} denatured salmon sperm DNA. 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 washing for 1 h 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, NY).

Preparation of inverted membrane vesicles (IMVs)

IMVs were prepared as described previously (Rosen 1986). Cells (1 liter) were grown in LBK medium to the middle of the exponential phase of growth (OD_{600} = 1.5) at 37°C and harvested by centrifugation at 5,000 × g for 10 min at 4°C. The cells were suspended in a minimum volume of a buffer that contained 10 mM Tris-HCl, pH 7.4, 140 mM choline chloride, 250 mM sucrose, and 0.5 mM dithiothreitol (TSCD buffer). The suspension was transferred to a weighted centrifuge tube and pelleted. The tube was wiped and weighted. The cells were suspended in 10 volumes of the TSCD buffer per gram of pellet

weight and lysed by passing twice through a French pressure cell (SLM Instruments, Inc., Urbana, IL) at 4,000 psi. $MgCl_2$ and DNase I were added to 5 mM and 10 $\mu g \cdot ml^{-1}$, respectively, and the mixture was incubated for 10 min at room temperature. Unbroken cells were removed by centrifugation once at 6,000 × g for 15 min at 4°C. The membrane vesicles were pelleted by centrifugation at 100,000 × g for 45 min at 4°C and washed once with the TSCD buffer. The pellet was suspended in 1 ml of the TSCD buffer and immediately used for measurement of Na^+/H^+-antiport activity or stored in small aliquots at -80°C.

Measurement of Na^+/H^+-antiport activity of IMVs

The Na^+/H^+-antiport activity of IMVs was estimated from the extent of the collapse of a preformed proton gradient (ΔpH) with a fluorescent amine, acridine orange, as the pH indicator (Fig. 2-3). Distribution of the amine across the membrane is related to proton distribution according to the following expressions. One can assume that the uncharged form of a weak base is freely permeable across the membrane, whereas the charged form does not cross the membrane. When a ΔpH, acid interior, is formed, the weak base that had penetrated the membrane, in the uncharged form, becomes protonated and accumulates inside the vesicles. At equilibrium the distribution will be proportional to the ΔpH (Schuldiner et al. 1972). Acridine orange basically distributes across the membrane as a weak base in accordance with the ΔpH, although it also undergoes some binding interaction with membrane sites in response to the ΔpH (Lee and Forte 1978). Fluorescence of

acridine orange is quenched during the accumulation. Therefore, changes in ΔpH are estimated from the changes in fluorescence intensity. 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). When indicated, 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-sec 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 sec^{-1} (mg protein) $^{-1}$]. IMVs from pBR322$^+$ cells (negative control) had low Na^+/H^+-antiport activity, which was considered 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.

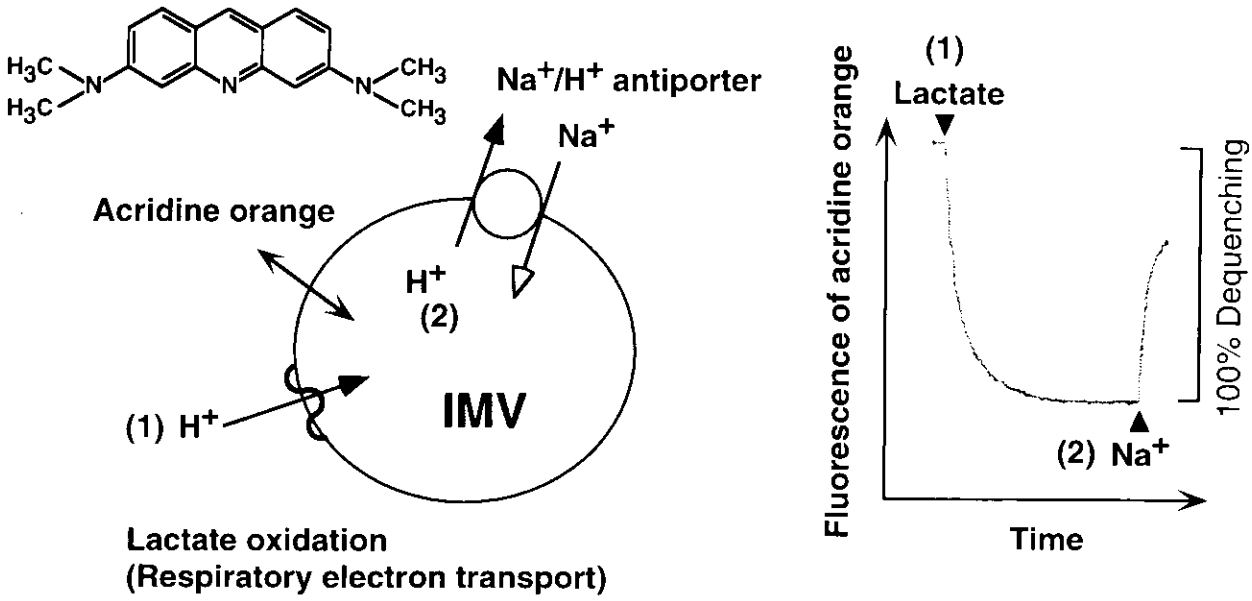

FIG. 2-3. Measurement of Na^+/H^+-antiport activity of IMVs. Fluorescence of an amine, acridine orange, was monitored to estimate changes in the transmembrane pH gradient (ΔpH). (1) Addition of tris-*D*-lactate energizes IMVs to form a ΔpH. Acridine orange that had penetrated the membrane in the uncharged form is protonated and accumulates inside the vesicles, resulting in quenching of the fluorescence. (2) Subsequent addition of NaCl or LiCl disrupts the ΔpH. The intravesiclar amine is uncharged and released from the membranes, resulting in restoration of the fluorescence. The initial rate of this dequenching, as measured during the 2-sec interval that followed the addition of NaCl or LiCl at various concentrations was taken as the Na^+/H^+-antiport activity.

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. Colony formation was examined after incubation for 24 h at 37°C.

Additional procedure

The concentration of proteins in IMVs was determined as described elsewhere (Bradford 1976).

2.4. Results

Expression of *nhaS* genes in *E. coli* TO114

I transformed *E. coli* TO114 cells with plasmids that contained individual *nhaS* genes (Fig. 2-2). The resultant transformed cells were grown on solid LBK medium supplemented with 50 μg ml^{-1} ampicillin. Colonies were obtained 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. I also obtained TO114 cells that harbored pBR322, pR*nhaA*, or p*nhaA*.

I attempted to analyze levels of products of *nhaS* genes in membrane fractions of transformed *E. coli* cells by SDS-polyacrylamide gel electrophoresis and silver staining. However, I failed to detect bands that corresponded unequivocally to the *Synechocystis* proteins either before or after induction by NaCl. Thus, to evaluate whether or not *nhaS* genes were at least transcribed in *E. coli* under control of the *nhaAp* promoter, I performed Northern blotting analysis 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. 2-4). Transcripts of the *nhaS1*, *nhaS3*, and *nhaS4* genes accumulated in *nhaS1*$^+$, *nhaS3*$^+$, and *nhaS4*$^+$ cells, respectively. By 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.

I 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 levels of transcripts of *nhaS1*, *nhaS2* and *nhaS4* genes in *nhaS1*$^+$, *nhaS2*$^+$, and *nhaS4*$^+$ cells, respectively, did not change significantly. This was probably due to the presence of the Na^+-dependent regulatory gene *nhaR* in the construct for expression of the *nhaS3* gene (Fig. 2-2), which might have promoted transcription of the *nhaS3* gene under high-salt conditions (Rahav-Manor et al. 1992).

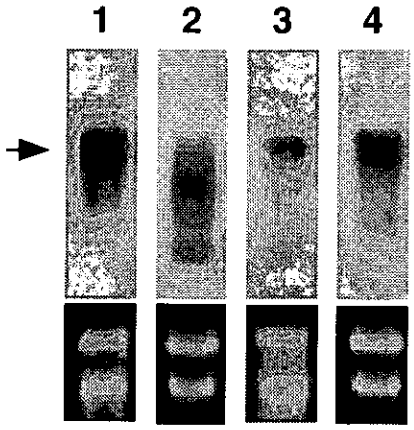

FIG. 2-4. Northern blotting analysis of the expression of *nhaS* genes in transformed *E. coli* TO114 cells. Total RNA was extracted from cells that had been grown in LBK medium. Results are shown for *nhaS1* transcripts in *nhaS1*$^+$ cells (lane 1), *nhaS2* transcripts in *nhaS2*$^+$ cells (lane 2), *nhaS3* transcripts in *nhaS3*$^+$ cells (lane 3), and *nhaS4* transcripts in *nhaS4*$^+$ cells (lane 4). An arrow indicates the positions of the expected transcripts. 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.

Na^+/H^+-antiport activity of IMVs

I measured 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 following 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), I used pR*nhaA*/TO114 cells in further experiments as the positive control, referring to them as *nhaA*$^+$ cells. I used pBR322/TO114 (pBR322$^+$) cells as the negative control.

Figure 2-5 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. 2-5A) or in the presence of 140 mM KCl (Fig. 2-5B; K^+-rich conditions). Under K^+-free conditions, IMVs from pBR322$^+$ cells had low Na^+/H^+-antiport activity (Fig. 2-5A). Such activity might have been due to a non-specific monovalent cation/H^+ antiport system (Pinner et al. 1993) that did not transport Na^+ ions under K^+-rich conditions (Fig. 2-5B). IMVs from *nhaS1*$^+$ and *nhaS3*$^+$ cells had significant Na^+/H^+-antiport activity under K^+-rich conditions, as did the IMVs from *nhaA*$^+$ cells (Fig. 2-5B). 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.

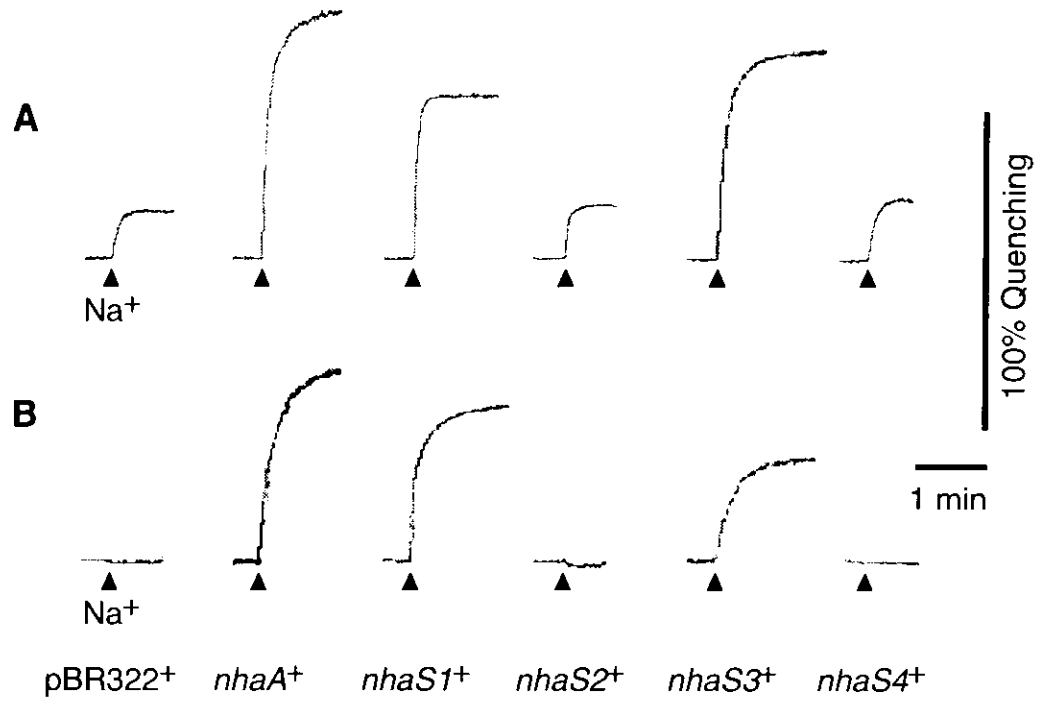

FIG. 2-5. Activities of the Na^+/H^+-antiport system in IMVs prepared from transformed cells. IMVs were prepared from cells that had been grown in LBK medium. Activity was assayed in a solution that consisted of 5 mM $MgCl_2$, 1 μM acridine orange, and 10 mM Tris titrated with Mes (pH 8.5) and supplemented with 140 mM choline chloride (A) or 140 mM KCl (B). Arrowheads indicate the time at which 5 mM NaCl was added to the assay solution.

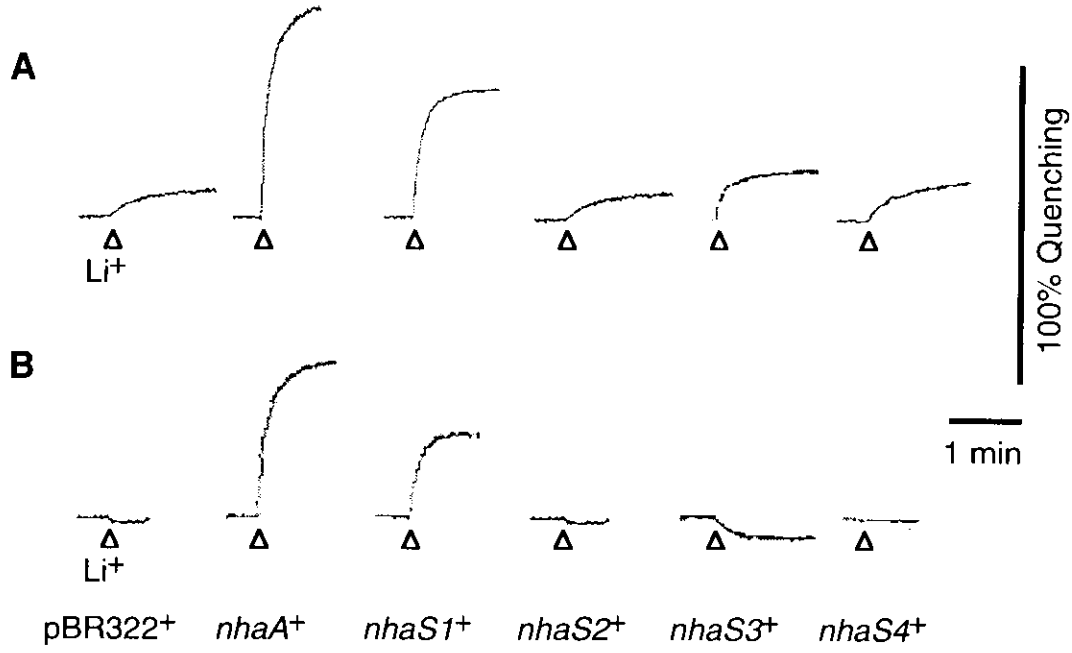

FIG. 2-6. Activities of the Li^+/H^+-antiport system in IMVs prepared from transformed cells. Experiments were carried out as described in the Figure 2-5 legend except that 5 mM LiCl was added to the assay solution instead of 5 mM NaCl.

