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A Putative Multisubunit Na^+/H^+ Antiporter from *Staphylococcus aureus*

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We cloned several genes encoding an Na^+/H^+ antiporter of *Staphylococcus aureus* from chromosomal DNA by using an *Escherichia coli* mutant, lacking all of the major Na^+/H^+ antiporters, as the host. *E. coli* cells harboring plasmids for the cloned genes were able to grow in medium containing 0.2 M NaCl (or 10 mM LiCl). Host cells without the plasmids were unable to grow under the same conditions. Na^+/H^+ antiport activity was detected in membrane vesicles prepared from transformants. We determined the nucleotide sequence of the cloned 7-kbp region. We found that seven open reading frames (ORFs) were necessary for antiporter function. A promoter-like sequence was found in the upstream region from the first ORF. One inverted repeat followed by a T-cluster, which may function as a terminator, was found in the downstream region from the seventh ORF. Neither terminator-like nor promoter-like sequences were found between the ORFs. Thus, it seems that the seven ORFs comprise an operon and that the Na^+/H^+ antiporter consists of seven kinds of subunits, suggesting that this is a novel type of multisubunit Na^+/H^+ antiporter. Hydropathy analysis of the deduced amino acid sequences of the seven ORFs suggested that all of the proteins are hydrophobic. As a result of a homology search, we found that components of the respiratory chain showed sequence similarity with putative subunits of the Na^+/H^+ antiporter. We observed a large Na^+ extrusion activity, driven by respiration in *E. coli* cells harboring the plasmid carrying the genes. The Na^+ extrusion was sensitive to an H^+ conductor, supporting the idea that the system is not a respiratory Na^+ pump but an Na^+/H^+ antiporter. Introduction of the plasmid into *E. coli* mutant cells, which were unable to grow under alkaline conditions, enabled the cells to grow under such conditions.

The Na^+/H^+ antiporter is widely distributed in cell membranes from bacteria to animals. The antiporter plays important roles in the Na^+ cycle across the cytoplasmic membrane of all living cells (22, 34, 54). In bacteria, the antiporter extrudes Na^+ or Li^+ in exchange for H^+. The driving force for this process is an electrochemical potential of H^+ across the membrane, which is established either by the respiratory chain or the H^+-translocating ATPase (22). However, in animals, an H^+ is extruded from cells in exchange for Na^+ via the antiporter (called the exchanger in animal cells). The driving force is an electrochemical potential of Na^+ which is established by the Na^+,K^+-ATPase.

The Na^+/H^+ antiporter has several roles in bacterial cells: (i) establishment of an electrochemical potential of Na^+ across the cytoplasmic membrane, which is the driving force for Na^+-coupled processes such as Na^+/solute symport (4, 11, 18, 46, 47) and Na^+-driven flagellar rotation (13); (ii) extrusion of Na^+ and Li^+, which are toxic if accumulated at high concentrations in cells (14, 31, 33, 37); (iii) intracellular pH regulation under alkaline conditions (22, 34); and (iv) cell volume regulation (10, 34). Mutants of *Escherichia coli* which lack the Na^+/H^+ antiporter activity have been isolated (9, 31). Such mutants facilitated cloning of the gene(s) encoding the Na^+/H^+ antiporter. So far, genes for three distinct Na^+/H^+ antiporters of *E. coli*, *nhaA* (19), *nhaB* (36), and *chaA* (17), have been cloned and sequenced. The NhaA and NhaB antiporters have been purified and biochemically characterized (38, 42). Furthermore, homologs of *nhaA* and *nhaB* have been found in several other bacteria. These homologous genes have been cloned and sequenced. They include *nhaA* in *Salmonella enteritidis* (35), *Vibrio parahaemolyticus* (24), and *Vibrio alginolyticus* (29) and *nhaB* in *V. parahaemolyticus* (31) and *V. alginolyticus* (30). Furthermore, it has become clear that homologs of the *nhaA* and *nhaB* genes are present in *Haemophilus influenzae*, of which the entire chromosomal DNA sequence has been determined (8). Genes for other Na^+/H^+ antiporters have also been cloned and sequenced and include *nhaC* in *Bacillus firmus* (16), *napA* in *Enterococcus hirae* (52), *nhaP* in *Pseudomonas aeruginosa* (51), and *nhaD* in *V. parahaemolyticus* (32). Only one gene, and therefore one protein, is involved in Na^+/H^+ antiport in all of these Na^+/H^+ antiporters. Recently, a unique antiporter, called TetA(L), has been reported in *Bacillus subtilis*. TetA(L) is both an Na^+/H^+ antiporter and a tetracycline/H^+ antiporter (5, 6). It should be stressed that only one protein [TetA(L)] mediates these two distinct antiport activities. For the two types of Na^+/H^+ antiporters, some similarities and diversities in primary structure and functional properties do exist. Thus, a comparison of the primary structures and the properties of many Na^+/H^+ antiporters would help to gain insight into their structure-function relationships.

