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nhaG Na^+/H^+ Antiporter Gene of *Bacillus subtilis* ATCC9372, Which Is Missing in the Complete Genome Sequence of Strain 168, and Properties of the Antiporter[1]

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We cloned a gene which enabled *Escherichia coli* mutant host cells lacking all of the major Na^+/H^+ antiporters to grow in the presence of 0.2 M NaCl from chromosomal DNA of *Bacillus subtilis* ATCC9372. An Na^+/H^+ antiport activity was observed with membrane vesicles prepared from *E. coli* cells possessing the cloned gene, but not with vesicles from the host cells. Lithium ion was also a substrate for the antiporter. We sequenced the cloned DNA and found one open reading frame (designated *nhaG*) preceded by a promoter-like sequence and a Shine-Dalgarno sequence, and followed by a terminator-like sequence. The deduced amino acid sequence of NhaG suggested that it consisted of 524 residues and that the calculated molecular mass was 58.1 kDa. None of the bacterial Na^+/H^+ antiporters so far reported, except NhaP of *Pseudomonas aeruginosa* and SynNhaP (NhaS1) of *Synechocystis* sp., showed significant sequence similarity with the NhaG. However, the NhaP, the SynNhaP, animal NHEs (Na^+/H^+ exchangers), and some hypothetical Na^+/H^+ antiporters of several organisms showed significant sequence similarities with the NhaG. Interestingly, the entire DNA region corresponding to the *nhaG* gene is missing in the reported complete genome sequence of *B. subtilis* strain 168. We detected a band that hybridized with the *nhaG* DNA in chromosomal DNA from *B. subtilis* ATCC9372 but not with that from strain 168. The missing DNA region (1,774 base pairs) is sandwiched by two identical sequences, TTTTCTT.

Key words: *Bacillus subtilis*, cloning, missing gene, Na^+/H^+ antiporter, *nhaG*.

Na^+ circulation across cell membranes in microorganisms is involved in energy transduction, extrusion of Na^+ that is toxic when present at high concentrations, intracellular pH regulation, and so on (*1–7*) The Na^+/H^+ antiporter plays central roles in such processes not only in microbial cells but also in animal cells (*8–10*).

It is known that there are several Na^+/H^+ antiporters in *Escherichia coli* (NhaA, NhaB, and ChaA) (*11–13*), in other microorganisms (NhaC, NhaD, NhaP, NapA, and Mrp) (*3, 14–17*) and in animal cells (*8–10*). Thus, it is very difficult to analyze one Na^+/H^+ antiporter of interest in microbial cells. Cloning the Na^+/H^+ antiporter gene of interest and expression in *E. coli* are useful for the analysis of such an antiporter. It is desirable to use cells which lack the major Na^+/H^+ antiporters as a cloning host. We constructed an *E. coli* mutant lacking all of the three major Na^+/H^+ antiporters (*ΔnhaA, ΔnhaB, ΔchaA*) and a restriction system (*hsd*$^-$). The restriction system in host cells is undesirable for cloning of gene(s) from other organisms because it cleaves DNA introduced from other organisms. The *E. coli* mutant has proven to be very useful, and it greatly facilitated the cloning of Na^+/H^+ antiporter genes from other bacteria. We have cloned several genes encoding Na^+/H^+ antiporters from several Gram-negative bacteria including *Pseudomonas aeruginosa* (*16*) and *Vibrio parahaemolyticus* (*15, 18*), sequenced them and characterized the antiporters. We then studied the structure–function relationships, the physiological significance and the evolutionary relationship of these antiporters (*15, 16, 18*). In addition, the *E. coli* mutant was proven useful for the cloning of Na^+/H^+ antiporter genes from Gram-positive bacteria. We cloned genes encoding a novel type of Na^+/H^+ antiporter from Gram-positive *Staphylococcus aureus* (*1*) and *Bacillus subtilis*. Recently the complete genome sequence of *B. subtilis* was reported (*19*). We demonstrate here, based on sequence analysis and Southern blot analysis, that the new *B. subtilis* Na^+/H^+ antiporter gene, *nhaG*, is not present in the reported complete genome sequence of *B. subtilis*.