Figure 2-6 shows profiles of Li^+/H^+-antiport activity, as determined upon addition of 5 mM LiCl under K^+-free conditions (Fig. 2-6A) and under K^+-rich conditions (Fig. 2-6B). The IMVs prepared from *nhaS1*$^+$ cells had high Li^+/H^+-antiport activity under K^+-rich conditions (Fig. 2-6B), 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. 2-6B) but they had considerably higher Li^+/H^+-antiport activity under K^+-free conditions than IMVs prepared from pBR322$^+$ cells (Fig. 2-6A). 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.

For calculation of kinetic parameters, the activity of Na^+/H^+- and Li^+/H^+-antiport of IMVs prepared from *nhaA*$^+$, *nhaS1*$^+$, and *nhaS3*$^+$ cells were assayed under K^+-free conditions. The initial rate of restoration of fluorescence that followed the addition of NaCl or LiCl at various concentrations was taken as the rate of the antiport activity (Fig. 2-7). The IMVs from pBR322$^+$ cells had low activity of Na^+/H^+- and Li^+/H^+-antiport under these conditions (Fig. 2-7), which was taken as the background activity. The antiport activity of IMVs prepared from pBR322$^+$ cells was subtracted from the activity of IMVs prepared from *nhaA*$^+$, *nhaS1*$^+$, and *nhaS3*$^+$ cells. Figures 2-8 and 2-9 show double reciprocal plots of the rate of the antiport activity after the subtraction. The apparent K_m values were

calculated by the intersection of the fitted line with abscissa.

Table 2-1 shows the kinetic parameters of the Na^+/H^+-antiport activity of IMVs prepared from *nhaA*$^+$, *nhaS1*$^+$, and *nhaS3*$^+$ cells. 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 value for Na^+ ions of the activity of IMVs from *nhaA*$^+$ cells was of the same order of magnitude as the value reported previously for purified NhaA (i.e., 0.1 mM at pH 8.6; Taglicht et al. 1991).

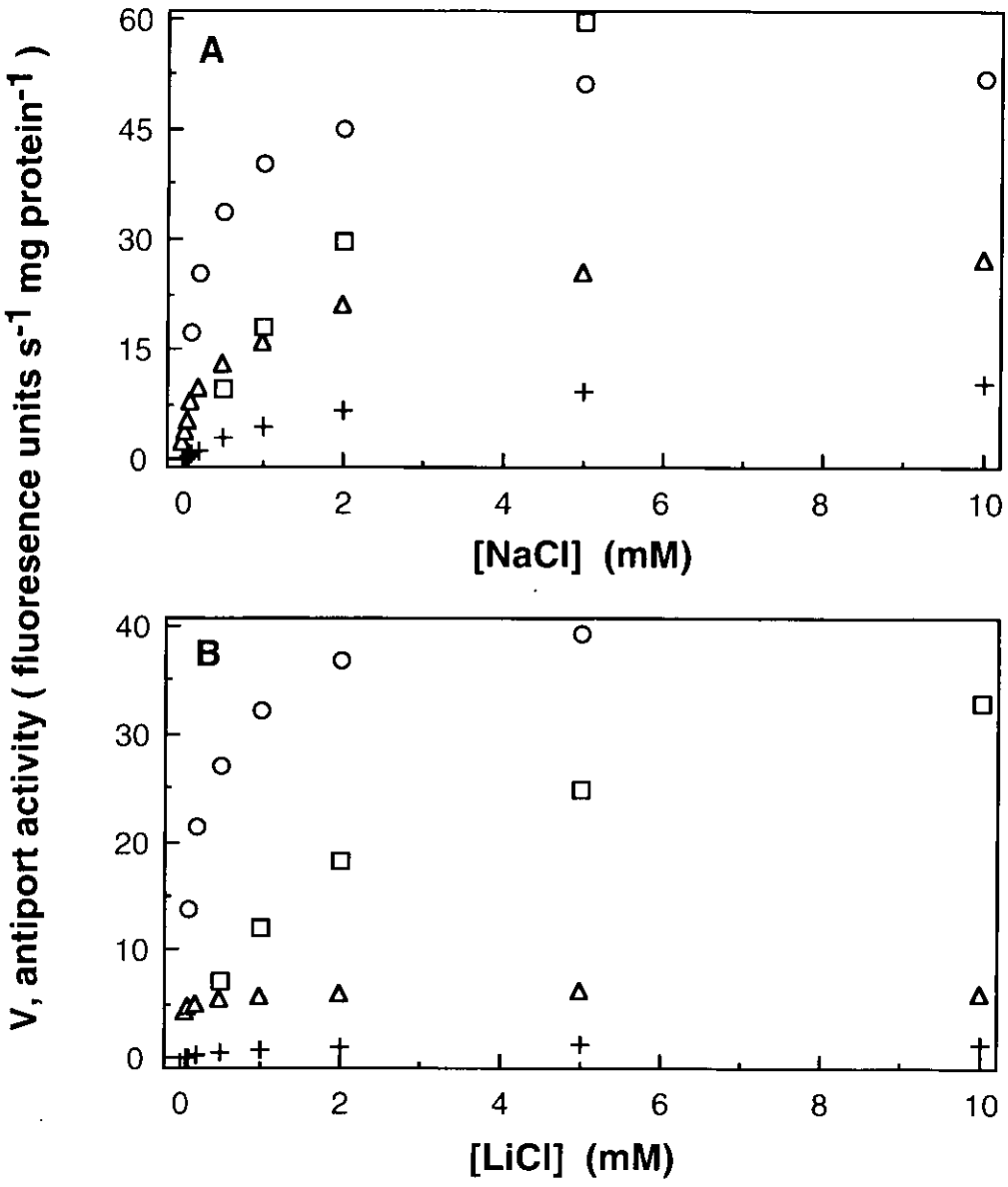

FIG. 2-7. Kinetics under K^+-free conditions of the activity of Na^+/H^+-antiport (A) and Li^+/H^+-antiport (B) of IMVs prepared from *nhaA*$^+$ cells (O), *nhaS1*$^+$ cells (□), *nhaS3*$^+$ cells (Δ), and pBR322$^+$ cells (+). The activity of IMVs from pBR322$^+$ cells was taken as backgound activity, as described in Materials and Methods.

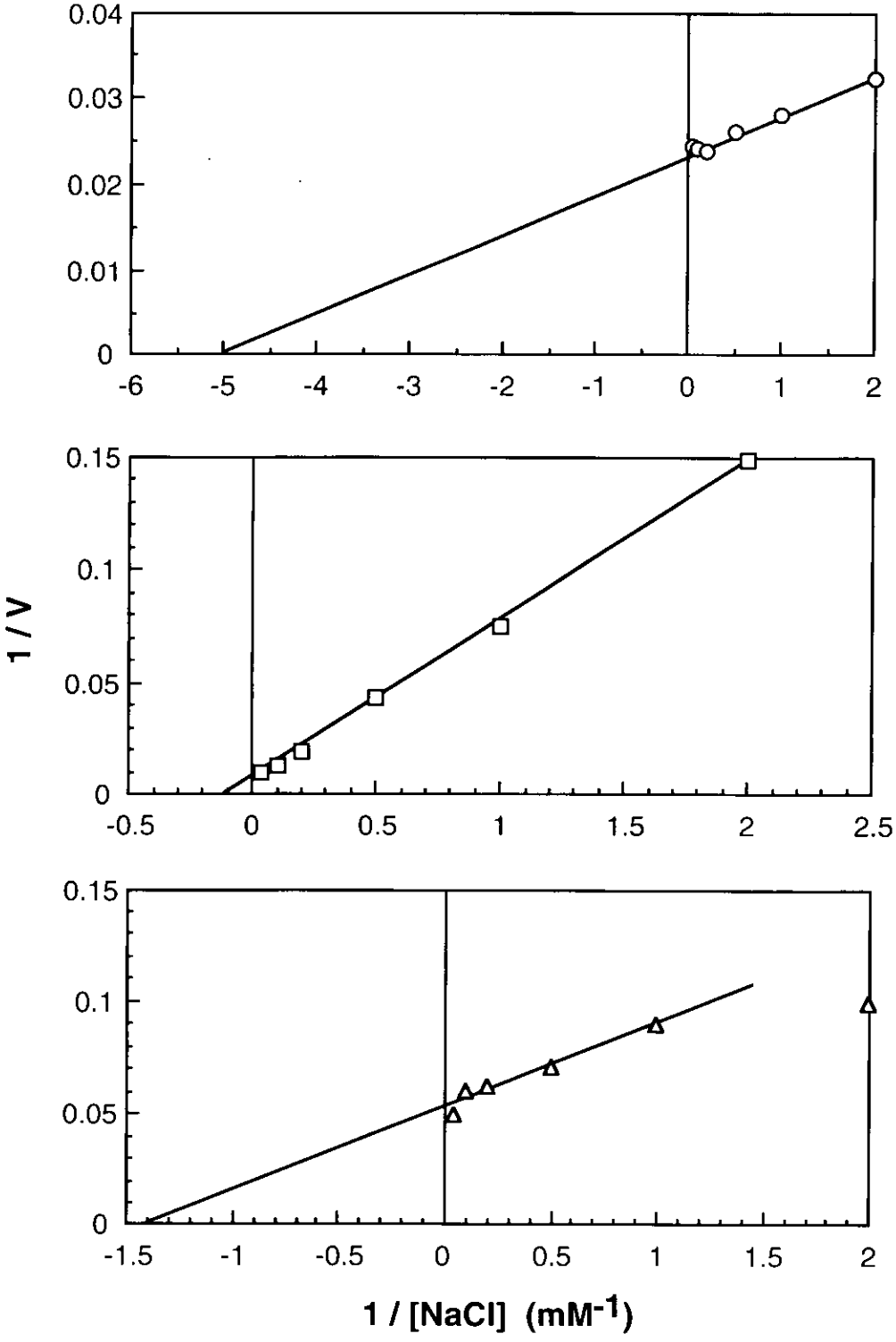

FIG. 2-8. Double reciprocal plots of the Na^+/H^+-antiport activity of IMVs prepared from *nhaA*$^+$ cells (○), *nhaS1*$^+$ cells (□), and *nhaS3*$^+$ cells (Δ). The apparent K_m values were calculated by the intersection of the fitted line with abscissa.

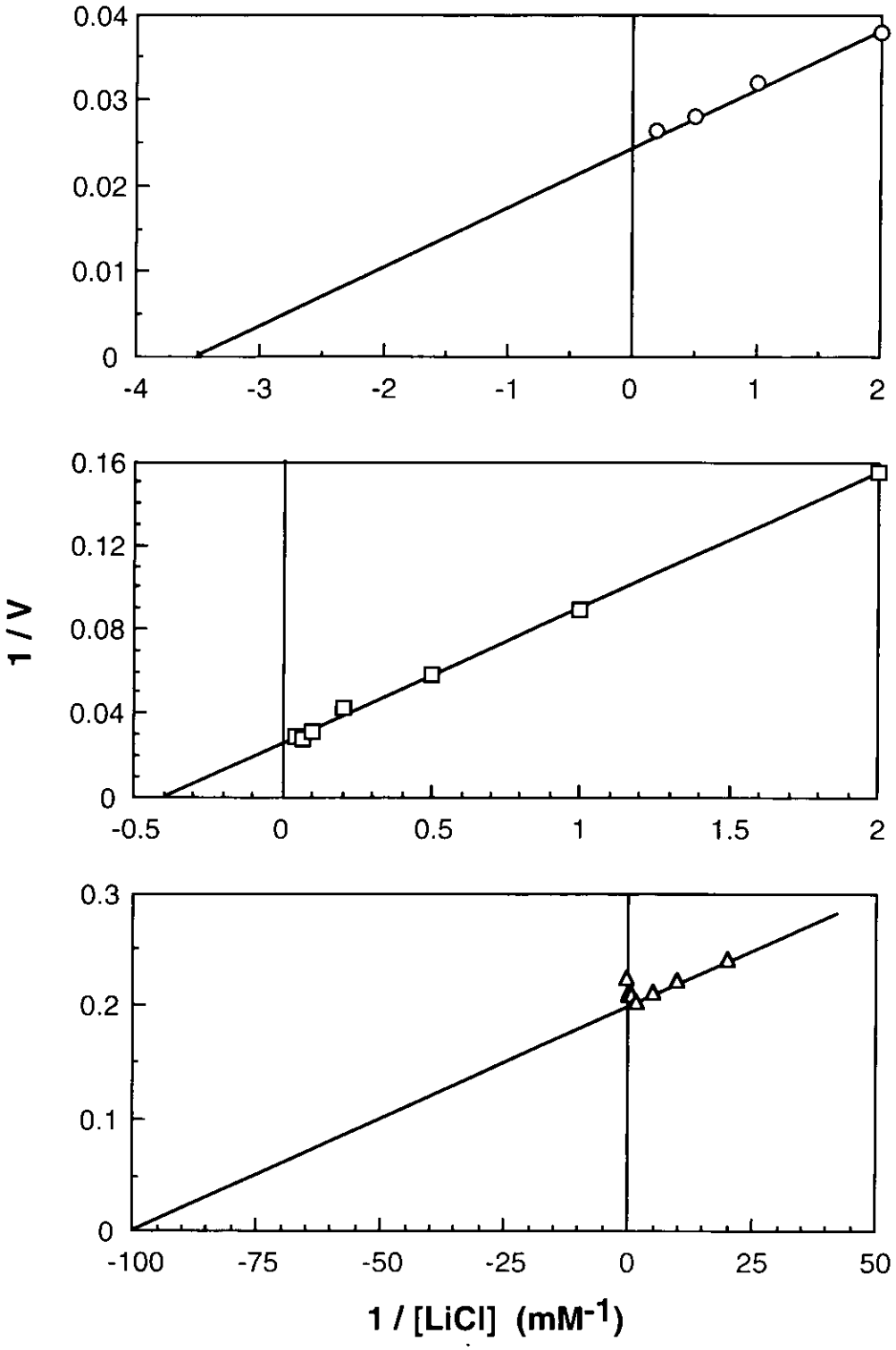

FIG. 2-9. Double reciprocal plots of the Li^+/H^+-antiport activity of IMVs prepared from *nhaA*$^+$ cells (O), *nhaS1*$^+$ cells (□), and *nhaS3*$^+$ cells (Δ). The apparent K_m values were calculated by the intersection of the fitted line with abscissa.

