Expression of functional Na^+/H^+ antiporters of *Helicobacter pylori* in antiporter-deficient *Echerichia coli* mutants

Hiroki Inoue[a,b], Tatsuya Sakurai[a], Satoshi Ujike[a], Tomofusa Tsuchiya[c], Hiroshi Murakami[a], Hiroshi Kanazawa[a,b,*]

[a] *Department of Biotechnology, Faculty of Engineering, Okayama University, Machikaneyama-cho 1-16, Toyonaka City, Osaka, Japan*
[b] *Department of Biological Sciences, Graduate School of Science, Osaka University, Machikaneyama-cho 1-16, Toyonaka City, Osaka, Japan*
[c] *Department of Pharmaceutical Sciences, Okayama University, Machikaneyama-cho 1-16, Toyonaka City, Osaka, Japan*

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Abstract An open reading frame with a sequence homologous to *Escherichia coli* Na^+/H^+ antiporter A (ENhaA) was found in the total genomic sequence of *Helicobacter pylori*, a pathogenic bacterium of gastric inflammation, and was named HNhaA. The primary sequences and the hydropathy profiles of ENhaA and HNhaA were very homologous except for one additional region found in HNhaA. This sequence has about 40 hydrophilic amino acid residues inserted at the position next to residue 235 of ENhaA which corresponds to residue 245 of HNhaA. HNhaA was expressed in *E. coli* mutants deficient in Na^+/H^+ antiporters and complemented the salt-sensitive phenotype of the mutants. Membrane vesicles prepared from these transformants of HNhaA using mutants deficient in the antiporters had the antiporter activities. Surprisingly, the antiporter activity in the transformant membranes was high at acidic and neutral pH, while ENhaA did not function at these pHs. A hydrophilic region around residue 235 in ENhaA and the additional hydrophilic region of about 40 residues in the same region found in HNhaA might be responsible for this difference in activity by acting as putative pH sensors.

Key words: Na^+/H^+ antiporter; *Helicobacter pylori*; *Escherichia coli*

1. Introduction

Na^+/H^+ antiporters are found in the cytoplasmic membranes of organisms from bacteria to humans and/or in their intracellular vesicles, where their function is to maintain intracellular pH, salt concentration, and osmolarity [1–3]. In *Escherichia coli*, three antiporters (NhaA, NhaB and ChaA) are known and their functional characteristics have been well described [4–8]. The growth of each of the three antiporter-deficient mutants was found to be inhibited by high concentrations of NaCl or the presence of LiCl [9].

The antiporter activity of *E. coli* NhaA (ENhaA) is enhanced at alkaline pH, and very low at neutral and acidic pH. ENhaA was overproduced and purified as an active form in vitro [6]. Its functionally important residues and topology in *E. coli* membranes have been analyzed [10–12]. We have identified three Asp residues that are essential for its antiporter activity [10]. Although the topological arrangement of ENhaA in the cell membranes has been estimated by *phoA* fusion experiments [12], its tertiary structure in membranes is not precisely known. Understanding the tertiary structure is essential for understanding the relationship between structure and function of ENhaA.

Since the activity of ENhaA depends on the pH outside membrane vesicles, surveying the characteristics of antiporters in other bacteria living in pH environments different from that of *E. coli* could provide some insights into the functioning of ENhaA including mechanisms of the hypothetical pH sensor. From this point of view, we surveyed a homologous sequence of NhaA in *Helicobacter pylori* [13] which is known as a pathogenic bacterium of gastric inflammation that lives under very acidic conditions. We found one open reading frame that has a sequence homologous to that of ENhaA (HNhaA). We have subcloned the sequence (HNhaA) into an expression vector of *E. coli* and introduced it into *E. coli* mutants deficient in the antiporters. HNhaA complemented the defective phenotype of an *E. coli* mutant whose growth is inhibited by LiCl and high concentrations of NaCl. HNhaA in *E. coli* membrane vesicles, unlike ENhaA, exhibited a high activity in the pH range from 6.0 to 8.5.