MATERIALS AND METHODS

Organisms and Growth—B. subtilis ATCC9372 and *E. coli* KNabc (*ΔnhaA*::Km^r, *ΔnhaB*::Em^r, *ΔchaA*::Cm^r) were used. *E. coli* KNabc was constructed from TG1 by P1 trans-

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duction. The *ΔnhaA*, *ΔnhaB*, and *ΔchaA* were transferred from NM81 (*5*), TO101 (*20*), and TO114 (*20*), respectively. *B. subtilis* cells were grown in L medium (*21*) unless otherwise indicated. *E. coli* cells were grown in L(K) medium (*1*), in which NaCl was replaced with KCl, at 37°C under aerobic conditions. When necessary, NaCl was added to the L(K) medium at indicated concentrations. Cell growth was monitored turbidometrically at 650 nm.

Cloning and Sequence Analysis—Chromosomal DNA was prepared from *B. subtilis* cells by the method of Weiss and Wake (*22*). The DNA was partially digested with the restriction enzyme *Sau*3AI, and fragments of 4 to 10 kbp were separated by sucrose density gradient centrifugation. The DNA fragments were ligated to pUC19 (which had been digested with *Bam*HI and dephosphorylated with bacterial alkaline phosphatase) by using T4 DNA ligase. Competent *E. coli* KNabc cells were transformed (*23*) with the ligated hybrid plasmids and spread on agar plates consisting of L(K) broth, 0.2 M NaCl, 40 μg/ml ampicillin, 40 μg/ml of kanamycin, and 1.5% agar. The plates were incubated at 37°C for 2 days and the colonies formed were picked up. Plasmids were prepared from the transformants, and competent *E. coli* KNabc cell were retransformed and spread on the plates again. The plates were incubated at 37°C for 2 days. Plasmids contained in the retransformants were prepared. We obtained four candidate hybrid plasmids. Judging from their restriction patterns, all of the four plasmids seemed to carry a common DNA region and its flanking regions. Two of the plasmids, pBUN21 and pBUN23, were further characterized. Deletion plasmids for sequencing were constructed using exonuclease III and mung bean nuclease from pBUN23. The nucleotide sequence was determined by the dideoxy chain termination method (*24*) using a DNA sequencer (Pharmacia Biotech, ALF express). Sequencing of both sense and antisense strands was completed.

Sequence data were analyzed with the GENETYX sequence analysis software (Software Development). The Protein-today database on the GenomeNet (http://www.genome.ad.jp/) was screened for sequence similarities

Southern Blot Analysis—Chromosomal DNA prepared from *B. subtilis* cells or plasmid DNA of pBUN23 was digested with restriction enzymes *Hin*dIII and *Eco*RV, and the fragments were separated by electrophoresis in a 1% agarose gel and blotted to a nylon membrane, Hybond-N (Amersham), by the capillary blotting method as suggested by the manufacturer. The probe (*Hin*dIII–*Eco*RV fragment, 1.2 kbp) was prepared from the *nhaG* gene region of pBUN23. The probe was hybridized with the DNA blot on the nylon membrane, and detected with the Enhanced Chemiluminescence (ECL) detection system (Amersham) as suggested by the manufacturer.

Preparation of Membrane Vesicles and Na^+/H^+ Antiport Assay—Membrane vesicles of *E. coli* KNabc cells or KNabc/pBUN23 cells were prepared by the French press method as described previously (*25*) and stored frozen at −80°C until use The Na^+/H^+ antiport activity was measured by the quinacrine fluorescence quenching method (*25*). The assay mixture contained 10 mM Tricine-KOH, pH 8.0, 140 mM KCl, 10 mM $MgSO_4$, 1 μM quinacrine, and 50 μg/ml protein of membrane vesicles. The fluorescence was measured with excitation at 420 nm and emission at 500 nm.