TABLE 2-1. Kinetic parameters under K^+-free conditions of the Na^+/H^+-antiport activity of IMVs prepared from transformed cells. The activity of IMVs from pBR322$^+$ cells [K_m = 1.5 mM and V_{max} = 12 units s^{-1} (mg protein)$^{-1}$ for Na^+ ions; K_m = 0.7 mM and V_{max} = 1.5 units s^{-1} (mg protein)$^{-1}$ for Li^+ ions] was taken as background activity. Two independent experiments yielded essentially the same results.

Type of cell	Na^+		Li^+	
	V_{max}[a]	K_m (mM)	V_{max}[a]	K_m (mM)
nhaA$^+$	42	0.2	42	0.30
nhaS1$^+$	120	7.7	40	2.50
nhaS3$^+$	22	0.7	5	0.01

[a]Presented in fluorescence units s^{-1} (mg protein)$^{-1}$.

Sensitivity of cell growth to high concentrations of Na^+ and Li^+ ions

Table 2-2 describes the maximum respective concentrations of Na^+ and Li^+ ions that allowed growth of transformed cells on solid LBn medium prepared with 5, 25, 105, and 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. By contrast, *nhaS1*$^+$ and *nhaS3*$^+$ cells were able to grow at 570 mM Na^+ ions and at 10 mM Li^+ ions, and at 420 mM Na^+ ions and at 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 of Na^+/H^+-antiport activity under K^+-rich conditions of the IMVs prepared from such cells. This sensitivity to Na^+ and Li^+ ions of pBR322$^+$, *nhaS2*$^+$, and *nhaS4*$^+$ cells decreased as the concentration of K^+ ions in the medium was increased from 5 to 105 mM. As described on the following page, this K^+ ion dependence could reflect the absence of Na^+/H^+-antiport activity in the membranes.

TABLE 2-2. Effects of K^+ ions on the maximum concentrations of Na^+ and Li^+ ions that allowed growth of transformed cells on solid LBn medium. Three independent experiments yielded essentially similar results.

Type of cell	K^+ in the medium (mM)	Maximum concentration for growth (mM)	
		Na^+	Li^+
pBR322$^+$	5	<20	0
	25	60	2
	105	120	3
	305	120	3
nhaA$^+$	5	1070	400
	25	1070	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

Sensitivity of cell growth to depletion of K^+ ions

The pBR322$^+$ cells did not grow in the presence of 5 mM K^+ ions, the background level, even in the absence of additional Na^+ and Li^+ ions (Table 2-2). This observation was consistent with previous reports on a *ΔnhaAΔnhaB* strain of *E. coli* (Harel-Bronstein et al. 1995; Verkhovskaya et al. 1998) 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^+ to Na^+ ions in the medium was low (Harel-Bronstein et al. 1995). To elucidate the effect of transformation on the K^+ depletion sensitivity, I examined the growth of transformed cells at various concentrations of K^+ ions (Table 2-3). 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 did pBR322$^+$ cells. By contrast, the requirement for K^+ ions of *nhaS2*$^+$ cells did not differ significantly from that of pBR322$^+$ cells. The sensitivity of growth to high concentrations of K^+ ions was not affected by the transformation.

TABLE 2-3. The minimum and maximum concentrations of K^+ ions, that allowed transformed cells to grow on solid LBn medium. Three independent experiments yielded essentially the same results.

Type of cell	Concentration of K^+ (mM)	
	Minimum	Maximum
pBR322$^+$	20	1150
nhaA$^+$	≤5	1150
nhaS1$^+$	≤5	950
nhaS2$^+$	25	1150
nhaS3$^+$	≤5	1150
nhaS4$^+$	6	1150

2.5. Discussion

Functional expression of the *nhaS1*, *nhaS3*, and *nhaS4* genes in *E. coli* under the control of the *nhaAp* promoter

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. By 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 could be 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. Instability of the heterologous transcripts could be related to inefficient translation, due to the presence of codons that are used at low frequencies in *E. coli* (Kane 1995). Inefficient translation can increase susceptibility of transcripts to RNases (Braun et al. 1998). 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 (our unpublished data). It has been suggested that NhaS2 might be required for the uptake of Na^+ ions in *Synechocystis* (Mikkat et al. 2000). The instability of *nhaS2* transcripts might have been a consequence

of the disturbed balance of ions in the transformed *E. coli* cells.

The 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 2-1) 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 value for Na^+ ions is close to that reported for AtNHX1 of *Arabidopsis thaliana* (Apse et al. 1999). The smaller value of the 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 to Li^+ ions of the host cells, while it dramatically increased the tolerance of host cells to Na^+ ions (Table 2-2). 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). This result suggests that Li^+ ions might be a better substrate of NhaS3 than Na^+ ions. The K_m value for Na^+ ions of NhaS3 was similar to the

value obtained for NapA of *Enterococcus hirae* that was expressed in *E. coli* (i.e., 1.0 mM; Strausak et al. 1993). However, the K_m value for Li^+ ions of NhaS3 was much smaller than that reported for NapA (i.e., 0.1 mM; Strausak et al. 1993). K^+ ions in the assay solution significantly inhibited the Li^+/H^+-antiport activity (Fig. 2-6). However, the tolerance of *nhaS3*$^+$ cells to Li^+ ions increased as the concentration of K^+ ions in the medium was increased (Table 2-2), suggesting that K^+ ions in the medium might have 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.

I 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. This observation suggests that the *nhaS4* gene might encode a membrane-bound protein that transports K^+ and/or Na^+ ions.

Chapter 3

Evaluation of effects of targeted mutagenesis of genes for Na^+/H^+ antiporter homologues in *Synechocystis*

3.1. Summary

Single and double mutants of *Synechocystis* in which individual *nhaS* genes were interrupted by insertion of an antibiotic-resistance gene cartridge were generated and effects of the mutations on growth phenotypes were examined. The disruption of the *nhaS1*, *nhaS2*, *nhaS4*, and *nhaS5* genes in all copies of the chromosomal DNA was verified by PCR. I failed to disrupt the *nhaS3* gene: homozygous null mutants were not recovered after the mutagenesis. The single mutants that I obtained did not show any phenotypic changes in terms of the sensitivity to growth inhibition by NaCl. Δ*nhaS1*Δ*nhaS2* cells grew slower than wild-type cells both in BG11 medium (the standard medium), that contained 18 mM Na^+, and in a high-salt medium, prepared by adding NaCl to 0.5 M to BG11 medium. The growth retardation of Δ*nhaS1*Δ*nhaS2* cells appeared to be greater in the presence of 0.5 M NaCl than in its absence. By contrast, Δ*nhaS4*Δ*nhaS5* cells grew as well as wild-type cells regardless of the presence or absence of 0.5 M NaCl. These results suggested that (i) the function of the *nhaS1* and *nhaS2* genes' products might be complementary, (ii) the *nhaS3* gene is essential for viability of *Synechocystis* cells, and (iii) products of the *nhaS4* and *nhaS5* genes may contribute little to high-salt stress tolerance.

3.2. Introduction

Gene knockout is an elegant approach to obtain insights into physiological roles of gene's product. Because of the ease of gene manipulation protocols, several Na^+/H^+ antiporters in bacteria and yeast have been studied with this approach. These include NhaA of *E. coli* (Padan et al. 1989) and *Vibrio parahaemolyticus* (Kuroda et al. 1994), NapA of *Enterococcus hirae* (Waser et al. 1992), NhaC of *Bacillus firmus* OF4 (Ito et al. 1997), NhaB of *E. coli* (Pinner et al. 1993), TetA(L) of *Bacillus subtilis* (Cheng et al. 1994), SOD2 of *Schizosaccharomyces pombe* (Jia et al. 1992) and *Saccharomyces cerevisiae* (NHA1; Prior et al. 1996), and NHX1 of *Saccharomyces cerevisiae* (Nass et al. 1997). Recently targeted mutagenesis has become a routine method of gene inactivation in mammals, and results from this approach are just emerging for NHE isoforms (Cox et al. 1997; Schultheis et al. 1998; Bell et al. 1999). In contrast, this approach has yet to become a choice of plant gene study, as established protocols are still lacking.

Synechocystis is naturally competent and easily modified by targeted mutagenesis (Golden et al. 1987). Targeted inactivation of a gene by insertion of a selectable marker is achieved via a double homologous recombination event on the chromosomes. Using this advantage, I disrupted the *nhaS* genes in *Synechocystis*. Based on growth characteristics of resultant mutants and results obtained from the expression in *E. coli* of the *nhaS* genes (Chapter 2), I discuss possible roles of the Na^+/H^+ antiporter homologues in *Synechocystis*.

3.3. Materials and Methods

Bacterial strain and growth conditions

The cyanobacterial strain *Synechocystis* sp. PCC 6803 was originally provided by Dr. J. G. K. Williams (DuPont de Nemours and Co., Wilmington, DE). Cells were grown at 34 °C in BG11 medium (Stanier et al. 1971) supplemented with 20 mM HEPES; the pH of the medium was adjusted to 7.5 with KOH. Cultures were supplied with illumination from incandescent lamps at 70 $\mu E\ m^{-2}\ s^{-1}$ and aerated with air that contained 1% CO_2. The growth of cells was monitored in terms of optical density at 730 nm.

Construction of plasmids

Plasmids used for targeted disruption of the *nhaS* genes in *Synechocystis* are shown in Figure 3-1. 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 Dr. S. S. Golden (Department of Biology, Texas A&M University, College Station, TX). The *nhaS* genes that had been amplified by PCR, as described in section 2.3, were subcloned into the TA cloning site of plasmid pT7Blue (Novagen, Madison, WI). 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 (Takara Shuzo). 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.

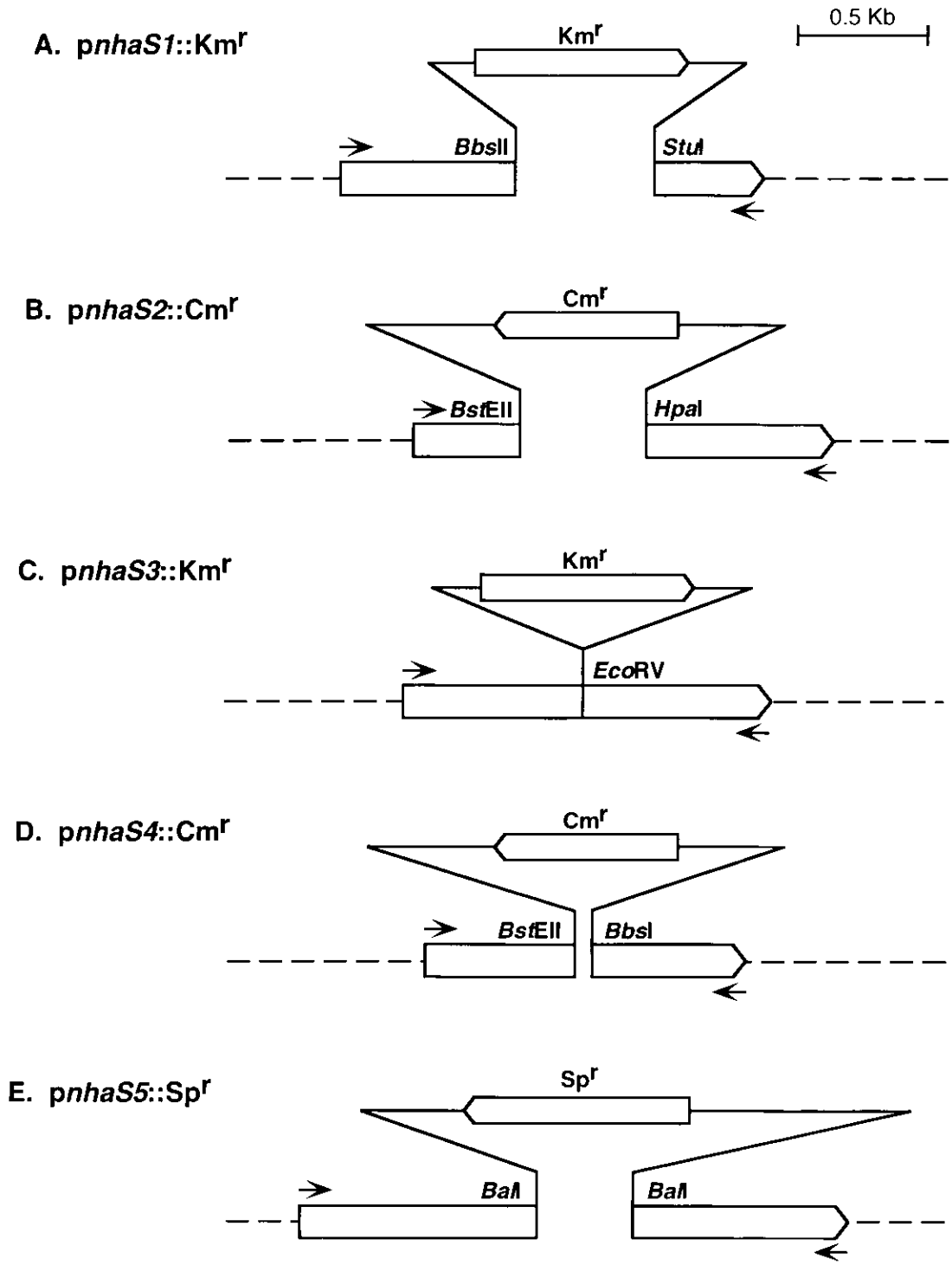

FIG. 3-1. Construction of plasmids used for the targeted mutagenesis of the *nhaS* genes in *Synechocystis*. Arrows indicate the primers used for PCR for evaluation of the state of the *nhaS* genes on the chromosomal DNA. Dotted lines represent the pT7Blue backbone. The gap in each open reading frame indicates the length of the substituted DNA region with the antibiotic resistance gene cartridge.