Staphylococcus aureus is a halotolerant bacterium (20). This microorganism can survive even in the presence of 3 M NaCl or 1 M LiCl. We detected Na^+/serine symport activity in *S. aureus* (1). *S. aureus* cells are able to grow under alkaline conditions, up to pH 9.5. Therefore, it seems that *S. aureus* possesses a strong Na^+/H^+ antiport activity. Indeed, in everted membrane vesicles prepared from cells of *S. aureus*, we detected Na^+/H^+ antiport activity (21). Here we report on a putative

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multisubunit Na^+/H^+ antiporter of *S. aureus* and its characteristics.

MATERIALS AND METHODS

Organisms, media, and growth. *S. aureus* 209P was grown in nutrient broth (0.5% beef extract, 1.5% polypepton, 0.5% K_2HPO_4, 85 mM NaCl). The *E. coli* strains TG1 and KNabc, which lacks three major Na^+/H^+ antiporters (NhaA, NhaB, and ChaA) (31), were grown in modified L medium (2) [NaCl in the original medium was replaced with KCl; hereafter called L(K) medium]. NaCl or LiCl was added at the indicated concentration to the medium when necessary. To test the effect of pH on growth, *E. coli* HITΔAB$^-$ (44) or HITΔAB$^-$/pNAS20 was grown in a minimal medium (100 mM Tris-HCl [at indicated pHs], 20 mM $(NH_4)_2SO_4$, 50 mM KCl, 1 mM K_2HPO_4, 0.3 mM $MgSO_4$, 0.01 mM $CaCl_2$) supplemented with 40 mM glycerol (15). Cells were grown under aerobic conditions at 37°C. Cell growth was monitored turbidimetrically at 650 nm.

Preparation of membrane vesicles. *E. coli* cells were grown in L(K) medium supplemented with 20 mM potassium lactate. Everted membrane vesicles were prepared by passing cells through a French press as described previously (24).

Na^+/H^+ antiport assay. Activity of the Na^+/H^+ antiporter was measured by the quinacrine fluorescence quenching method with everted membrane vesicles as described previously (24) by use of a Hitachi F2000 fluorescence spectrophotometer.

Na^+ extrusion assay. *E. coli* cells grown in L(K) medium were washed twice with a buffer containing 0.2 M 3-[*N*-morpholino]propanesulfonic acid (MOPS) and 5 mM $MgSO_4$ (pH adjusted to 7.5 with tetramethylammonium hydroxide) and then suspended in a buffer containing 0.2 M *N*-tris[(hydroxymethyl)methyl] glycine (Tricine) and 5 mM $MgSO_4$ (pH adjusted to 8.0 with tetramethylammonium hydroxide). Movement of Na^+ out of cells was measured with an Na^+-selective electrode as described previously (47).

Cloning of genes. *E. coli* KNabc is a mutant lacking all three major Na^+/H^+ antiporters and a restriction system (*hsdΔ5*) (31). Therefore, this mutant is useful for the cloning of an Na^+/H^+ antiporter gene(s) from another organism (31, 32, 51). *S. aureus* 209P cells were grown in nutrient broth. Ampicillin (final concentration, 100 μg/ml) was added to the culture medium at the exponential phase of growth to weaken its peptidoglycan layer. Chromosomal DNA was prepared from *S. aureus* cells by the method of Ausubel et al. (3). The DNA was partially digested with *Sau*3A1. Restriction fragments between 4 to 10 kbp were separated by sucrose density gradient centrifugation. The DNA fragments were ligated into either pUC19 or pBR322 (which had been digested with *Bam*HI and dephosphorylated with bacterial alkaline phosphatase) by using T4 DNA ligase. Competent cells of *E. coli* KNabc were transformed with the ligated recombinant plasmids and spread onto 1.5% agar plates containing L(K) medium, 0.2 M NaCl (or 10 mM LiCl), and 100 μg of ampicillin per ml. The plates were incubated at 37°C for 1 day. The clones formed were picked. Plasmid DNA was prepared from the transformants. Competent cells of *E. coli* KNabc were retransformed and spread onto the same plates again. The plates were incubated at 37°C for 1 day. Many colonies appeared on the plates. Plasmids that were harbored by the retransformants were prepared. We obtained 25 candidate plasmids (13 candidates from the NaCl plate and 12 candidates from the LiCl plate; 20 candidates from the pUC19 vector and 5 candidates from the pBR322 vector). All of the plasmids possessed common restriction fragments in addition to several different ones. We therefore concluded that all of the plasmids carry a common portion of chromosomal DNA of *S. aureus*. We selected one of the plasmids, pNAS20, which carried the shortest DNA insert, for further analysis.