2. Materials and methods

2.1. E. coli strains and culture conditions

The following *E. coli* strains were used: HITΔAB$^-$ (Δ*lacY*, Δ*nhaA*, *nhaB*$^-$) [15], KNabc(Δ*nahA*::Km^r, Δ*nhaB*::Em^r, Δ*chaA*:::Cm^r, *sup*E, *hsd*Δ5, *thi*, Δ(*lac-pro*AB)/F′ (*tra*Δ36, *pro*AB$^+$, *lacI*q, *lac*ΔM15 [9]) and JM103 [16]. These *E. coli* strains were cultured in L broth (LB) containing 87 mM KCl (LBK) instead of NaCl or Tanaka minimal medium [17]. For agar plates, 1.5% agar was added to these media. For selection of transformants by an expression vector, an appropriate antibiotic was added to the medium if it was necessary. For analyses of salt tolerance of HITΔAB$^-$ or KNabc with various plasmids, various concentrations of NaCl or LiCl were added to the LB plate containing 87 mM NaCl and the plate was incubated at 37°C.

2.2. Construction of expression plasmid

An open reading frame (HP1552) whose sequence is homologous to that of *E. coli* NhaA exists between nt 1 632 576 and 1 631 263 of the total genomic sequence of *H. pylori* [13,14]. This region was cloned to λ vector AE000653 and the putative NhaA gene was divided into two parts, 1 630 251–1 631 944 in plasmid GHPEI49 and 1 631 941–1 633 702 in plasmid GHPBC16 [13,14]. Therefore, four base pairs, 1 631 941–1 631 944, overlapped in the two plasmids. To join these separated regions, we applied two-step PCR. The first PCR was done as described previously [18]. When GHPEI49 was the template, primers M13 RV and HPnhaA–M2 (5′-GTTCATTCCCTA-CTCGCTCGCGTATA-3′), and *Tth* or *Pfu* DNA polymerase were used. Under these conditions, a 1693 bp insert DNA was amplified. When GHPBC16 was the template, primers HPnhaA-M1 (5′-GG-GATGAGCGAGCGCATATTCAGGCG-3′) and M13 FW, and *Tth* DNA or *Pfu* polymerase were used. Under these conditions, a 1761 bp insert DNA was amplified. In the second PCR, these amplified DNAs were used as the templates and HPnhaA–C and HPnhaA–N were used as the primers. HPnhaA–C includes a *Sph*I site and HPnhaA–N includes an *Eco*RI site. Under these conditions, DNA

*Corresponding author. Fax: (81) (6) 850-5817.
E-mail: kanazawa@chaos.bio.sci.osaka-u.ac.jp

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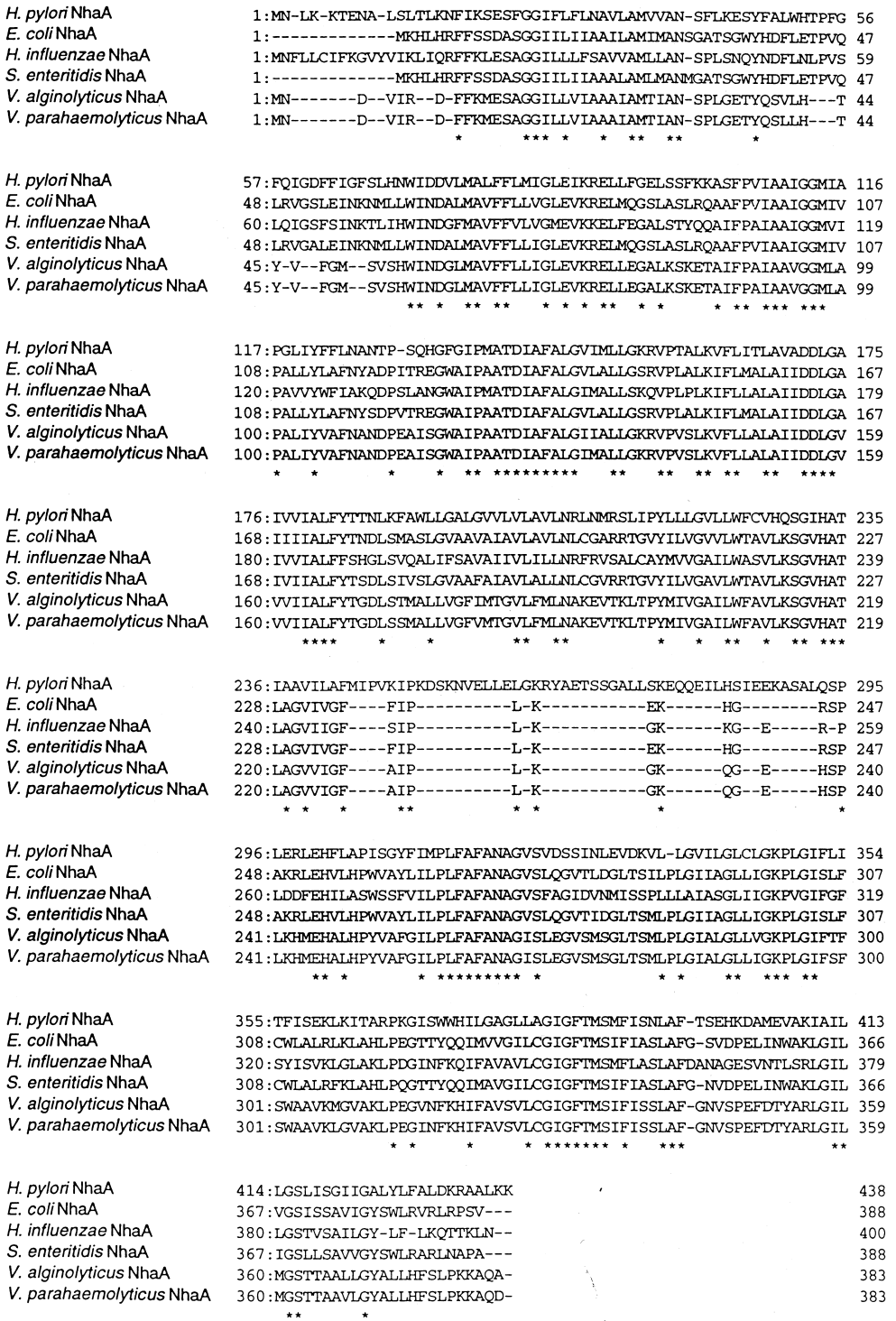