Transformation of *Synechocystis* and serial passaging

Wild-type cells of *Synechocystis* were transformed with the individual plasmids to generate Δ*nhaS* cells, as described previously (Tasaka et al. 1996). Cultures at OD_{730} of 0.33 were harvested by centrifugation at 2,500 × g for 15 min at room temperature. The cells were washed once with BG11 medium and resuspended in BG11 medium to give the cell density of 1.5 × 10^9 cells per ml. An aliquot of 100 µl was withdrawn and transferred to a centrifugation tube. 0.1 µg plasmid DNA was added to the cell suspension. The mixture was incubated at 34°C in the light at 35 µE m^{-2} s^{-1} for 12 h, and then spread onto filter membranes (Gene Screen Plus; Du Pont/NEN Research Products, Boston, MA) that had been placed on plates prepared with BG11 medium and 1.5% agar. The plates were incubated at 34°C in the dark for 16 h, and then in the light at 35 µE m^{-2} s^{-1} until the green lawn of the cells appeared. Filter membranes having the green lawn of the cells were transferred onto new agar plates that contained kanamycin, spectinomycin, or chloramphenicol at 5 µg ml^{-1}. Single colonies which were resistant to the antibiotic were selected and transferred to new agar plates that contained kanamycin, spectinomycin or chloramphenicol at 25, 15, and 15 µg ml^{-1}, respectively. Several transfers of single colonies to agar plates containing the antibiotic were completed. For the construction of the double mutants, Δ*nhaS1*Δ*nhaS2* and Δ*nhaS4*Δ*nhaS5*, I transformed Δ*nhaS1* cells with p*nhaS2*::Cm^r and Δ*nhaS4* cells with p*nhaS5*::Sp^r, respectively.

PCR and Southern blotting analyses

Disruption with the antibiotic-resistance cartridges of the *nhaS* genes on all copies of the chromosome was examined by PCR. Cells were lysed by incubation with 0.5% Triton-X100 at 95°C for 10 min and each lysate was used directly as the template. Segregation of mutated and native chromosomes of cells transformed with p*nhaS3*::Km^r was determined by Southern blotting. Chromosomal DNA was isolated as described (Golden et al. 1987). Two μg of chromosomal DNA were digested with *Sma*I, and the DNA fragments were fractionated on a 1.0% agarose gel. Bands of DNA were transferred to a nylon membrane and subjected to hybridization with the DNA probe, as described in section 2.3.

3.4. Results

Disruption of *nhaS* genes in *Synechocystis*

I created single and double mutants of *Synechocystis* in which individual *nhaS* genes were disrupted by insertion of an antibiotic-resistance gene cartridge. As *Synechocystis* maintains about twelve copies of the genome per cell (Labarre et al. 1989), segregation of mutated and native chromosomes in transformed cells was examined by PCR (Fig. 3-2). The disruption of the *nhaS1*, *nhaS2*, *nhaS4*, and *nhaS5* genes in all copies of the chromosome was verified (Fig. 3-2A, B, and D). In contrast, transformation of cells with p*nhaS3*::Km^r did not cause disappearance of the native *nhaS3* gene (Fig. 3-2C). In the efforts to disrupt the *nhaS3* gene, I 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)]; and media prepared by adding different concentrations of NaCl (100 μM to 100 mM) to the low-salt medium. To estimate proportion of the mutated copies of the *nhaS3* gene in transformed cells, I performed Southern blotting analysis. The result indicated that only about 10% of the copies of the chromosome were replaced by mutated chromosomes (Fig. 3-3). Transformed cells that had been grown mixotrophically in the presence of 5 mM glucose also retained a similarly high content of the native chromosome (data not

shown), excluding the possibility of a specific function of the *nhaS3* gene in photoautotrophic growth.

Growth characteristics of the mutants

The single mutants in which either *nhaS1*, *nhaS2*, *nhaS4*, or *nhaS5* genes had been completely disrupted did not show any phenotypic changes in terms of sensitivity to high concentrations of NaCl (data not shown). Δ*nhaS1*Δ*nhaS2* cells grew slower than wild-type cells both in BG11 medium and in a high-salt medium prepared by adding NaCl, to 0.5 M, to the BG11 medium (Fig. 3-4A). The retardation of growth Δ*nhaS1*Δ*nhaS2* cells, as compared to the growth of wild-type cells, appeared to be greater in the presence of 0.5 M NaCl than in its absence. By 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. 3-4B). Growth was severely inhibited in cells that contained both native and disrupted *nhaS3* genes even in the absence of 0.5 M NaCl (Fig. 3-4C).

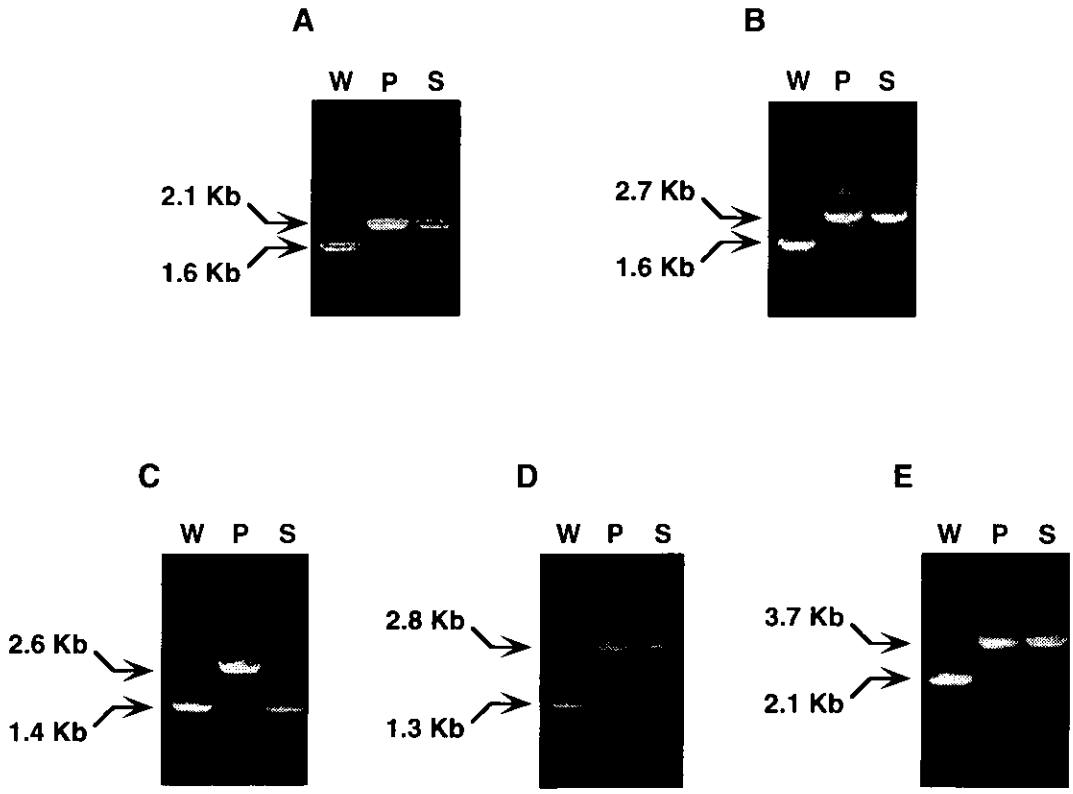

FIG. 3-2. Analysis by PCR of disruption of the *nhaS* genes with an antibiotic-resistance gene cartridge on the chromosome of wild-type cells and cells transformed with either p*nhaS1*::Km^r (A), p*nhaS2*::Cm^r (B), p*nhaS3*::Km^r (C), p*nhaS4*::Cm^r (D), or p*nhaS5*::Sp^r (E). **W**, the product of PCR obtained with DNA from wild-type cells as template; **P**, the product of PCR obtained with the plasmid used for the disruptional mutagenesis as template; **S**, the product of PCR obtained with DNA from transformed cells as template.

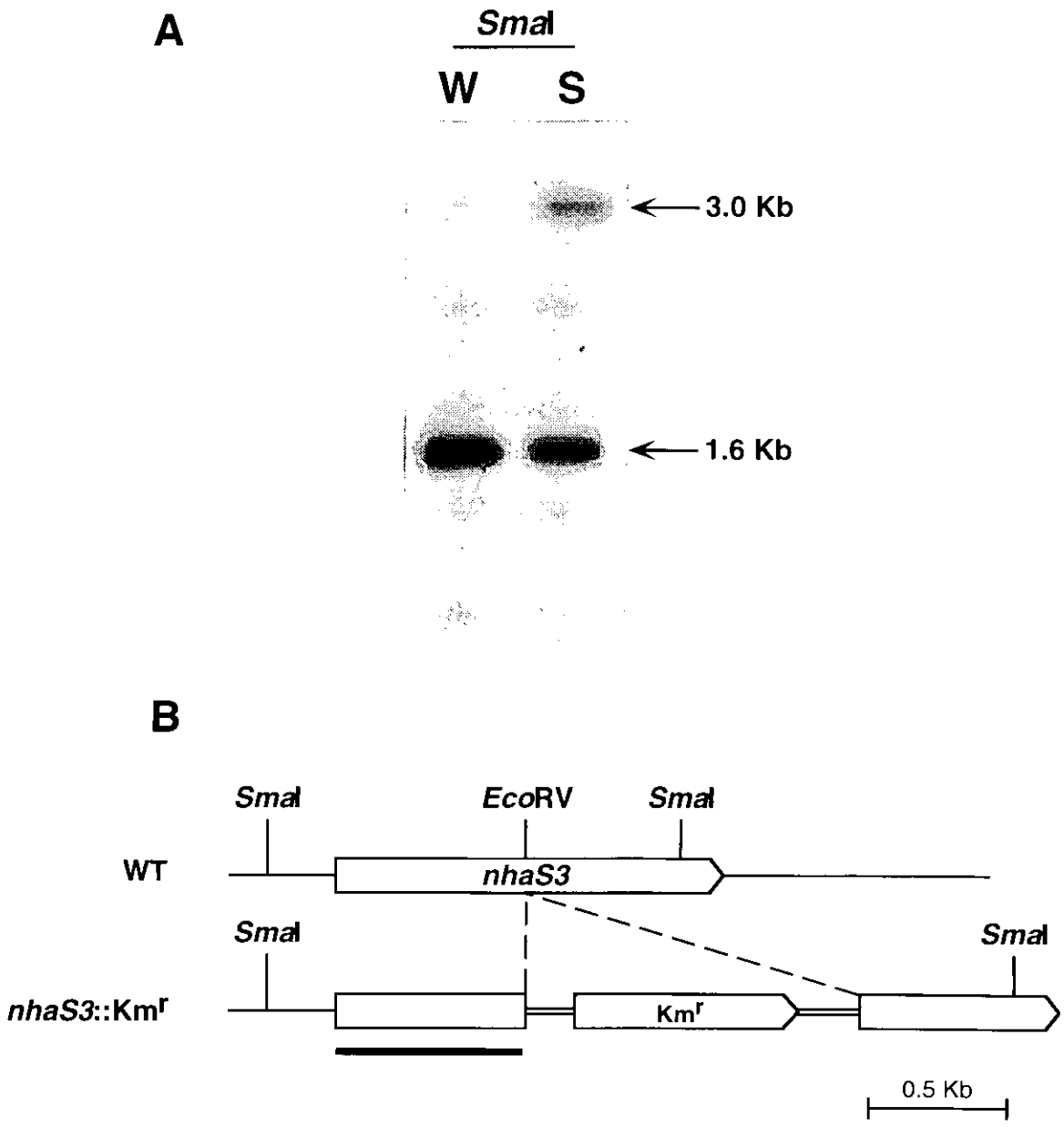

FIG. 3-3. Disruption of the *nhaS3* gene. (A) Segregation of the disrupted *nhaS3* gene was analyzed by Southern blotting. **W**, DNA from wild-type cells; **S**, DNA from cells transformed with p*nhaS3*::Km^r. (B) Schematic representation of the chromosomes with the native *nhaS3* gene (WT) and disrupted *nhaS3* gene by insertion of the kanamycin-resistance gene cartrige (*nhaS3*::Km^r). The thick line indicates the region used to generate the DNA probe.

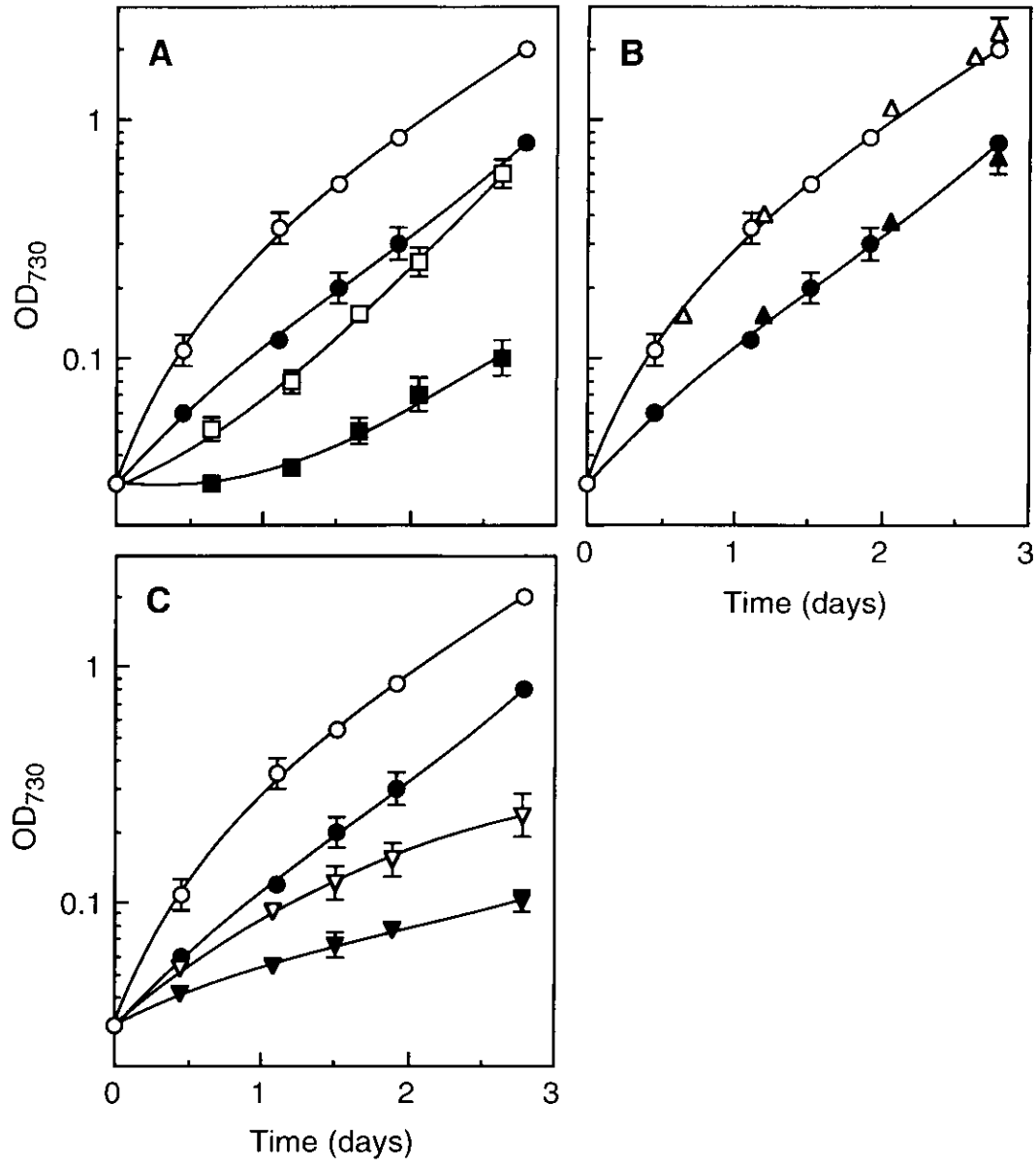

FIG. 3-4. Growth curves for *Synechocystis* in BG11 medium that contained 18 mM Na^+ ions (open symbols) or in high-salt medium prepared by increasing the concentration of NaCl in BG11 medium to 0.5 M (closed symbols). (A) Wild-type cells (○ and ●) and mutant cells with disrupted *nhaS1* and *nhaS2* genes (Δ*nhaS1*Δ*nhaS2*; □ and ■). (B) Wild-type cells (○ and ●) and mutant cells with disrupted *nhaS4* and *nhaS5* genes (Δ*nhaS4*Δ*nhaS5*; Δ and ▲). (C) Wild-type cells (○ and ●) and mutant cells with native and disrupted *nhaS3* gene (*nhaS3**; ∇ and ▼). The results were obtained from three independent determinations for each line of cells.