Southern analysis. Southern hybridization analysis was performed with the Enhanced Chemiluminescence Detection System of Amersham Corp., as suggested by the manufacturer. The probe used was an *Eco*RV-*Mlu*I fragment (1.0 kbp) derived from the *mnhD* gene of *S. aureus*. Chromosomal DNA prepared from *S. aureus* or *E. coli* was digested with *Eco*RV and *Mlu*I, separated by electrophoresis in a 1% agarose gel, and blotted onto a nitrocellulose membrane. The probe was hybridized with the resulting Southern blot.

Sequencing. A series of deletion plasmids for sequencing were constructed by using exonuclease III and mung bean nuclease from pNAS20. The nucleotide sequence was determined by the dideoxy chain termination method (40) using a DNA sequencer (ALF Express; Pharmacia Co.). Sequencing of both the sense and antisense strands was completed.

Computer analysis of sequence data. Sequence data were analyzed with the GENETYX sequence analysis software (Software Development Co.). The SwissProt database was screened for sequence similarities.

Other assays and materials. Protein content was determined by the method of Lowry et al. with bovine serum albumin as a standard (28). Reagents for DNA manipulation, sequencing, bacteriological media, and other chemicals were obtained from the usual commercial sources.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the DDBJ, GenBank, and EMBL databases under accession no. AB015981.

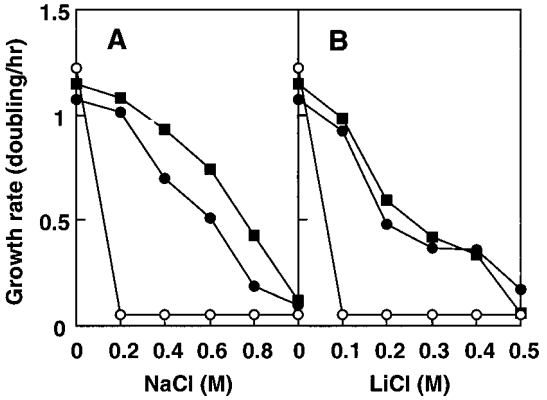

FIG. 1. Effect of Na^+ and Li^+ on the growth of cells. *E. coli* TG1 (■), KNabc (○), and KNabc/pNAS20 (●) were grown in L(K) medium containing various concentrations of NaCl (A) or LiCl (B) under aerobic conditions at 37°C.

RESULTS

Cloning of genes. *E. coli* KNabc cells were not able to grow in the presence of concentrations equal to or higher than 0.2 M NaCl or 10 mM LiCl (Fig. 1). The parental cells from strain TG1, grew well under such conditions. We tried to clone a gene(s) for an Na^+/H^+ antiporter(s) or an Na^+-extruding system(s) of *S. aureus*. By employing the shotgun method of cloning, we obtained 25 candidate plasmids carrying fragments of *S. aureus* chromosomal DNA that enabled the host cells, from strain KNabc, to grow in the presence of 0.2 M NaCl or 10 mM LiCl. However, restriction mapping suggested that all 25 candidate plasmids carried a common region of the *S. aureus* chromosome. KNabc cells harboring each one of the candidate plasmids showed similar growth capabilities in the presence of 0.2 M NaCl (data not shown). Thus, we chose one of them, pNAS20, which carried the shortest DNA insert. KNabc cells containing pNAS20 restored growth in the presence of 0.2 M NaCl or 0.1 M LiCl (Fig. 1). The transformed cells were able to grow even in the presence of 0.8 M NaCl or 0.4 M LiCl. Thus, it is likely that plasmid pNAS20 carries a gene(s) for Na^+/H^+ antiporter activity in *S. aureus*. In addition, it is likely that the putative antiporter gene(s) of *S. aureus* has been expressed in *E. coli* cells and that the gene product(s) has been synthesized in a functional form. It is unlikely that the gene product(s) activated the inactive *E. coli* Na^+/H^+ antiporters NhaA, NhaB, and ChaA, because KNabc is a deletion mutant of *nhaA*, *nhaB*, and *chaA* (31).

To test whether the pNAS20 really carries the Na^+/H^+ antiporter gene(s), we measured Na^+/H^+ antiport activity with everted membrane vesicles from KNabc/pNAS20 cells. Indeed, we detected Na^+/H^+ antiport activity in membrane vesicles of KNabc/pNAS20 but not in those of KNabc (Fig. 2). A weak Li^+/H^+ antiport activity was also detected in KNabc/pNAS20 (Fig. 2). The antiport activities were observed at pH 7.0. This suggests that the Na^+/H^+ antiporter of *S. aureus* is not of the NhaA type, the principal Na^+/H^+ antiporter of *E. coli* or *V. parahaemolyticus*, the activity of which is not measurable at pH 7.0 but very high at pH 8.5 (24, 33).