Fig. 1. Homology of NhaA primary structures of various bacteria. From top to bottom: *Helicobacter pylori* [13], *Escherichia coli* [4], *Haemophilus influenzae* [30], *Salmonella enteritidis* [28], *Vibrio alginolyticus* [25], *Vibrio parahaemolyticus* [29]. Sequences are spaced to maximize homology. Asterisks indicate residues that are conserved among the species. An extra 40 residues which are not present in any of the species except *H. pylori* are shown in the row starting with residue 236 of *H. pylori*.

encoding the reading frame of HNhaA was amplified. This product was then integrated into the unique *Eco*RI and *Sph*I sites in the expression vector, a pBR322 derivative described previously [11], with a FLAG epitope tag sequence inserted next to the *Sph*I site (pBR322 FLAG). DNA sequences of the cloned ENhaA and HNhaA, with the FLAG tag sequence, were verified by DNA sequencing by the dideoxy chain termination method [20] with an automatic DNA sequencer (ALF express, Pharmacia Biotech). GHPEI49 and GHPBC16 were kindly provided by the Institute of Genetic Research (TIGR).

2.3. Preparation of membrane vesicles and the Na^+/H^+ antiporter assay

E. coli transformant cells with various expression plasmids of NhaA were cultured in 300 ml of LBK at 37°C with vigorous shaking. After

harvesting, washing and disrupting cells by a French press, membrane vesicles were prepared by centrifugation as described previously [17]. Membrane vesicles (200 μg) were suspended and incubated in 2 ml buffer [17]. Proton flow was measured by monitoring ACMA (9-amino-6-chloro-2-methoxyacridine) fluorescence quenching after addition of lactate as a substrate of the electron transport respiratory chain [9]. After addition of NaCl or LiCl, antiporter activity was measured using fluorescence dequenching by a fluorescence photometer (Hitachi F-4500).

2.4. Immunodetection of the tagged antiporters in the membranes

Aliquots (2 μg) of the membrane vesicles from various NhaA transformants were subjected to SDS polyacrylamide gel electrophoresis as described previously [17]. The separated proteins were blotted onto a GHVP filter (Millipore) [18] and reacted with anti-FLAG M2 monoclonal antibody (Kodak). The reacted proteins were visualized by an ABC Vecta stain kit as described previously [18].