3.5 Discussion

Possible roles of the Na^+/H^+ antiporter homologues 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 may 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 *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, homologues of eukaryotic Na^+/H^+ antiporters, may be complementary and that each may 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 slower 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. Transformed cells with p*nhaS3*::Km^r retained a high content of the native *nhaS3* gene, and this transformation severely inhibited cell growth. These results suggest that lower levels of the NhaS3 protein may be present in the cell, and a decreasing level due to the decrease in the copy number of the *nhaS3* gene might have caused detrimental effects. 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 may function to monitor changes in intracellular concentrations of ions and might be involved in the appropriate adjustment of such concentrations.

Disruption of both the *nhaS4* and *nhaS5* genes had no effects on phenotypes in terms of high-salt tolerance, an observation that suggests that NhaS4 and NhaS5 may contribute little to high-salt stress tolerance. The results from the heterologous expression of the *nhaS4* gene suggested that its product might be a transporter of K^+ and/or Na^+ ions (Chapter 2). Sequence similarity of NhaS4 and NhaS5 to the NapA family of Na^+(or K^+)/H^+ antiporters might also suggest that NhaS4 and NhaS5 could be involved in transport of K^+ ions.

It remains to be determined whether the various Na^+/H^+ antiporters are localized on the plasma membrane, the thylakoid membrane, or both. Their locations could prove helpful in our clarifying their physiological roles in *Synechocystis*.

Chapter 4

Phylogenetic analysis of Na^+/H^+ antiporter homologues: from cyanobacteria to higher plants

4.1. Summary

Phylogenetic relationships among the Na^+/H^+ antiporters and putative Na^+/H^+ antiporter homologues were analyzed. BLAST search results indicated that NhaS1 and NhaS2 are similar to NhaP of *Pesudomonas aeruginosa* and eukaryotic Na^+/H^+ antiporters, while NhaS3, NhaS4, and NhaS5 are similar to NapA of *Enterococcus hirae*. Comparison of deduced amino acid sequences of the NhaS proteins to those of the Na^+/H^+ antiporters revealed significant similarities within the putative fifth and sixth transmembrane segments, of the NhaS proteins, and corresponding regions of the Na^+/H^+ antiporters. A phylogenetic tree based on the evolutionary distances revealed that 127 Na^+/H^+ antiporter homologues identified from various organisms, including animals, plant, yeast, eubacteria, and archaea, cluster with two groups, which are named NhaP/NHE and NapA families. The ubiquitous distribution of the members of the two families throughout the biological kingdoms indicates that the two types of proteins diverged before the divergence of major lineages in prokaryotes. Na^+/H^+ antiporter homologues from various cyanobacteria form five distinct groups, namely, NhaS1-5 subfamilies. The NhaS1 and NhaS2 subfamilies belong to the NhaP/NHE family, while the others belong to the NapA family. The findings that NhaS1 and NhaS3 are low-affinity and high-affinity Na^+/H^+ antiporters, respectively (Chapter 2), are consistent with the common kinetic properties of each type of Na^+/H^+ antiporters. NhaS3 orthologs exist in all cyanobacteria where the entire genome sequence is available, suggesting that NhaS3 might be of particular importance to cyanobacteria. This

is in agreement with the fact that the *nhaS3* gene is essential for the viability of *Synechocystis* (Chapter 3). The *Arabidopsis* proteins cluster with six groups; SOS1 and AtNHX1 belong to the NhaP/NHE family, while AtNapA1-3 and KEA1 belong to the NapA family. SOS1 and NhaS1 form a monophyletic group with a bootstrap value less than 50% and are closely related to NhaS2. These results could suggest that the *Arabidopsis* genes were acquired from the ancestor of plastids, although the relationship between these proteins is not well resolved.

4.2. Introduction

Prokaryotic Na^+/H^+ antiporters form several groups, namely, the NhaA, NhaB, NhaC, NhaD, NapA, and NhaP families, while NHE isoforms of animals, NHX1 of yeast, and AtNHX1 and SOS1 of *Arabidopsis thaliana* form the large family of eukaryotic Na^+/H^+ antiporters. NhaP and the eukaryotic Na^+/H^+ antiporters show significant similarity to each other and collectively form the NhaP/NHE family. With the on-going success of genome sequencing, many putative genes for Na^+/H^+ antiporter homologues have been identified from various organisms, including animals, plants, yeast, eubacteria, and archaea. Each can be sorted into one of the Na^+/H^+ antiporter families, based on the amino acid sequence similarities.

In this chapter, I describe phylogenetic analyses of the Na^+/H^+ antiporters and homologues. The purposes of this study are (i) to infer phylogenetic relationships among these proteins, (ii) to obtain clues to the functions of the cyanobacterial Na^+/H^+ antiporter homologues, and (iii) to trace the ancestry and evolution of the *Arabidopsis* genes.

4.3. Methods

Secondary structures of the Na^+/H^+ antiporter homologues were predicted from hydropathy profiles, as determined by the algorithm of Kyte and Doolittle (1982).

The deduced amino acid sequences of Na^+/H^+ antiporters were obtained from GeneBank/EMBL/DDBJ DNA database (http://www.ncbi.nlm.nih.gov/entrez/). Preliminary sequences of Na^+/H^+ antiporter homologues were obtained by protein BLAST search using the following databases: GeneBank/EMBL/DDBJ DNA database (http://www.ncbi.nlm.nih.gov/blast/), The Institute for Genomic Research (http://www.tigr.org/), Munich Information Centre for Protein Sequences (http://mips.gsf.de/), Kazusa DNA Research Institute (http://www.kazusa.or.jp/), The Sanger Centre (http://www.sanger.ac.uk/), GenoList (http://genolist.pasteur.fr/), University of Wisconsin (http://www.genome.wisc.edu/) and Doe Joint Genome Institute (http://www.jgi.doe.gov/tempweb/JGI_microbial/html/).

The amino acid sequences of the Na^+/H^+ antiporters and Na^+/H^+ antiporter homologues were aligned using the CLUSTAL W, version 1.6, program (Thompson et al. 1994) and then were revised manually. For the construction of a tree of the 127 proteins, including 52 NhaP/NHE-type proteins and 75 NapA-type proteins (Fig. 4-2), the 350 residues corresponding to positions 117-466 from the initial Met of human NHE1 were aligned. Of the 350 residues, the 145 residues corresponding to positions 163-185, 189-203, 228-248, 251-281, 333-364, and 377-404 from the initial Met of human NHE1 were used to calculate the evolutionary distances. The regions used for phylogenetic

analyses included large part of the six transmembrane segments of human NHE1, namely, M4, M5, M5a, M5b, M7, and M8 (Fig. 1-2B; Wakabayashi et al. 2000). For the construction of a tree of the proteins belonging only to one of the two families (Figs. 4-3 and 4-4), the overall sequences were aligned. The regions used for phylogenetic analyses were as follows: the 263 residues of the NhaP/NHE-type proteins corresponding to positions 105-139, 155-213, 221-276, 294-302, 316-367, 376-402, and 478-508 from the initial Met of human NHE1, which included large part of the transmembrane segments M2-M8 and M10 of human NHE1; the 193 residues of the NapA-type proteins corresponding to positions 57-80, 84-102, 114-136, 139-157, 213-150, 257-287, 296-318, and 322-333 from the initial Met of NapA of *Enterococcus hirae*, which included large part of the putative transmembrane segments I-V and VII-XI of this protein (Waser et al. 1992). Phylogenetic inference was completed using the PROTDIST, NEIGHBOR, SEQBOOT, and CONSENSE programs in the PHYLIP, version 3.573c, software package (Felsenstein 1996). The evolutionary distances were calculated with the PROTDIST program using the Dayhoff and PAM matrix model (Dayhoff 1878), and phylogenetic trees were constructed with the neighbor-joining method (Saitou and Nei 1987). Statistical supports for internal branches were estimated by bootstrap analyses with one-hundred replications using the SEQBOOT and CONSENSE programs.

4.4. Results and Discussion

Sequence similarities of the *Synechocystis* NhaS proteins to the NhaP/NHE and NapA Na^+/H^+ antiporters

BLAST search results revealed that NhaS1 and NhaS2 are similar to the NhaP of *Pseudomonas aeruginosa* (Utsugi et al. 1998) and eukaryotic Na^+/H^+ antiporters, whereas NhaS3, NhaS4, and NhaS5 are similar to NapA of *Enterococcus hirae* (Strausak et al. 1993; Waser et al. 1992). Fig. 4-1 shows sequence relations of the NhaS proteins to the human NHE1 (Sadat et al. 1989), NhaP of *Pseudomonas aeruginosa*, SOS1 of *Arabidopsis thaliana* (Shi et al. 2000), and NapA of *Enterococcus hirae*. The NhaS proteins appear to contain eleven transmembrane segments (Fig. 4-1A). NhaS1, NhaS2, and NhaS5 contain a large hydrophilic extension at the carboxyl terminus, as do the eukaryotic Na^+/H^+ antiporters. The transmembrane regions (~400 amino acids) of the NhaS proteins exhibit moderate sequence similarities, i.e., 20-33% identity, to each other and to NHE1, NhaP, SOS1, and NapA. The highest similarity was found within the putative fifth and sixth transmembrane segments, of the NhaS proteins, and the corresponding regions of the Na^+/H^+ antiporters (M5a and M5b of human NHE1; Fig. 4-1B). Asp-267 of human NHE1 in the segment M5a, the importance of which for the Na^+/H^+ exchange was demonstrated (Fafournoux et al. 1994), is conserved in all the proteins sampled. These findings suggest that the fifth and sixth transmembrane segments, particularly the Asp residue, might play a crucial role in the activity of the NhaS proteins.

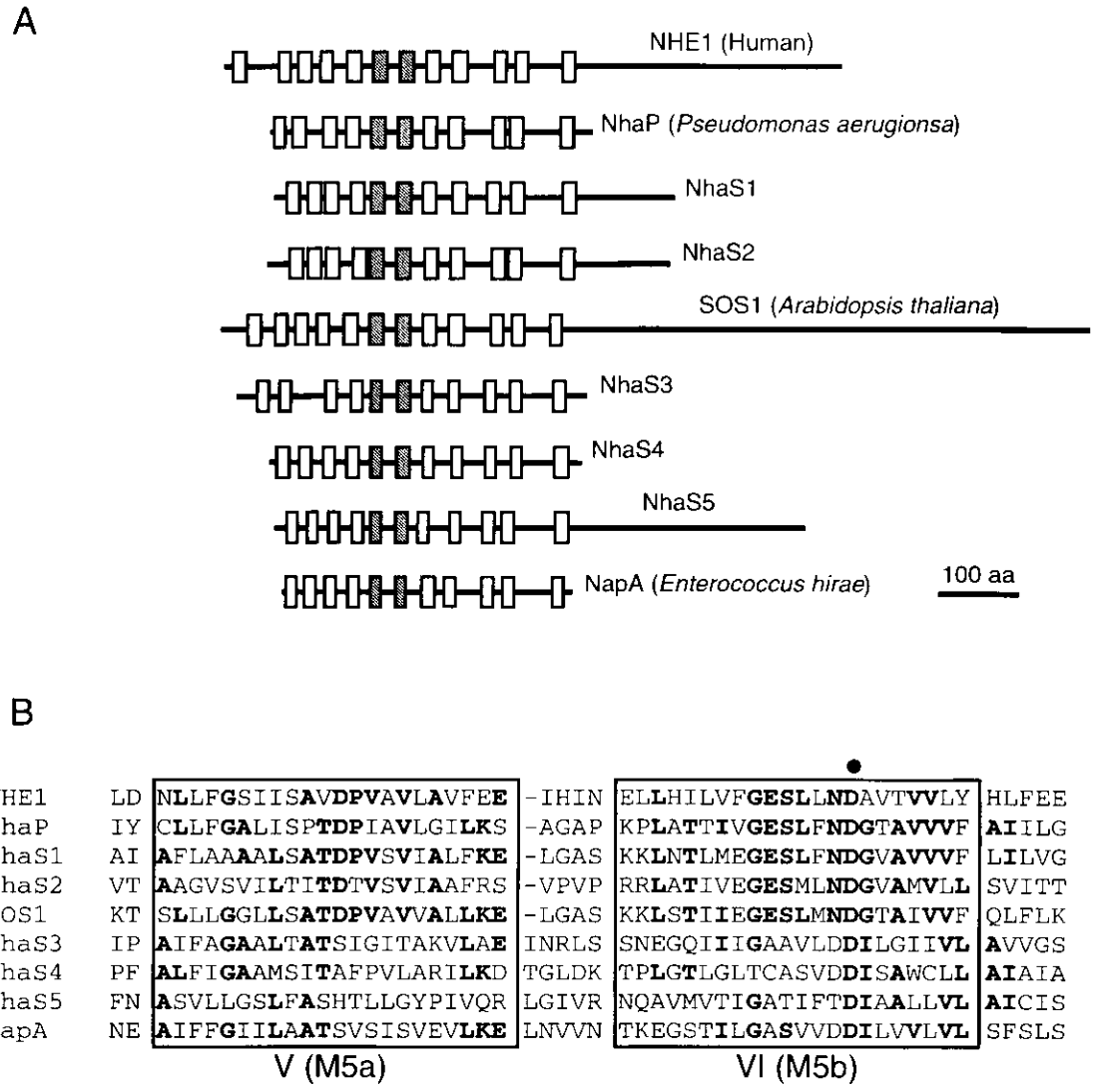

FIG. 4-1. Sequence relations of NhaS proteins of *Synechocystis* to other Na^+/H^+ antiporter homologues. (A) Secondary structures predicted from hydropathy profiles. Putative transmembrane segments are boxed. Segments that exhibit more than 30% sequence identity, namely, the putative fifth (V) and sixth (VI) segments, of NhaS proteins, which correspond respectively to the sixth (M5a) and seventh (M5b) segments of human NHE1, are shaded. (B) Alignment of amino acid sequences of the conserved segments. Identical residues are indicated by bold letters and conserved Asp residues are indicated by a dot.