Therefore, we tested the effect of pH on the Na^+/H^+ antiporter activity in membrane vesicles of KNabc/pNAS20. The activity was at its maximum at pH 7.0 to 7.5 (data not shown). Thus, the pH profile for the Na^+/H^+ antiporter activity derived from *S. aureus* is obviously different from that of NhaA of *E. coli* (33) or *V. parahaemolyticus* (24). In addition, the pH profile is not similar to that of the NhaB Na^+/H^+ antiporter of *E. coli* (14, 33) or *V. parahaemolyticus* (31). Activity of the

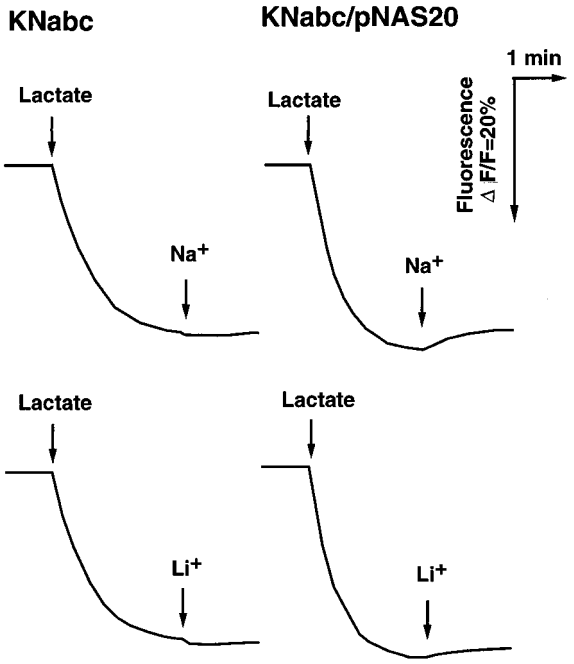

FIG. 2. Na^+/H^+ antiport and Li^+/H^+ antiport activities in membrane vesicles. Everted membrane vesicles were prepared from *E. coli* KNabc and KNabc/pNAS20 cells by the French press method. Antiport activities were measured by the quinacrine fluorescence quenching method. At the time point indicated by the first arrow on the left, potassium lactate (final concentration, 5 mM) was added to the assay mixture to initiate respiration. At time point indicated by the second arrow from the left, NaCl (final concentration, 5 mM) or LiCl (final concentration, 5 mM) was added.

NhaB system is measurable at pH 7.0 and higher at alkaline pHs such as pH 8.0 or 8.5. The ChaA system of *E. coli* utilizes Ca^{2+} as an efficient substrate (17). We detected no significant difference in Ca^{2+}/H^+ antiport activity between membrane vesicles prepared from cells of KNabc/pNAS20 and those from cells of KNabc (data not shown). Thus, the properties of the Na^+/H^+ antiporter derived from pNAS20 are different from those of NhaA, NhaB, and ChaA.

Sequences. We constructed many deletion derivatives from pNAS20 in order to localize the region of the DNA insert where the antiporter gene(s) is located (Fig. 3). The length of the insert derived from chromosomal DNA of *S. aureus* in the pNAS20 was about 7 kbp. This is too long for an Na^+/H^+ antiporter possessing about 12 transmembrane domains. It has been shown that the length of the gene for NhaA (19), NhaB (35), or ChaA (17) (these antiporters possess about 12 transmembrane domains) is about 1.5 kbp. Deletion plasmids were constructed and introduced into KNabc cells. The growth capability in the presence of 0.2 M NaCl was tested. Surprisingly, we found that most (about 6 kbp) of the DNA insert carried on the pNAS20 plasmid was necessary for growth in the presence of 0.2 M NaCl (Fig. 3). One deletion plasmid, pNAS2081, which lacks an internal portion of the DNA insert, did not enable KNabc cells to grow in the presence of 0.2 M NaCl (Fig. 3). These results suggest that either the Na^+/H^+ antiporter of *S. aureus* consists of multiple subunits, multiple components are involved in regulation of the antiporter, or the antiporter protein is a giant protein with roughly 2,000 amino acid residues.

We determined the sequence of 6,995 nucleotides of the DNA insert of pNAS20 (DDBJ/EMBL/GenBank accession no. AB015981). We found eight open reading frames (ORFs) and a part of another ORF in the sequenced region. All of the ORFs were preceded by Shine-Dalgarno sequences (41). Amino acid sequences were deduced from these ORFs. Analysis of a series of deletion plasmids revealed that the first ORF and the incomplete ORF found in the last portion of the DNA insert were not necessary for the Na^+/H^+ antiporter function (Fig. 3). KNabc cells harboring plasmid pNAS2003, carrying the seven ORFs except the first ORF (or the last incomplete ORF), grew in the presence of 0.2 M NaCl (Fig. 3). Furthermore, membrane vesicles prepared from such cells showed Na^+/H^+ antiport activity (data not shown). Thus, we conclude that the seven ORFs are necessary and sufficient for Na^+/H^+ antiporter function. A promoter-like sequence (-35 region and -10 region) was found in the upstream region from the seven ORFs (data not shown). An inverted repeat followed by T-cluster was found in the downstream region from the seven ORFs (data not shown). Neither a terminator-like nor a promoter-like sequence was found between the seven ORFs. Therefore, it seems that the seven ORFs comprise an operon. We designated the operon *mnh*, and the genes included in this operon were designated *mnhA* to *mnhG* (from upstream to downstream).