RESULTS

Cloning of Na^+/H^+ Antiporter Gene—*E. coli* KNabc cells, which lack all of the major Na^+/H^+ antiporters, are unable to grow in the presence of 0.2 M NaCl or 10 mM LiCl (Fig. 1) because they can not efficiently extrude Na^+ or Li^+, which are toxic when present at certain concentrations in cells. We cloned a gene from chromosomal *B. subtilis* ATCC9372 DNA that enabled *E. coli* KNabc to grow in the presence of 0.2 M NaCl (Fig. 1A) or 10 mM LiCl (Fig. 1B). Growth of *E. coli* possessing the cloned gene in the presence of 10 mM LiCl was low, whereas growth in the presence of 0.2 M NaCl was good. Since Na^+ and Li^+ inhibit multiple biochemical processes in bacterial cells (*2*), it is most likely that the cloned gene encodes an Na^+ (Li^+) extrusion pump. Perhaps Li^+ efflux activity of the putative extrusion pump is not high enough for cells to escape from Li^+ toxicity.

We tested whether Na^+/H^+ antiport activity could be observed with membrane vesicles prepared from cells into which the cloned gene was introduced Membrane vesicles were prepared from *E. coli* KNabc or KNabc cells harboring a plasmid pBUN23, which carries our gene of interest. Indeed, we detected Na^+/H^+ antiport activity in membrane vesicles from KNabc/pBUN23 but not in vesicles from KNabc (Fig. 2). We also detected some Li^+/H^+ antiport activity in membrane vesicles prepared from KNabc/pBUN23 (data not shown). Thus, it is highly likely that the cloned gene encodes an Na^+ (Li^+)/H^+ antiporter. The pH optimum for Na^+/H^+ antiport activity and that for Li^+/H^+ antiport activity measured in membrane vesicles prepared from KNabc/pBUN23 were 8.0–9.0 and 8 0, respectively (data not shown). Lastly, Na^+ seemed to be a better substrate for the antiporter than Li^+ (data not shown).

Restriction Mapping and Sequencing—*E. coli* KNabc cells harboring either pBUN21 or pBUN23 showed similar growth in the presence of 0.2 M NaCl (data not shown). Na^+/H^+ antiport activity was very similar in membrane vesicles prepared from either of these two types of cells (data not shown). Furthermore, it seemed that these two plas-

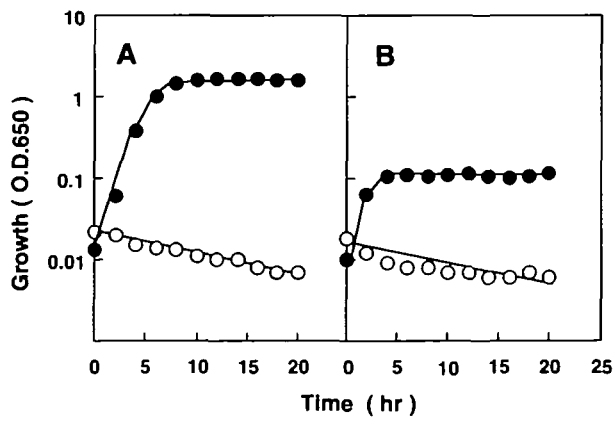

Fig 1 **Growth of *E. coli* KNabc and KNabc/pBUN23 in the presence of NaCl or LiCl.** Cells of *E coli* KNabc (○) or KNabc/pBUN23 (●) were shaken in the L(K) medium in the presence of either 0 2 M NaCl (A) or 10 mM LiCl (B) at 37°C under aerobic conditions Cell growth was monitored turbidometrically at 650 nm

mids carried a common DNA region of *B. subtilis* as judged from the restriction fragments detected after digestion with several restriction enzymes. We partially sequenced the DNA insert in the two plasmids. We found that a short overlapping region was present in the two plasmids. It became clear that the overlapping region corresponded to portions of putative *yxaC* and *yxaB* genes and their flanking region as judged by comparison with the reported genome sequence (Fig. 3A) (*19*). Curiously, however, there was no complete open reading frame in the common DNA region of the two plasmids as judged from the reported genome sequence We constructed a restriction map of the DNA insert in the two plasmids (Fig. 3B) We found a discrepancy in the restriction maps between the one we constructed using the two plasmids and the one predicted from the whole genome sequence of *B. subtilis*. There was a 2 4-kbp overlapping region in the two plasmids according to our map, which is enough for one gene encoding a membrane transport protein (Fig. 3B). There was a *Hin*dIII restriction site and an *Eco*RV restriction site between the putative *yxaC* gene and the putative *yxaB* gene according to our map (Fig. 3B).