2.5. DNA manipulation and sequencing

Preparation of plasmids, digestion by restriction endonucleases and ligation by T4 DNA ligase of the DNA fragments and other techniques related to handling of DNA were performed according to the published procedures [19]. The nucleotide sequences cloned on the various expression plasmids in this study were determined by the dideoxynucleotide chain termination reaction with [α-^{35}S]deoxy-CTP (37 TBq/mol, Amersham) by T7 DNA polymerase [20], and subsequent autoradiography or with fluorescent primers by an automatic sequencer (Pharmacia Biotech).

2.6. Reagents and enzymes

Restriction endonucleases, T4 DNA ligase, *Tth* and *Pfu* DNA polymerases, and T7 DNA polymerase were purchased from Toyobo Co., New England Biolabs, and Takara Co. Oligonucleotides were synthesized by Pharmacia Biotech. Other reagents and materials were of the highest grade commercially available.

3. Results

3.1. NhaA (HNhaA) of H. pylori and construction of expression vectors of the gene in E. coli

We searched for sequences homologous to the sequence of *E. coli* NhaA (ENhaA) in the complete genomic sequence of the gastric pathogen *H. pylori* determined by Tomb et al. [13]. One open reading frame (HP1552) of 1314 bp was found to be highly homologous to ENhaA which was also mentioned by Tomb et al. This putative NhaA (HNhaA) also conserved

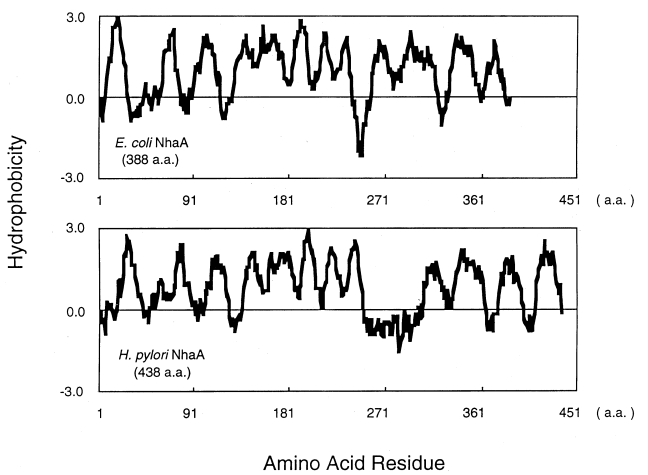

Fig. 2. Hydropathy profiles of NhaA from *E. coli* and *H. pylori*. Profiles are based on the primary structures according to the procedure by Kyte and Doolittle [31]. For the calculation, 13 consecutive residues are taken as one unit. Vertical and horizontal scales indicate the hydrophobicity index and residue numbers, respectively.

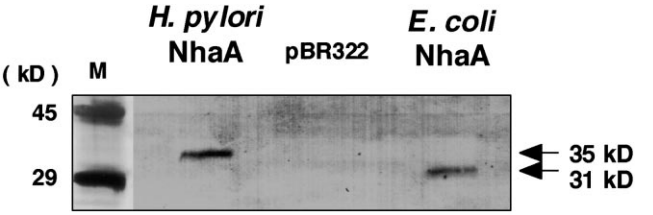

Fig. 3. Immunological detection of ENhaA and HNhaA with epitope tag. Membrane proteins (2 μg) of ENhaA or HNhaA with FLAG tag transformants of KNabc were subjected to SDS polyacrylamide gel electrophoresis (12.5% acrylamide). As a negative control, membrane proteins of the transformant with pBR322 were also analyzed. After electrophoresis, the proteins were blotted to a GVHP filter [18] and reacted with anti-FLAG monoclonal antibody M2 (Kodak) and the bands were visualized [18]. Molecular size markers (M) were stained with Coomassie brilliant blue.

functionally essential residues Asp-133, Asp-163, and Asp-164 of ENhaA reported previously (Fig. 1) [10]. Although this sequence is also homologous to other NhaAs from other bacteria (*Haemophilus influenzae*, *Salmonella enteritidis*, *Vibrio alginolyticus*, and *Vibrio parahaemolyticus*) as shown in Fig. 1, only one additional sequence with approximately 40 residues among the sequences in Fig. 1 was found in HNhaA. This sequence was inserted at the position corresponding to residue 236 in ENhaA. Hydropathy analyses of ENhaA and HNhaA revealed that this additional sequence was highly hydrophilic and looked like an extension of the corresponding hydrophilic region in ENhaA around residue 235 (Fig. 2). The other regions flanking this hydrophilic region in HNhaA and ENhaA were very homologous to each other except for about 10 residues at the amino-terminal region (Fig. 1).