As a result of a Southern hybridization analysis using a DNA

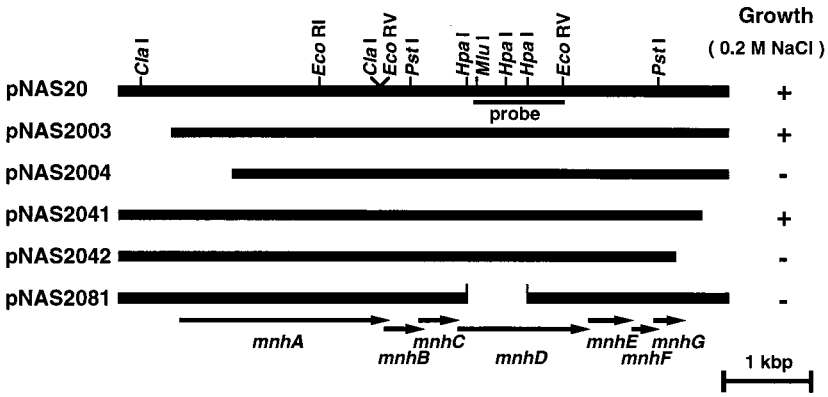

FIG. 3. Plasmids and restriction map. Physical maps of the DNA insert derived from the *S. aureus* chromosome in each plasmid are shown. The DNA inserts are aligned. Restriction sites are present at the same horizontal position in each insert. Locations and directions of each ORF (*mnhA* to *mnhG*) which were revealed by sequencing are shown at the bottom. The probe used for the Southern analysis is also shown. The growth capability of *E. coli* KNabc harboring each plasmid in L(K) medium supplemented with 0.2 M NaCl is shown on the right. Symbols: +, cell growth occurred; −, no cell growth occurred.

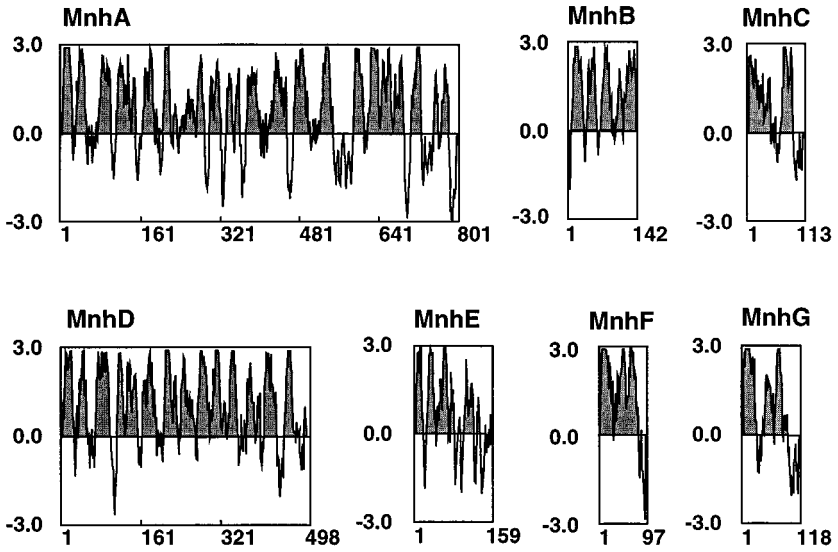

FIG. 4. Hydropathy patterns of deduced Mnh proteins. Hydropathy values were calculated by the method of Kyte and Doolittle (28) for the deduced amino acid sequences of MnhA to MnhG. The values were plotted from the NH_2 terminus to the COOH terminus. The portions above and below the midpoint line indicate hydrophobic and hydrophilic regions, respectively. The hydrophobic regions are shaded.

fragment derived from *mnhD* as a probe (Fig. 3), we detected a dense band in a sample of *S. aureus* chromosomal DNA but not in a sample of *E. coli* chromosomal DNA (data not shown).

Characteristics of the primary structure. Judging from the deduced amino acid sequences, each putative protein (MnhA to MnhG) consists of 801, 142, 113, 498, 159, 97, and 118 amino acid residues, respectively. Calculated molecular weights for MnhA to MnhG are 89,385, 15,682, 12,260, 54,755, 18,319, 10,616, and 12,818, respectively. These proteins are all of low polarity (58 to 71% hydrophobic residues).

Hydropathy profiles calculated by the method of Kyte and Doolittle (26) and plotted along the amino acid sequences suggested that all subunits have hydrophobic domains which seem to be membrane-spanning regions (Fig. 4). In particular, MnhA and MnhD possess many hydrophobic domains. It seems that all of the Mnh proteins are intrinsic membrane proteins.