We determined the nucleotide sequence of the overlapping region (2.4 kbp) of the two plasmids. We found that a 1.8-kbp DNA region of the 2.4-kbp DNA was absent in the whole genome sequence (*19*). The sequence of another region of the 2.4-kbp DNA (0.6-kbp) was very similar to that reported in the whole genome sequence (*19*) Within the 1.8-kbp DNA region, we found one open reading frame, which was preceded by two promoter-like sequences plus a Shine-Dalgarno sequence and was followed by a terminator-like sequence. The sequence of one of the promoter-like sequences (GTTTAT at –35 region and GGGTAA at –10 region) was identical to that of the promoter of the *katE* gene of *B. subtilis* (*26*). The other sequence was similar to that of promoters of *E. coli* (CTGTCT at –35 region and TATCAT at –10 region). Therefore, it seems that this sequence functions as a promoter for the *nhaG* in *E. coli* cells. We designated the open reading frame *nhaG*. The deduced amino acid sequence suggested that NhaG consisted of 524 residues. The calculated molecular mass was 58,105 Da. The amino acid sequence of NhaG showed 37% identity

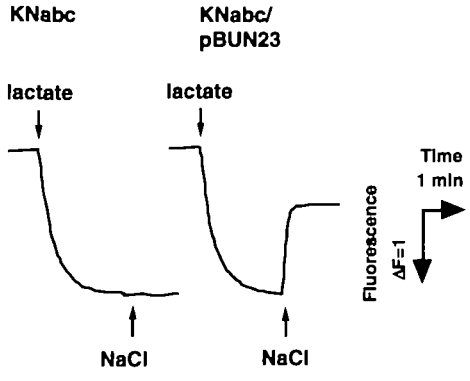

Fig 2. **Na⁺/H⁺ antiport activity in membrane vesicles measured by fluorescence quenching.** Membrane vesicles were prepared from cells of *E coli* KNabc or KNabc/pBUN23 by a French press method. At the time points indicated by downward arrows, potassium lactate (final concentration 5 mM) was added to initiate fluorescence quenching of quinacrine due to respiration Then, at the time points indicated by upward arrows, NaCl (final concentration 5 mM) was added to the assay mixture The pH of the assay mixture was 8 0 Fluorescence intensity is shown in arbitrary units.

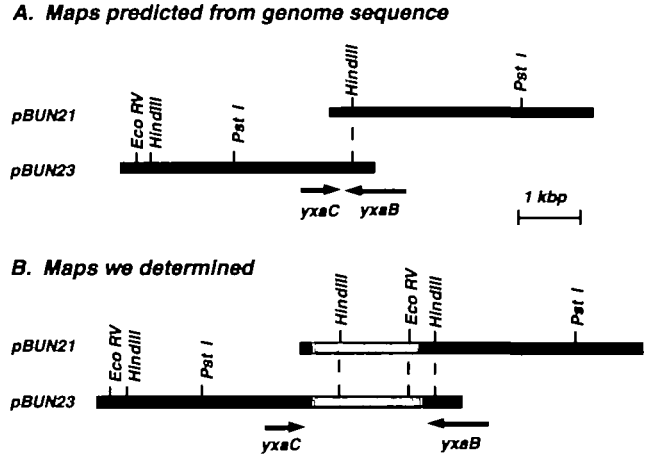

Fig 3 **Discrepancy in restriction maps.** (A) Restriction maps of chromosomal DNA region carried in plasmids pBUN21 and pBUN23 were prepared from the nucleotide sequence that was determined by the genome project of *B subtilis*. There is no common complete ORF in the two plasmids, and not enough space for an ORF between the *yxaC* and *yxaB* genes (B) Restriction maps of the DNA inserts in pBUN21 and pBUN23 that we determined A DNA segment of about 1 8 kbp (shown as hatched area) carrying a *Hin*dIII site and an *Eco*RV site is present between the *yxaC* and *yxaB* genes

TABLE I **Sequence similarities in NhaG and other Na⁺/H⁺ antiporters or hypothetical proteins.**