In contrast, sequence similarity was not found in the carboxy-terminal hydrophilic region of NhaS1, NhaS2, NhaS5, and the eukaryotic Na^+/H^+ antiporters. In NHE isoforms, the carboxy-terminal hydrophilic region mediates the response of the exchanger to various stimuli (Wakabayashi et al. 1997). Therefore, the carboxy-terminal region of NhaS1, NhaS2, and NhaS5 may also each play a role in the regulation of the activity.

Phylogenetic relationships among the NhaP/NHE1- and NapA-type proteins

To avoid bias in the phylogenetic analysis, I collected amino acid sequences of Na^+/H^+ antiporters and Na^+/H^+ antiporter homologues as many as possible, namely, 222 polypeptides from widely divergent 53 organisms. These proteins appeared to fall into one of the NhaA, NhaB, NhaC, NhaD, NapA, and NhaP/NHE families, based on their amino acid sequence similarities. I aligned overall sequences of all these proteins. The sequences of the NhaP/NHE- and NapA-type proteins could be aligned well, while the other types of proteins formed distinct clusters with little similarity to the two types of proteins (data not shown). Therefore, I used only the NhaP/NHE- and NapA-type proteins, listed in Table 4-1, in further analyses.

TABLE 4-1. Na^+/H^+ antiporter homologues from various organisms. Asterisks (†) indicate the organisms where the entire genome sequence has been determined. The genes used for phylogenetic analyses are indicated by bold letters.

Organism	Gene name	Accession No.	Length (aa)	Family
†*Homo sapiens* (Human)	**NHE1**	P19634	815	NHE/NhaP
	NHE2	AAD41635	812	NHE/NhaP
	NHE3	P48764	834	NHE/NhaP
	NHE5	Q14940	896	NHE/NhaP
	NHE6	Q92581	669	NHE/NhaP
	FLJ20623	BAA91295	524	NapA
Rattus norvegicus (Rat)	**NHE1**	AAA98479	820	NHE/NhaP
	NHE2	AAA75406	813	NHE/NhaP
	NHE3	AAA41702	831	NHE/NhaP
	NHE4	AAA41703	717	NHE/NhaP
	NHE5	Q9Z0X2	898	NHE/NhaP
Oncorhynchus mykiss	**βNHE**	Q01345	759	NHE/NhaP
Xenopus laevis	**XLNHE**	CAA69925	781	NHE/NhaP
†*Arabidopsis thaliana*	**AtNHX1**	AAF21755	585	NHE/NhaP
	T9J14.2	AAD56988	546	NHE/NhaP
	F24P17.16	AAF08577	552	NHE/NhaP
	MTE17.18	BAB08564	529	NHE/NhaP
	SOS1	AAF76139	1146	NHE/NhaP
	T5E21.14	AAF63173	755	NHE/NhaP
	MBK23.12	BAB11467	810	NapA
	MKP6.19	BAB02053	800	NapA
	F9D16.170	CAB79325	820	NapA
	F5K20.20	CAB88334	842	NapA
	F22C12.7	AAF24561	868	NapA
	T10F5.16	AAD22684	821	NapA
	F20M17.5	AAD32281	735	NapA
	T8K14.18	AAD30236	783	NapA
	F3O9.18	AAD34690	785	NapA
	MQJ2.6	BAB10261	857	NapA
	MJG14.5	BAB11358	840	NapA
	T9D9.5	AAC16929	831	NapA

	F10K1.31	AAF82222	829	NapA
	F7A7.200	CAB82284	780	NapA
	MRN17.13	BAB10611	822	NapA
	F28D10.90	CAC03540	817	NapA
	F28D10.100	CAC03541	705	NapA
	F28D10.120	CAC03543	731	NapA
	MRN17.14	BAB10612	800	NapA
	F24D13.3	AAC98448	822	NapA
	F4F15.190	CAB41328	732	NapA
	F28D10.110	CAC03542	671	NapA
	F24D13.4	AAC98449	617	NapA
	F7F7.210	CAB82285	732	NapA
	T23G18.2	AAF18257	2658	NapA
	T6D22.24	AAF79832	2621	NapA
	F3F20.3	AAD30610	855	NapA
	T4B21.3	AAD03448	735	NapA
	MIO24.16	BAB11240	562	NapA
	F6N23.15	AAC13638	617	NapA
	KEA1	AAD01191	618	NapA
	T22P22.190	CAB87698	601	NapA
	At2g19600	AAD10158	574	NapA
	F10F5.6	AAG51773	420	NhaD
†*Saccharomyces cerevisiae*	**NHX1**	S69734	633	NHE/NhaP
	KHA1	S46584	873	NapA
	NHA1	S59330	985	SOD2
†*Escherichia coli* K-12	**KefB**	A65129	601	NapA
	KefC	AAC73158	620	NapA
	YbaL	E64778	558	NapA
	YcgO	P76007	578	NHE/NhaP
	YjcE	P32703	549	NHE/NhaP
	NhaA	AAA23448	388	NhaA
	NhaB	BAA36041	513	NhaB
†*Haemophilus influenzae*	**KefX**	P44933	618	NapA
	NhaA	AAC21894	400	NhaA
	NhaB	AAC22086	522	NhaB
	NhaC	AAC22762	468	NhaC
†*Vibrio cholerae*	**KefB1**	AAF95747	656	NapA
	KefB2	AAF94153	528	NapA
	NhaP	AAF93562	444	NHE/NhaP
	NhaA	AAF94778	382	NhaA
	NhaB	AAF95049	530	NhaB
	NhaC	AAF96106	447	NhaC

	YqkI	AAF95185	481	NhaC
	NhaD1	AAG48354	428	NhaD
	NhaD2	AAF96911	477	NhaD
†*Pseudomonas aerugionsa*	**KefB1**	AAG04596	613	NapA
	KefB2	AAG08903	567	NapA
	YbaL	AAG08914	585	NapA
	NhaP	BAA31695	424	NHE/NhaP
	YjcE	AAG07048	581	NHE/NhaP
	YcgO	AAG08406	538	NHE/NhaP
	NhaB	AAG05209	500	NhaB
†*Helicobacter pylori*	**NapA**	AAD08229	383	NapA
	KefB	AAD07539	416	NapA
	NhaA	AAD08592	438	NhaA
†*Campylobacter jejuni*	**KefB1**	CAB73485	541	NapA
	KefB2	CAB73670	391	NapA
	NhaA1	CAB73643	382	NhaA
	NhaA2	CAB73642	389	NhaA
†*Mesorhizobium loti*	**Mlr2268**	BAB49439	737	NapA
	Mlr3234	BAB50172	594	NapA
	Mll3064	BAB50044	517	NHE/NhaP
	Mlr3752	BAB50575	627	NHE/NhaP
	Mlr8398	BAB54289	591	NHE/NhaP
	Mlr5309	BAB51783	398	NhaA
†*Rickettsia provazekii*	**KefB**	CAA15236	575	NapA
Magnetospirillum sp.	**MagA**	BAA06982	434	NapA
†*Bacillus subtilis*	**YjbQ**	CAB13021	614	NapA
	YhaU	CAB12825	408	NapA
	NhaG	BAA89487	524	NHE/NhaP
	YvgP	CAB15347	670	NHE/NhaP
	YheL	CAB12807	453	NhaC
	YqkI	CAB14288	468	NhaC
†*Bacillus halodurans* C-125	**NapA**	BAB06563	686	NapA
	YcgO	BAB07757	490	NHE/NhaP
	YheL	BAB07665	473	NhaC
Bacillus cereus	**GerN**	AAF91326	387	NapA
Bacillus megaterium	**GrmA**	AAB40041	386	NapA

†*Lactococcus lactis*	**NapA**	CAA51756	379	NapA
	YdiF	AAK04481	680	NHE/NhaP
†*Staphylococcus aureus*	**YjbQ**	BAB57172	614	NapA
	YvgP	BAB44682	692	NHE/NhaP
	NhaC1	BAB58463	466	NhaC
	NhaC2	BAB58488	459	NhaC
Enterococcus hirae	**NapA**	AAA24781	383	NapA
†*Mycobacterium tuberculosis*	**KefB1**	CAB08337	385	NapA
	kefB2	CAA16665	355	NapA
	YjcE	CAB00981	542	NHE/NhaP
Amycolatopsis orientalis	**NapA**	ABB45049	409	NapA
Streptomyces coelicolor	**YcgO1**	CAB45470	514	NHE/NhaP
	YcgO2	CAB90969	515	NHE/NhaP
	YjcE1	CAC05973	530	NHE/NhaP
	YjcE2	CAB51980	528	NHE/NhaP
	NhaA1	CAC03650	410	NhaA
	NhaA2	CAB38501	474	NhaA
	NhaA3	CAB54170	629	NhaA
†*Borrelia burgdorferi*	**NapA**	AAC66821	701	NapA
	NhaC1	AAB91530	449	NhaC
	NhaC2	AAB91529	462	NhaC
†*Deinococcus radiodurans*	**KefB1**	AAF11914	575	NapA
	KefB2	AAF10721	383	NapA
	NhaP1	AAF11941	458	NHE/NhaP
	NhaP2	AAF12552	422	NHE/NhaP
	NhaP3	AAF10227	446	NHE/NhaP
†*Aquifex aeolicus*	**NapA1**	AAC06696	387	NapA
	NapA2	AAC07034	408	NapA
	NapA3	AAC07780	422	NapA
†*Methanococcus jannaschii*	**NapA**	AAB99281	388	NapA
	NhaP1	AAB99540	422	NHE/NhaP
	NhaP2	AAB98037	426	NHE/NhaP
†*Methanobacterium*	**NapA**	AAB85263	399	NapA
thermoautotrophicum	KefB	AAB84864	512	NapA

†*Archaeoglobus fulgidus*	**NapA1**	AAB91016	377	NapA
	NapA2	AAB89998	338	NapA
	Nhe2	AAB90396	494	NHE/NhaP
†*Pyrococcus abyssi*	**NapA1**	CAB49058	375	NapA
	NapA2	CAB49490	426	NapA
	NapA3	CAB50569	380	NapA
	NapA4	CAB50128	390	NapA
	NhaP	CAB50204	443	NHE/NhaP
†*Thermoplasma acidophilum*	**NapA**	CAC12310	381	NapA
	NhaP	CAC12029	432	NHE/NhaP
†*Aerophyrum pernix* K2	**NapA**	BAA81441	412	NapA
†*Synechocystis* sp. PCC 6803	**NhaS1**	BAA17925	527	NHE/NhaP
	NhaS2	BAA18490	540	NHE/NhaP
	NhaS3	BAA10332	461	NapA
	NhaS4	BAA16991	410	NapA
	NhaS5	BAA10378	698	NapA
†*Anabaena* sp. PCC 7120	**NhaS1**	—	520	NHE/NhaP
	NhaS2	—	517	NHE/NhaP
	NhaS3A	—	461	NapA
	NhaS3B	—	470	NapA
	NhaS5A	—	712	NapA
	NhaS5B	—	681	NapA
Nostoc punctiforme	**NhaS1A**	—	523	NHE/NhaP
ATCC 29133	**NhaS1B**	—	515	NHE/NhaP
	NhaS3	—	509	NapA
	NhaS4A	—	429	NapA
	NhaS4B	—	736	NapA
	NhaS5	—	712	NapA
Synechococcus sp. WH 8120	**NhaS3**	—	458	NapA
†*Prochlorococcus marinus* MED4	**NhaS3**	—	455	NapA

Figure 4-2 shows a neighbor-joining tree of the 127 Na^+/H^+ antiporter homologues, including 53 NhaP/NHE-type proteins and 75 NapA-type proteins, from 39 organisms. They form two distinct groups, the NhaP/NHE and NapA families, as expected from the BLAST search results. The ubiquitous distribution of the members of the two families throughout the biological kingdoms indicates that the two types of proteins diverged before the divergence of major lineages in prokaryotes. This suggests that the different functions of the two types of Na^+/H^+ antiporters might have originated early in evolution. In fact, they appear to have distinct properties. NHE isoforms catalyze the electoneutral exchange of Na^+ ions for protons, being activated by internal protons (Wakabayashi et al. 1997). It has been proposed that NHX1 of *Saccharomyces cerevisiae* might be activated by decreases in cytoplasmic pH (Nass et al. 1997). NHE isoforms have rather high K_m values for Na^+ ions, which range from 4.7 to 59 mM (Orlowski 1993). A high K_m (7 mM) for Na^+ ions was also reported for vacuoles of *Arabidopsis thaliana* that overexpressed the *AtNHX1* gene (Apse et al. 1999). In contrast, it was reported that NapA has a relatively low K_m (1.0 mM) for Na^+ ions (Strausak et al. 1993).