We searched for homology between the deduced amino acid sequences of MnhA to MnhG and reported sequences in a protein sequence database (SwissProt) (Table 1). We found putative homologs of MnhA to MnhG in *B. subtilis*, for which the complete nucleotide sequence of the genome has been determined (23). A comparison of sequences of the primary structures of MnhA and YufT, MnhB and YufU, MnhC and YufV, MnhD and YufD, MnhF and YufC, and MnhG and YufB revealed 39 to 54% identities and 82 to 89% similarities. Although an ORF corresponding to MnhE has not been identified in the sequence of *B. subtilis* (23), a homolog of MnhE emerges if we remove one nucleotide from the reported sequence of the corresponding region of the *B. subtilis* DNA. The amino acid sequence of the resulting hypothetical homolog showed 38% identity and 79% similarity with the MnhE sequence. Comparison of amino acid residues between MnhA and YufT revealed that the MnhA has 27 additional residues at the NH_2 terminus. We checked whether there is an initiation codon preceded by a Shine-Dalgarno sequence in the corresponding upstream region from the proposed *yufT* gene (EMBL/GenBank/DDBJ accession no. Z93937). In fact, we found a TTG codon preceded by a Shine-Dalgarno sequence in the same frame as the original *yufT*. If this TTG is the initiation codon for the *yufT* gene, the product YufT consists of 801 amino acid residues, exactly the same number of residues that MnhA has. The newly added 27 residues in the YufT sequence had a high sequence similarity with the corresponding region of MnhA. The numbers of amino acid residues in all of the corresponding homologs were similar (data not shown). Thus, the Yuf proteins may form a complex and function as an Na^+/H^+ antiporter in *B. subtilis*. MnhA, MnhB, and MnhC showed sequence similarities (38 to 48% identities and 78 to

TABLE 1. Identities and similarities of amino acid sequence between Mnhs and homologs

S. aureus Na^+/H^+ antiporter	% Identity/% similarity (homolog)				
	B. subtilis hypothetical homolog	*Bacillus* sp. strain C-125 Na^+/H^+ antiporter[a]	*R. meliloti* Pha	*E. coli* NDH-1	Bovine complex I
MnhA	54/86 (YufT)	49/84 (ORF1)	36/76 (PhaA)	31/71 (NuoL)	24/67 (ND5)
MnhB	45/82 (YufU)	39/81 (ORF2)	43/83 (PhaB)	NA[b]	NA
MnhC	52/89 (YufV)	47/88 (ORF3)	42/75 (PhaC)	NA	NA
MnhD	49/86 (YufD)	NA	32/71 (PhaD)	22/67 (NuoN)	18/64 (ND2)
MnhE	38/79 (YufX)[c]	NA	24/73 (PhaE)	NA	NA
MnhF	51/88 (YufC)	NA	25/74 (PhaF)	NA	NA
MnhG	39/82 (YufB)	NA	28/73 (PhaG)	NA	NA

[a] Genes responsible for Na^+/H^+ antiporter (12).
[b] NA, not available.
[c] The deduced product emerged when one nucleotide was removed from the reported sequence (41).

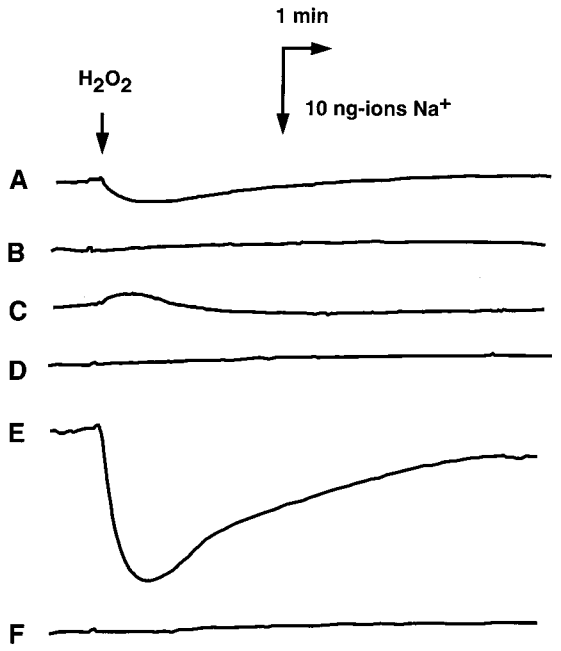

FIG. 5. Na^+ extrusion from cells elicited by respiration. Extrusion of Na^+ from cells of *E. coli* TG1 (A and B), KNabc (C and D), and KNabc/pNAS20 (E and F) was measured with an Na^+ electrode. At the time point indicated by the arrow labeled H_2O_2, a small amount (5 μl of a 0.5% solution) of H_2O_2 was added to the cell suspension (2.5 ml) to supply O_2 and to initiate respiration. In three cases (B, D, and F), CCCP was present at 100 μM in the assay medium. A downward deflection represents Na^+ extrusion from the cells.