Antiporter or hypothetical protein (%)	Accession number	Amino acid residues Full length	Identity (%)	Similarity
Na⁺/H⁺ antiporters				
NhaP (*P. aeruginosa*)	AB010827	424	37	77
SynNhaP (*Synechocystis* sp)	S75063	527	31	72
NHE1 (human)	P19634	815	24	70
Hypothetical proteins				
NHE2 (*A. fulgidus*)	F69355	494	35	70
C75631 (*D radiodulans*)	C75631	422	30	71
NhaP (*P multocida*)	PM0365	441	29	70
SynNhaP2 (*Synechocystis* sp)	S76231	540	29	71
H75278 (*D. radiodulans*)	H75278	458	28	70
YjcE (*M. tuberculosis*)	Q50678	542	27	68
NAH (*C elegans*)	P35449	609	26	69
Mj1521 (*M. jannaschii*)	Q58916	422	25	66
VC0389 (*V cholerae*)	Q9KUX0	444	25	62

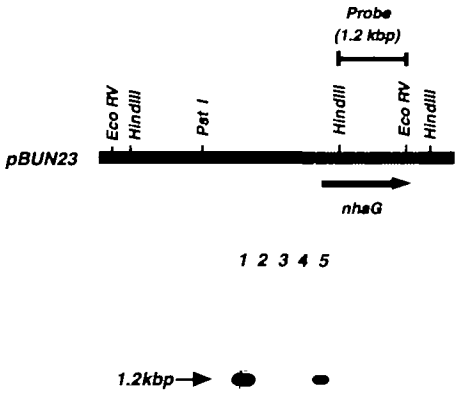

Fig 4 **Southern blot analysis.** Relevant restriction sites in the DNA insert of pBUN23 are shown. The DNA region missing in the sequence determined in the genome project of *B. subtilis* is shown as a hatched area. The probe (1.2-kbp *Hin*dIII–*Eco*RV fragment) used for the Southern blot analysis is shown. The position and direction of the *nhaG* gene are shown by a thick arrow. The DNA digested with *Hin*dIII and *Eco*RV was electrophoresed in an agarose gel, blotted and hybridized. Results of the hybridization are shown in the lower panel. Lane 1, pBUN23 DNA, lane 2, chromosomal DNA of strain 168 obtained from one source, lane 3, chromosomal DNA of strain 168 obtained from another source, lane 4, chromosomal DNA of strain 160, lane 5, chromosomal DNA from strain ATCC9372.

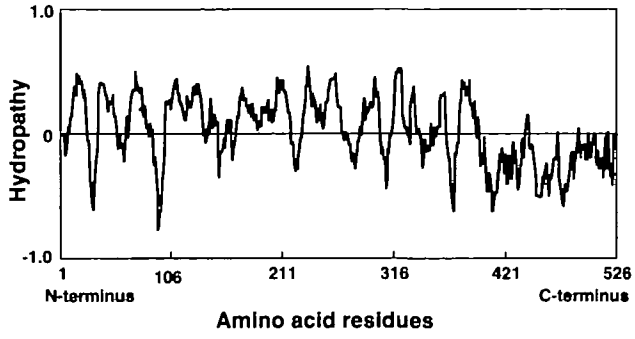

Fig 5 **Hydropathy pattern of the NhaG Na^+/H^+ antiporter.** Hydropathy values were calculated by the method of Eisenberg *et al.* (*38*) along the deduced amino acid sequence of NhaG. The values were plotted from the N-terminus to the C-terminus. Portions above and below the midpoint line indicate hydrophobic and hydrophilic regions, respectively.

and 77% similarity to the NhaP Na^+/H^+ antiporter of *P. aeruginosa* that we reported previously (*16*) (Table I). A homology search in a sequence database (Protein-today) revealed that NhaG showed sequence similarity with SynNhaP in cyanobacterium *Synechocystis* sp. (*27, 28*), animal Na^+/H^+ exchangers such as human NHE1 (*29*) and some hypothetical proteins predicted from genome sequences (Table I). No Na^+/H^+ antiporter from Gram-positive bacteria so far reported (*1, 3, 14, 17*) showed significant sequence similarity with NhaG. Thus, it seems that NhaG is a unique bacterial Na^+/H^+ antiporter found in a Gram-positive bacterium. Some hypothetical proteins suggested in *Archaeoglobus fulgidus* (*30*), *Deinococcus radiodulans* (*31*), *Pasteurella multocida* (*32*), *Synechocystis* sp. (*33*), *Mycobacterium tuberculosis* (*34*), *Caenorhabditis elegans* (*35*), *Methanococcus jannaschii* (*36*), and *Vibrio cholerae* (*37*) showed significant sequence similarity with NhaG (Table I).