The open reading frame HP1552 was divided into two segments, which were present in clones GHPEI49 and GHPBC16, respectively, in the original clone bank [13,14]. We rejoined these two parts into one continuous coding sequence and cloned it into the pBR322 FLAG vector between unique *Eco*RI and *Sph*I restriction sites. At the carboxy-terminus, an epitope tag sequence recognized by an anti-FLAG monoclonal antibody (M2) was also inserted for later analysis of HNhaA expression in the transformed cells.

3.2. Complementation of the salt sensitive phenotype of E. coli HITΔAB$^-$ and KNabc, which is deficient in the antiporters, by the HNhaA expression vector

We introduced expression vectors with HNhaA or ENhaA into KNabc (a mutant strain with deleted *nhaA, nhaB* and *chaA*), or HITΔAB$^-$ (a mutant with a deleted *nhaA* and defective *nhaB*). Since both HNhaA and ENhaA used in this experiment have an epitope tag at the C-terminus, ENhaA without the tag was also introduced as a positive control. It is known that 0.3 M NaCl at pH 8.0 and 0.03 M LiCl at pH 7.5 are the minimal concentrations that inhibit the growth of

Table 1

Plasmid	Growth of transformant	
	0.7 M NaCl	0.3 M LiCl
HNhaA	++	+
ENhaA	++	+
ENhaA (−tag)	++	+
pBR322	−	−

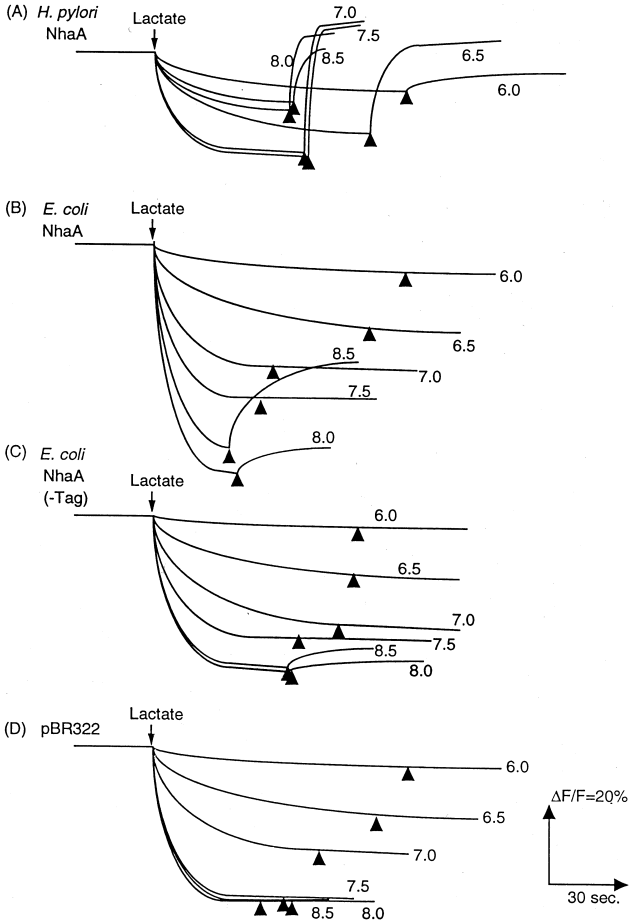

Fig. 4. Na^+/H^+ antiporter activities in the transformants of various NhaA. Membrane vesicles from transformants of KNabc with HNhaA with FLAG tag (*H. pylori* NhaA), ENhaA (*E. coli* NhaA), ENhaA without FLAG tag (*E. coli* NhaA (−Tag)), or pBR322 were prepared as described previously [10]. Membrane vesicles (200 μg) were suspended in 2 ml buffer (10 mM Tricine, 140 mM KCl) with 2 μM ACMA and respiration was started by addition of potassium lactate (5 mM, pH 7.0). pH was adjusted to the desired value with KOH. The change in ACMA fluorescence was monitored with a Hitachi F-4500 fluorophotometer at 420 nm for excitation, and at 500 nm for emission. To measure Na^+/H^+ antiporter activities, 5 mM NaCl was added at the times marked by closed triangles.

the antiporter-deficient *E. coli* mutant, KNabc. ENhaA with and without the tag sequence, and also HNhaA with the tag sequence complemented the salt-sensitive phenotype of KNabc (Table 1) at concentrations of NaCl (0.7 M) and LiCl (0.3 M) above the minimal conditions. When we used HITΔAB$^-$ as a host strain, the results were essentially the same as those obtained with KNabc (data not shown).