The NhaS1-5 subfamilies of cyanobacterial Na^+/H^+ antiporter homologues

DNA sequences of various strains of cyanobacterium are now available. These include two strains of filamentous nitrogen-fixing cyanobacteria, *Anabaena* sp. PCC 7120 and *Nostoc punctiforme* ATCC 29133, and two closely related marine

unicellular cyanobacteria, *Synechococcus* sp. WH8102 and *Prochlorococcus marinus* MED4. Na^+/H^+ antiporter homologues from the cyanobacteria form five distinct groups, named NhaS1-5 subfamilies, and the monophyly of each subfamily was supported by high bootstrap values. This means that the five kinds of proteins may have appeared early in evolution and been retained in various cyanobacteria. This could also suggest that each subfamily has a distinct role in cyanobacterial cells. The NhaS1 and NhaS2 subfamilies belong to the NhaP/NHE family, while the others belong to the NapA family. In accordance with the distinct kinetic properties observed for each type of Na^+/H^+ antiporters, the present study of the expression in *E. coli* of cyanobacterial genes demonstrated that NhaS1 is a low-affinity Na^+/H^+ antiporter with a K_m value of 7.7 mM for Na^+ ions, whereas NhaS3 is a high-affinity Na^+/H^+ antiporter with a K_m value of 0.7 mM for Na^+ ions (Chapter 2). This study provided the first example, to my knowledge, of the functional identification of the two types of Na^+/H^+ antiporters in a single organism.

Anabaena sp. PCC 7120 and *Nostoc punctiforme* ATCC 29133 have several NhaS proteins as does *Synechocystis* (Table 4-1). In contrast, *Prochlorococcus marinus* MED4, the whole genome sequence of which has been determined, has only an ortholog of NhaS3 but no other members of Na^+/H^+ antiporters. *Synechococcus* sp. WH8102, whose genome sequencing is almost finishing, appears to have only an NhaS3 ortholog. This might reflect the fact that the latter two strains are marine cyanobacteria, in which a primary Na^+-translocating system plays a predominant role in Na^+ extrusion. NhaS3 orthologs exist in all the

cyanobacteria where the entire genome sequence is available, suggesting that NhaS3 might be of particular importance to cyanobacteria. This is in good agreement with the fact that the *nhaS3* gene is essential for the viability of *Synechocystis* (Chapter 3).

The ancestry and evolution of the *Arabidopsis* genes for Na^+/H^+ antiporter homologues

Arabidopsis thaliana has many homologues of NhaP/NHE- and NapA-type Na^+/H^+ antiporters. These include AtNHX1 and three AtNHX1 homologues, SOS1 and an SOS1 homologue, and twenty-five NapA homologues. *Arabidopsis thaliana* also has an NhaD homologue, which was not used in this study. Putative open reading frames that encode these homologues distribute over the five chromosomes. The phylogenetic analyses revealed that the *Arabidopsis* proteins cluster with six groups; SOS1 and AtNHX1 belong to the NhaP/NHE family, while AtNapA1-3 and KEA1 belong to the NapA family (Fig. 4-2). Fig. 4-3 shows a tree of the proteins belonging only to the NhaP/NHE family. SOS1 and NhaS1 form a monophyletic group with a bootstrap value less than 50% and are closely related to NhaS2. These results could suggest that the *Arabidopsis* nuclear genes were acquired from the ancestor of plastids, although the relationship between these proteins are not well resolved. In contrast, it is evident that AtNHX1 is a more close relate of NHX1 of *Saccharomyces cerevisiae*, an observation that excludes the possibility of its symbiotic origin.

Figure 4-4 shows a tree of the proteins belonging only to the NapA family. The NhaS4 proteins and three of the four groups of the NapA-type *Arabidopsis* homologues, AtNapA1-3, form a monophyletic group with a bootstrap value less than 50%. KHA1 of *Saccharomyces cerevisiae* (Ramírez et al. 1998) is positioned at the base of the monophyletic group. This suggest that these *Arabidopsis* genes might have evolved from a unique ancestor followed by gene family diversification. However, whether or not the *Arabidopsis* genes were acquired from the ancestor of plastids is not clear since the relationships between these proteins are not well resolved. The other group of *Arabidopsis* homologues (KEA1 and F6N23.15) are sisters of bacterial homologues of the K^+/H^+ antiporter of *Escherichia coli*, KefC (Munro et al. 1991). This could suggest that these *Arabidopsis* genes were originated from a unique bacterial ancestor.

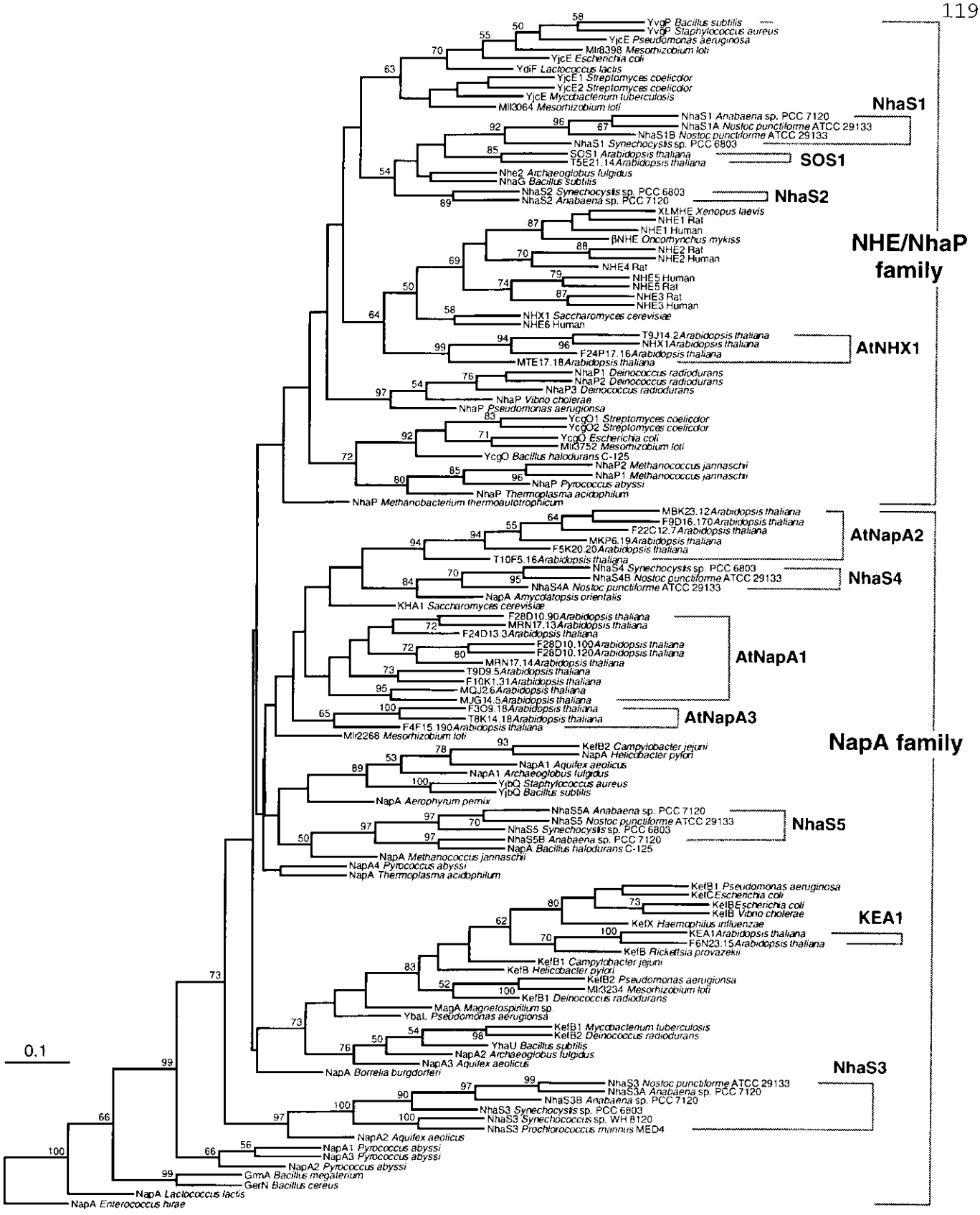

FIG. 4-2. A neighbor-joining tree of NHE/NhaP- and NapA-type Na^+/H^+ antiporter homologues. This tree is rooted with NapA of *Enterococcus hirae* as an outgroup. Bootstrap values are indicated for branches supported by more than 50% of 100 bootstrap replicates. The number on the scale bar is the estimated number of amino acid substitutions per residue. Five groups of cyanobacterial proteins (NhaS1-5) and six groups of *Arabidopsis* proteins (SOS1, AtNHX1, AtNapA1-3, and KEA1) are indicated by brackets.

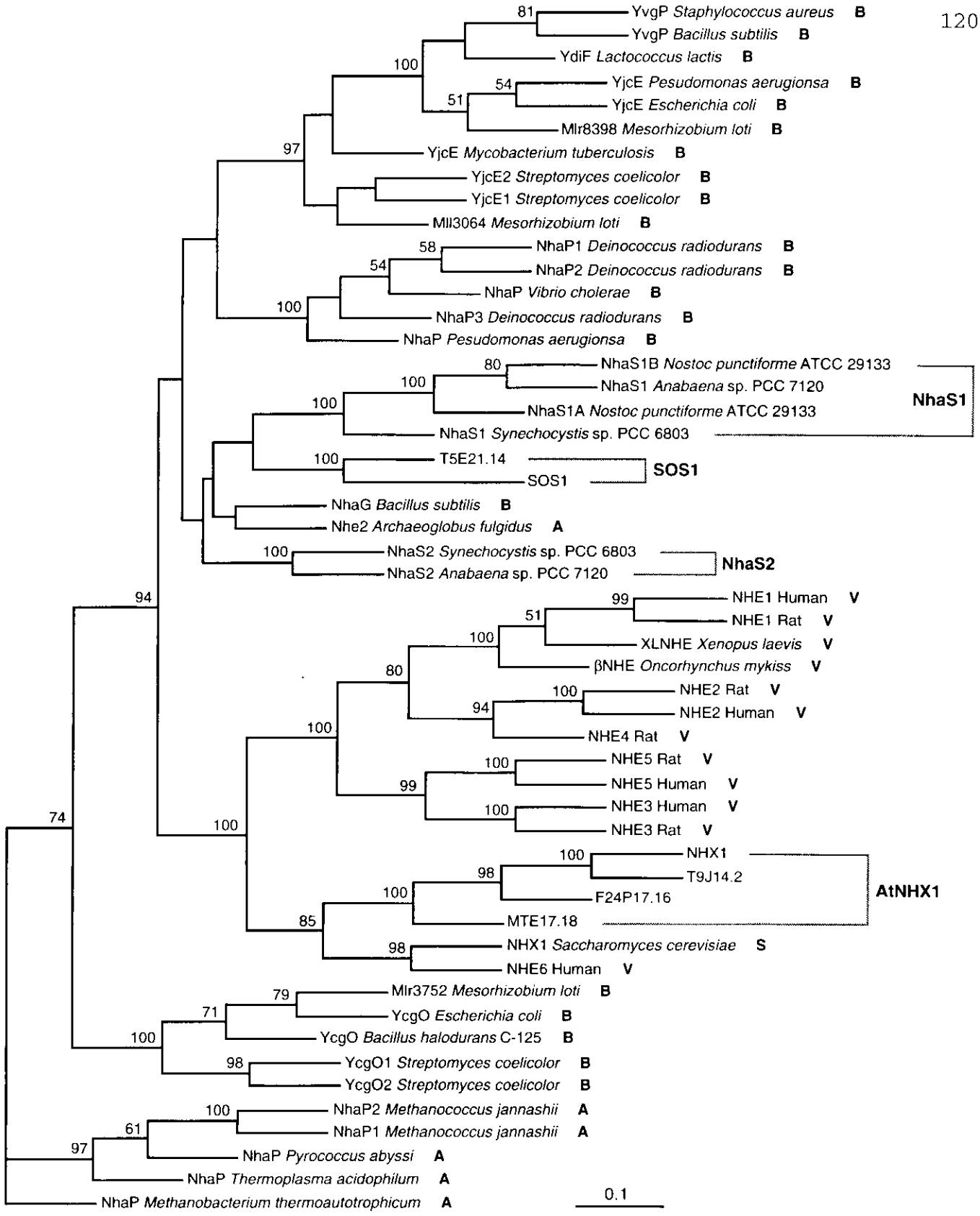

FIG. 4-3. A neighbor-joining tree of NHE/NhaP-type Na^+/H^+ antiporter homologues. This tree is rooted with NhaP of *Methanobacterium thermoautotrophicum* as an outgroup. Bootstrap values are indicated for branches supported by more than 50% of 100 bootstrap replicates. Groups of cyanobacterial proteins (NhaS1-2) and *Arabidopsis* proteins (SOS1 and AtNHX1) are indicated by brackets. Proteins from other organisms are indicated by bold letters after the names of proteins: vertebrates (**V**), yeast (**S**), eubacteria (**B**), and archaea (**A**).