Southern Blot Analysis—Interestingly, the DNA region missing in the complete genome sequence is sandwiched by two identical sequences, TTTTCTT. Thus, there are two possibilities: (i) that the *nhaG* gene is present in *B. subtilis* ATCC9372 but not in 168, (ii) that the reported genome sequence of *B. subtilis* 168 contains an error (*19*). We tested these possibilities by Southern blot analysis. Chromosomal DNA was prepared from *B. subtilis* ATCC9372 (the strain we used), *B. subtilis* 168 (the strain used in the genome project), and plasmid DNA pBUN23. The probe used was a DNA fragment derived from the coding region of *nhaG* (Fig. 4). A 1.2-kbp DNA band was detected from pBUN23 digested with *Hin*dIII and *Eco*RV (Fig. 4, lane 1). We also detected a 1.2-kbp DNA band in the chromosomal DNA of ATCC9372 digested with the same restriction enzymes (Fig. 4, lane 5). However, no hybridized band was detected with chromosomal DNA from strain 168 (Fig. 4, lanes 2 and 3). We obtained strain 168 from two independent sources, and both gave the same results on Southern blot analysis. Thus, it became clear that the *nhaG* region is present in strain ATCC9372 but not in strain 168. Furthermore, we tested whether the *nhaG* region was present in another *B. subtilis* strain, 160. No hybridized band was detected with chromosomal DNA of this strain (Fig. 4, lane 4).

Hydropathy Analysis—Hydropathy analysis of the deduced NhaG sequence by the method of Eisenberg *et al.* (*38*) revealed that there are 12 hydrophobic domains which may be transmembrane domains (Fig. 5). A hydrophilic stretch with more than 100 amino acid residues is present at the carboxyl terminal region of NhaG. Such a long hydrophilic stretch is not present in any other microbial Na^+/H^+ antiporter except for SynNhaP in *Synechocystis*. On the other hand, a long hydrophilic region has been reported in animal Na^+/H^+ exchangers, NHEs (*29, 39–41*). This fact also supports the notion that NhaG is similar to the Na^+/H^+ exchangers of eukaryotes, rather than to bacterial Na^+/H^+ antiporters.

DISCUSSION

We cloned a gene, *nhaG*, encoding an Na^+/H^+ antiporter from *B. subtilis* ATCC9372, and found that the gene is missing in *B. subtilis* 168, the genome sequence of which has been completely determined (*19*). The gene was found also to be absent in chromosomal DNA of *B. subtilis* strain 160. The missing DNA region was sandwiched by two identical sequences TTTTCTT in the ATCC9372 chromosome. Thus, this sequence may be a signal for transposition or insertion. It seems possible that the DNA region present in ATCC9372 but absent in 168 and 160 came from another organism by transposition. The following facts suggest that the DNA is from an organism which is rather closely related to *B. subtilis*: (i) G+C content of the *nhaG* DNA region was 45%, and that in whole genome of 168 was 44%, (ii) a promoter-like sequence for the *nhaG* gene showed close similarity to promoter sequences of *B. subtilis* genes (*26*).

In *B. subtilis*, some Na^+ extrusion systems have been identified. Previously, we suggested that the *B. subtilis yuf*