87% similarities) with putative genes (ORF1, ORF2, and ORF3) responsible for an Na^+/H^+ antiporter of alkaliphilic *Bacillus* sp. strain C-125 (12) (Table 1). Also, we found homologs of MnhA to MnhG in *Rhizobium meliloti* (EMBL/GenBank/DDBJ database accession no. X93358) (39). They are designated PhaA to PhaG. Sequence similarities between MnhA and PhaA, MnhB and PhaB, MnhC and PhaC, MnhD and PhaD, MnhE and PhaE, MnhF and PhaF, and MnhG and PhaG were demonstrated (21 to 41% identities and 58 to 73% similarities) (Table 1). Interestingly, the sequence of the N-terminal portion (80 residues) of PhaB showed significant similarity (43% identity and 83% similarity) with the C-terminal sequence of MnhA, in addition to similarity between the C-terminal portion (125 residues) of PhaB and the entire region of MnhB (26% identity and 73% similarity). The molecular weights of PhaA to PhaG, except PhaB, roughly correspond to those of MnhA to MnhG, except MnhB.

We also found significant sequence similarities between MnhA and NuoL (53) of *E. coli* (20% identity and 46% similarity), MnhA and ND5 (1a) of bovine mitochondria (19% identity and 53% similarity), MnhD and NuoN (53) of *E. coli* (18% identity and 55% similarity), and MnhD and ND2 (1) of bovine mitochondria (13% identity and 46% similarity). NuoL, NuoN, ND5, and ND2 are components of the respiratory chain (1a, 53). Thus, it seemed possible that the protein products MnhA to MnhG are components of the respiratory chain of *S. aureus* and that Na^+ extrusion via the Mnh complex is directly driven by respiration, similar to the case of the respiratory Na^+ pump reported in *V. alginolyticus* (45) and *V. parahaemolyticus* (49). We tested these possibilities. Previously, we reported Na^+ extrusion from *E. coli* that is driven by an electrochemical potential of H^+ across the membranes by the Na^+/H^+ antiport mechanism (48) and from *V. parahaemolyticus* that is directly driven by the respiratory chain, the respiratory Na^+ pump (49). A key experiment to distinguish between these two mechanisms is to test the effect of an H^+ conductor on the respiration-driven Na^+ extrusion from cells. If the Na^+ extrusion is mediated by an Na^+/H^+ antiporter, an H^+ conductor should inhibit the Na^+ extrusion (48). On the other hand, if Na^+ extrusion is mediated by the respiratory complex, an H^+ conductor should not inhibit the Na^+ extrusion (49). In control experiments, we observed Na^+ extrusion from cells of wild-type *E. coli* (Fig. 5A), which was completely inhibited by an H^+ conductor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Fig. 5B). We detected no Na^+ extrusion in KNabc cells, which lack all of the major Na^+/H^+ antiporters. Thus, the Na^+ extrusion observed with wild-type *E. coli* is mediated by Na^+/H^+ antiporters. We detected some Na^+ uptake driven by respiration in KNabc cells (Fig. 5C). Perhaps this Na^+ uptake is due to the membrane potential established by respiration. We observed a large Na^+ extrusion from cells of KNabc/pNAS20 which was elicited by respiration (Fig. 5E). This large Na^+ extrusion was completely inhibited by CCCP (Fig. 5F). Thus, it seems that the Mnh complex is not a respiratory Na^+ pump but an Na^+/H^+ antiporter.

pH resistance in growth. Previously we reported the isolation of a mutant of *E. coli* HIT-1 which showed a defect in the Na^+/H^+ antiporter and a defect in growth under alkaline conditions (15). It is not clear yet, however, whether another mutation is present in the mutant. We constructed a series of Na^+/H^+ antiporter mutants (44). The mutant strain HITΔAB$^-$ is one of the mutants lacking both NhaA and NhaB antiporter activities (44). We tested whether the Na^+/H^+ antiporter genes from *S. aureus* confer upon *E. coli* HITΔAB$^-$ the ability to grow under alkaline conditions, in addition to exhibiting Na^+/H^+ antiporter activity. The wild-type *E. coli* grew well at pHs up to 8.5 (15). As shown in Fig. 6, the growth rate of HITΔAB$^-$ was greatly reduced under alkaline conditions (especially at pHs above 8.0) compared with that at neutral pH. Introduction of the plasmid pNAS20 into HITΔAB$^-$ cells restored growth at pHs between 7.6 and 8.3 (Fig. 6). It has been reported that *pha* genes of *R. meliloti* are involved in pH adaptation (39).