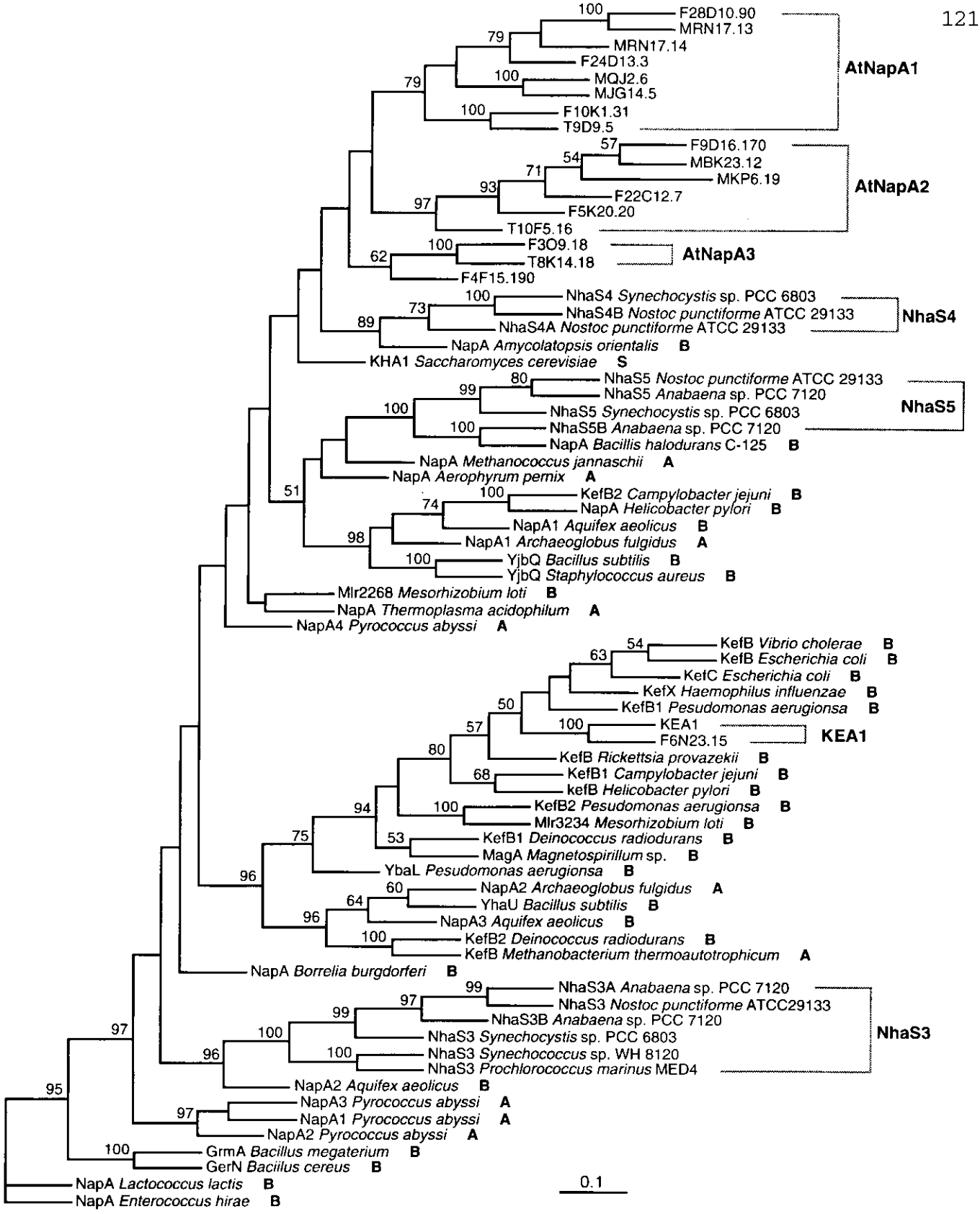

FIG. 4-4. A neighbor-joining tree of NapA-type Na^+/H^+ antiporter homologues. This tree is rooted with NapA of *Enterococcus hirae* as an outgroup. Bootstrap values are indicated for branches supported by more than 50% of 100 bootstrap replicates. Groups of cyanobacterial proteins (NhaS3-5) and *Arabidopsis* proteins (AtNapA1-3 and KEA1) are indicated by brackets. Proteins from other organisms are indicated by bold letters after the names of proteins: vertebrates (**V**), yeast (**S**), eubacteria (**B**), and archaea (**A**).

Concluding remarks

Table 4-2 shows characteristics of *Synechocystis* Na^+/H^+ antiporter homologues that have been revealed from the present research. In summary, two of the five homologues of Na^+/H^+ antiporters, NhaS1 and NhaS3, were functionally characterized. The five kinds of proteins distribute in various cyanobacteria and form NhaS1-5 subfamilies. The NhaS1 and NhaS2 subfamilies belong to the NhaP/NHE family, while the others belong to the NapA family. The findings that NhaS1 and NhaS3 are low-affinity and high-affinity Na^+/H^+ antiporters, respectively, are consistent with the common properties of each type of Na^+/H^+ antiporters. NhaS3 orthologs exist in all the strains where the entire genome sequence is available, suggesting that NhaS3 might be of particular importance to cyanobacteria. This is in agreement with the fact that the *nhaS3* gene is essential for the viability of *Synechocystis*. The indispensability of the *nhaS3* gene could be attributed to the unique features of cyanobacteria, since all the Na^+/H^+ antiporters characterized to date in heterotrophic bacteria are dispensable under low-salt conditions. Locations of the NhaS proteins on which membranes, the plasma membrane or thylakoids, should be determined to clarify their physiological roles. DNA microarray technique has been improved rapidly and will also provide us with a powerful tool in obtaining insights into the roles of each NhaS protein in cyanobacteria. *Arabidopsis thaliana* has many genes for Na^+/H^+ antiporter homologues, most of which have yet to be characterized. Phylogenetic relationship between Na^+/H^+ antiporter homologues from various organisms suggested

that some of the *Arabidopsis* genes were acquired from the cyanobacterial ancestor of plastids. This might shed light on the evolution of various Na^+/H^+ antiporters in plant cells.

TABLE 4-2. Characteristics of Na^+/H^+ antiporter homologues in *Synechocystis*.

	I	II	III
Name	*In vitro* expression	Targeted inactivation	Phylogenetic analysis
NhaS1	Low-affinity Na^+/H^+ antiporter	Involved in salt tolerance	NhaP/NHE-type Probable ancestor of SOS1 of *A. thaliana*
NhaS2		Involved in salt tolerance	NhaP/NHE-type
NhaS3	High-affinity Na^+/H^+ antiporter	Essential for viability	NapA-type
NhaS4	K^+ and/or Na^+ transporter	Little contribution to salt tolerance	NapA-type Probable ancestor of AtNapA1-3 of *A. thaliana*
NhaS5		Little contribution to salt tolerance	NapA-type

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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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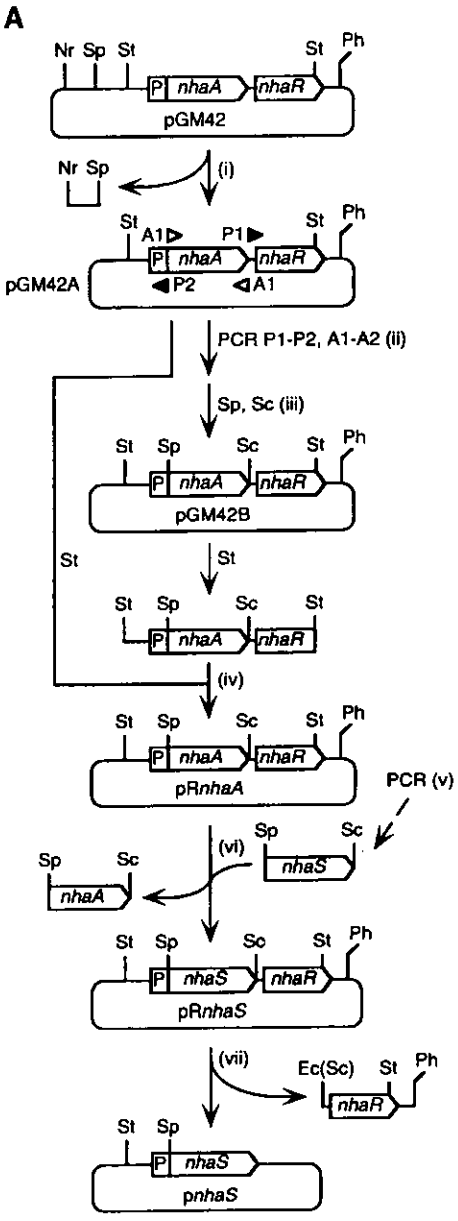

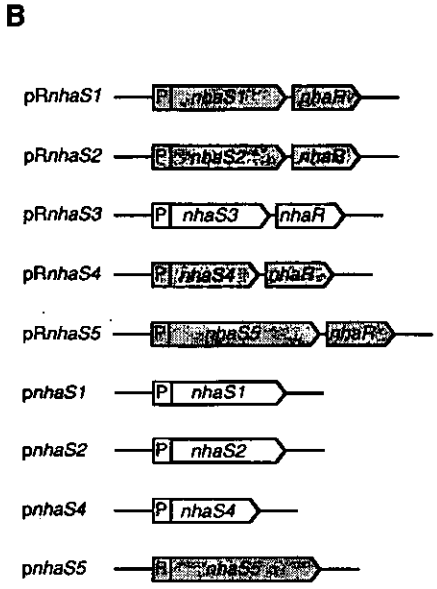

FIG. 1. Plasmids used for expression of *nhaS* genes in *E. coli* TO114. (A) Construction of vector plasmids. Solid lines represent the pBR322 backbone and the flanking regions of the *nhaA* and *nhaR* genes of *E. coli*. P, Na^+-inducible promoter of the *nhaA* gene (*nhaAp*). Restriction sites: Nr, *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.

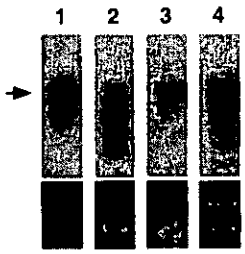

FIG. 2. Northern blotting analysis of the expression of *nhaS* genes in transformed *E. coli* TO114 cells. Total RNA was extracted from cells that had been grown in LBK medium. Results are shown for *nhaS1* transcripts in *nhaS1*$^+$ cells (lane 1), *nhaS2* transcripts in *nhaS2*$^+$ cells (lane 2), *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

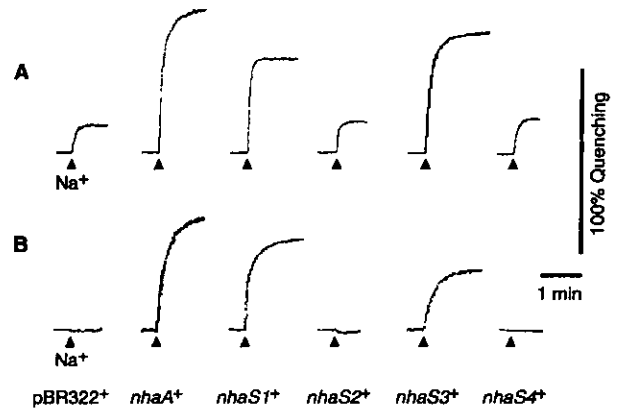

FIG. 3. Activities of the Na$^+$/H$^+$ antiport system in IMVs prepared from transformed cells. IMVs were prepared from cells that had been grown in LBK medium. Activity was assayed in a solution that consisted of 5 mM $MgCl_2$, 1 μM acridine orange, and 10 mM Tris titrated with MES (pH 8.5) and supplemented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

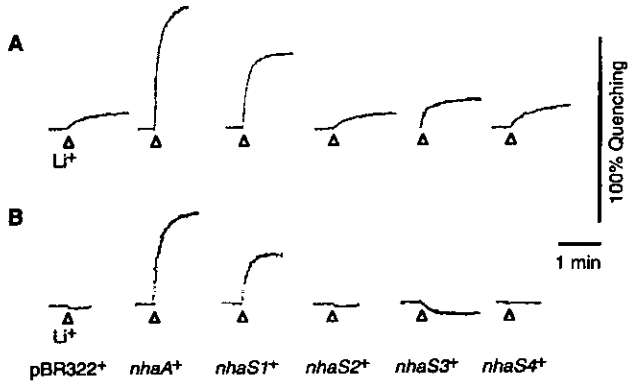

FIG. 4. Activities of the Li^+/H^+ antiport system in IMVs prepared from transformed cells. Experiments were carried out as described in the legend to Fig. 3 except that 5 mM LiCl was added to the assay solution instead of 5 mM NaCl.

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).

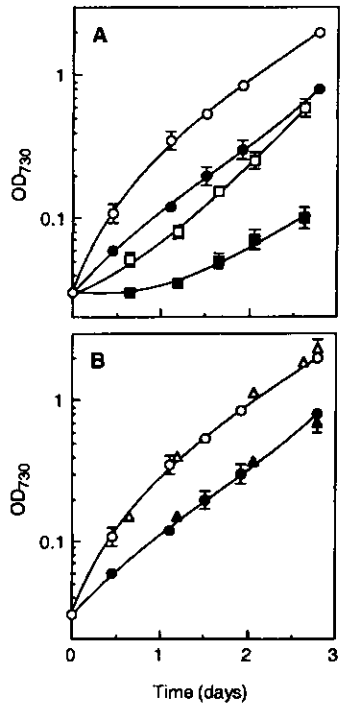

FIG. 5. Growth curves for *Synechocystis* in BG11 medium that contained 18 mM Na^+ ions (open symbols) or in high-salt medium prepared by increasing the concentration of NaCl in BG11 medium to 0.5 M (closed symbols). (A) Wild-type cells (circles) and mutant cells with disrupted *nhaS1* and *nhaS2* genes (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

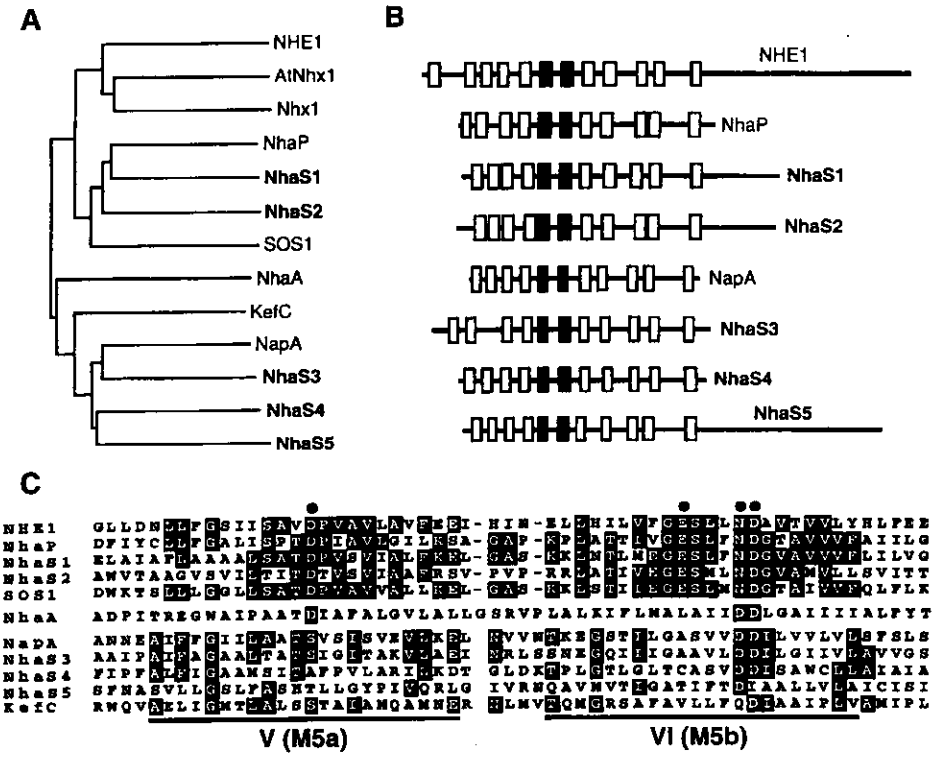

FIG. 6. Relationships of NhaS proteins to other Na^+/H^+ antiporters and related proteins. (A) Phylogenetic relationships as determined with the CLUSTAL W multiple sequence alignment algorithm (45). (B) Secondary structures predicted from hydropathy profiles, as determined by the algorithm of Kyte and 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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