genes, which were deduced from the whole genome sequence, would encode an Na^+/H^+ antiporter based on the fact that the *yuf* genes showed high sequence similarity with *mnh* genes encoding the multi-subunit type Na^+/H^+ antiporter of *S. aureus* (*1*). In fact, it has been reported recently that products of the *yuf* (*mrp*) genes are for an Na^+/H^+ antiporter, and that this antiporter is involved in resistance to cholate and in pH homeostasis under alkaline conditions (*3*). It has been also reported that the tetracycline/H^+ antiporter TetA(L), which is encoded by a chromosomal gene of *B. subtilis*, also functions as an Na^+/H^+ antiporter (*42–44*). An ABC (ATP binding cassette)-type Na^+ extrusion pump, NatAB, which is energized by ATP, has been reported in *B. subtilis* (*45*). Moreover, it has been implied that NhaC (YheL) plays a role in Na^+ uptake rather than Na^+ extrusion (*46*). We have also cloned the *mrp* genes from *B. subtilis* ATCC9372 (unpublished data). Ito *et al.* showed that an *mrp* deletion mutant became extremely sensitive to NaCl in the medium (*3*) Absence of the *nhaG* gene in *B. subtilis* 168 suggests that NhaG is not essential for the normal level of Na^+ resistance in this organism. Since the putative promoter sequences of the *nhaG* gene are similar to those for stress- or starvation-related genes, it is possible that *nhaG* is a stress-related gene, especially under the condition of higher or lower Na^+.

A homology search using the primary sequence of NhaG revealed that NhaP of *P. aeruginosa* (*16*) and the SynNhaP of *Synechocystis* sp. (*27, 28*) are bacterial and cyanobacterial Na^+/H^+ antiporters that showed significant sequence similarity to the NhaG. No significant similarity was found with NhaA (*11, 25*), NhaB (*13, 18*), NhaD (*15*), ChaA (*12*), or NapA (*17*). Although no significant similarity through the entire sequence was found with NhaC of *B. firmus* (*14*) and NhaC (YheL) of *B. subtilis* (*46*), partial sequence similarity was found with NhaG. Several hypothetical proteins from several organisms, which have been suggested from genome sequences, also showed significant sequence similarity with NhaG. Thus, this group of Na^+/H^+ antiporters may be present in a rather wide range of organisms. The Na^+/H^+ exchangers of animal cells, SynNhaP of *Synechocystis* and some hypothetical Na^+/H^+ antiporters listed in Table I [NHE2 (*A. fulgidus*), SynNhaP2 (*Synechocystis* sp), YjcE (*M. tuberculosis*), and NAH (*C. elegans*)], possess a hydrophilic stretch at the carboxyl termini. It has been reported that the hydrophilic carboxyl terminal region in animal NHEs is involved in regulation of function (*49–51*). This suggested that the hydrophilic region of the NhaG carboxyl terminus may be involved in regulation of its function. However, we were unable to find sequence similarity in the carboxyl terminal hydrophilic regions between NhaG and NHEs. Therefore, the mechanism of regulation in NhaG, if present, may be different from that in NHEs.

In addition, we constructed a plasmid carrying a truncated *nhaG* gene lacking the 3′-region corresponding to 26 amino acid residues. *E. coli* KNabc cells harboring the plasmid were still sensitive to 0.2 M NaCl (data not shown). Thus, the lack of 26 amino acid residues from the carboxyl terminus of NhaG severely damaged its function. It has also been reported that SynNhaP of *Synechocystis* from which the carboxyl terminal 55 amino acid residues were truncated could not fully complement the Na^+- and Li^+-sensitive phenotype (*27*). Very recently, an Na^+/H^+ antiporter of halotolerant cyanobacterium *Aphanothece halophytica* (ApNhaP) has been cloned, sequenced and characterized (*52*). Like SynNhaP, ApNhaP could not complement the Li^+-sensitive phenotype in *Escherichia coli* TO114, a mutant deficient in the Na^+/H^+ antiporters. Growth of *E. coli* KNabc possessing the *nhaG* gene was low in the presence of 10 mM LiCl (Fig. 1B). This result suggests that the ability of NhaG to recognize and/or transport Na^+ and Li^+ is more similar to that of ApNhaP than that of SynNhaP. It has also been reported that the carboxyl terminal hydrophilic tail of ApNhaP is important for its ion specificity (*52*). We found some sequences in the carboxyl tail structure of NhaG that were similar to those of SynNhaP, but not ApNhaP. In addition, the net charge of the carboxyl tail of NhaG was quite similar to that of SynNhaP [NhaG: acidic (Asp+Glu) 23, basic (Arg+Lys) 15, SynNhaP. acidic 24, basic 15]. Thus, it is likely that not only the long hydrophilic stretch at the carboxyl terminus but also the transmembrane region is important for the ion specificity in NhaG.

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