DISCUSSION

We found putative Na^+/H^+ antiporter genes in *S. aureus*. Our data suggest that the putative Na^+/H^+ antiporter consists of seven kinds of subunits. All of the Na^+/H^+ antiporters or

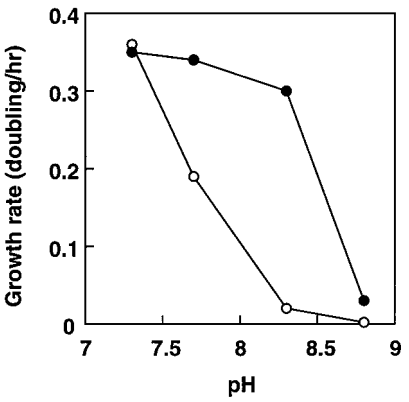

FIG. 6. Effects of pH on cell growth of *E. coli* HITΔAB$^-$ and HITΔAB$^-$/pNAS20. *E. coli* HITΔAB$^-$ (○) and HITΔAB$^-$/pNAS20 (●) were grown in minimal medium supplemented with glycerol at the indicated pHs at 37°C under aerobic conditions.

Na^+/H^+ exchangers so far identified in microbial or animal cells are of a single subunit, although we do not yet know whether they function as monomers or homo-oligomers. Several examples of Na^+ extrusion systems which consist of multiple components present in cell membranes are known; these include the respiratory Na^+ pump of halophilic bacteria (50), the F_0F_1-type Na^+-translocating ATPase of *Propionigenium modestum* (27), the Na^+-translocating ATPase of *Enterococcus hirae* (43), and a two-gene ATP-binding cassette (ABC)-type Na^+ extrusion system of *B. subtilis* (7). No similarity was found in the primary structure of components of the *S. aureus* Na^+/H^+ antiporter and components of these Na^+ extrusion systems. No ABC motif was found in any of the subunits of the Na^+/H^+ antiporter of *S. aureus* (data not shown).

We have found homologs of the *mnh* genes in *B. subtilis*, namely, the *yuf* genes. Recently, we cloned a DNA fragment containing the *yuf* region from the *B. subtilis* genome. *E. coli* KNabc cells harboring a plasmid carrying this DNA region restored Na^+/H^+ antiport activity (8a). It is also very likely that a similar Na^+/H^+ antiporter exists in the alkaliphilic *Bacillus* sp. strain C-125 (12). Hamamoto et al. reported a mutant of the alkaliphilic *Bacillus* in which the activity of the putative Na^+/H^+ antiporter was lost (12).

The overall GC content of the cloned gene was 33%, which is close to that of the genomic DNA of *S. aureus* (34%) (20). On the other hand, *R. meliloti* is a GC-rich bacterium in which the GC content in the *pha* operon (EMBL/GenBank/DDBJ accession no. X93358) is 63%. *R. meliloti* is a gram-negative and rod-shaped bacterium. *S. aureus* is a gram-positive coccus. It is interesting that genes of the same type are present both in *S. aureus* and in a quite different bacterium, *R. meliloti*.

Growth of KNabc/pNAS20 was observed in the presence of 0.8 M NaCl (or 0.4 M LiCl) (Fig. 1). This suggested that *S. aureus* Na^+/H^+ antiporter activity produced in *E. coli* is fairly high. In fact, we observed very high Na^+ extrusion activity in cells due to the Na^+/H^+ antiporter in KNabc/pNAS20 (Fig. 5). However, Na^+/H^+ antiporter activity was very low, as measured by the fluorescence quenching method, in everted membrane vesicles prepared from KNabc/pNAS20 (Fig. 2). At present, we do not know the reason for this discrepancy. Although cells of *S. aureus* can grow in the presence of a high concentration of NaCl or at alkaline pHs, which suggests the presence of a strong Na^+/H^+ antiporter in this microorganism, we detected weak (or moderate) Na^+/H^+ antiporter activity in membrane vesicles (21). Thus, some factor(s) present in the cytoplasm may be necessary for the full activity of the Mnh Na^+/H^+ antiporter. Addition of ATP to the mixture of the fluorescence quenching assay showed no effect (data not shown). Another possibility is that some component(s) of the Mnh system is unstable in membrane vesicles.

We obtained 25 candidate plasmids that enabled *E. coli* KNabc transformants to grow in the presence of 0.2 M NaCl or 10 mM LiCl. All of them seemed to carry a common DNA region of the *S. aureus* chromosome. This suggests that the Mnh Na^+/H^+ antiporter is the sole major Na^+ (and Li^+) extrusion system in *S. aureus*. Another possibility is that another Na^+/H^+ antiporter(s) of *S. aureus* is not expressed in *E. coli*.

We observed some Na^+ uptake driven by respiration in *E. coli* KNabc which lacks all of the major Na^+/H^+ antiporters, although Na^+ was extruded from parental cells. This suggests the presence of a membrane potential-driven Na^+ influx system in *E. coli*, perhaps a Na^+ channel. Recently, we developed a patch clamp method which was applicable to the measurement of ion flux through bacterial ion channels or ion pumps (25). This method could be utilized to analyze not only ion channels of bacterial cells but also the putative multisubunit Na^+/H^+ antiporter, Mnh, if the antiport process is electrogenic. In fact, it has been reported that the Na^+/H^+ antiporter probably consisting of ORF1 to ORF3 (and perhaps some others) of *Bacillus* sp. strain C-125 functions as an electrogenic transporter (12).

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