Next, at the minimal concentrations of NaCl and LiCl, the effects of pH were tested. ENhaA with and without the epitope tag and also HNhaA complemented the growth of cells up to pH 8.5 (data not shown). These results indicate that HNhaA could be expressed in *E. coli* mutants deficient in the Na^+/H^+ antiporters and complemented the salt-sensitive phenotypes.

3.3. Expression of ENhaA and HNhaA with the epitope tag in E. coli

Membrane vesicles from KNabc transformed with ENhaA or HNhaA with the epitope tag that is recognized by monoclonal antibody M2 were prepared and membrane proteins were blotted to a GVHP filter (Millipore). As shown in Fig. 3, HNhaA and ENhaA with the tag exhibited 35 kDa and 31 kDa bands, respectively, at the expected positions which were visualized after immunoreaction with the antibody, while no bands were observed for pBR322 (Fig. 3). These results indicate that both NhaA proteins with the tag were integrated into *E. coli* membranes and were as active as that of ENhaA without the tag.

3.4. The Na^+/H^+ and Li^+/H^+ antiporter activities in the membrane vesicles of transformants with NhaA

ACMA fluorescence was quenched by addition of lactate as reported previously [9]. The Na^+/H^+ antiporter activities in the everted membrane vesicles from transformants of KNabc were estimated by measuring the dequenching of ACMA fluorescence caused by addition of NaCl or LiCl. Fig. 4 shows the results with KNabc as a host strain. For pBR322 no activity was observed at any pH (Fig. 4D), while for ENhaA with and without the epitope tag sequence (Fig. 4B,C) activity was observed at pH 8.0 and pH 8.5 but not below pH 7.5 as reported previously [9,11]. In the case of tagged NhaA, the activities were slightly higher than those obtained without the tag sequence, i.e. with the wild-type sequence alone. The reasons for this slight difference are unclear. Striking differences were observed for the antiporter activities of HNhaA transformant vesicles (Fig. 4A). The antiporter activities were observed even at pHs lower than pH 7.5 and also in the range between pH 7.5 and pH 8.5. The relative activities of the antiporter based on the results of Fig. 4 are shown in Table 2. The profiles of pH-dependent Na^+/H^+ antiporter activities between HNhaA and ENhaA are clearly different. HNhaA is active even at pH 6.0 whereas ENhaA shows almost no activity at pH 7.0. Since respiratory chain activity driven by lactate changed depending on pH values, a comparison of the absolute activities of the antiporter at different pH values is difficult. Therefore, we compared the relative activities of the Na^+/H^+ antiporter as shown in Table 2. These results indicate that the Na^+/H^+ antiporter activities of *H. pylori* are relatively similar within the pH range from pH 6.0 to pH 8.5.

The Li^+/H^+ antiporter activities for HNhaA and ENhaA exhibited essentially the same profiles (data not shown), as was the case for the Na^+/H^+ antiporter activities (Fig. 4; Table 2). When we used HITΔAB$^-$ as the host strain of the ENhaA and HNhaA transformants, results similar to those shown in Fig. 4 were obtained (data not shown).

Table 2

Plasmid	Percent dequenching of ACMA fluorescence after addition of NaCl					
	pH					
	6.0	6.5	7.0	7.5	8.0	8.5
HNhaA	38.2	88.0	100	100	90.5	88.0
ENhaA	0	0	0	0	8.0	31.0
ENhaA (−tag)	0	0	0	0	1.9	18.8
pBR322	0	0	0	0	0	0

4. Discussion

The preceding results clearly show that proteins encoded by an open reading frame in *H. pylori* (HP1552) with a sequence homologous to the Na^+/H^+ antiporter of *E. coli* NhaA had Na^+/H^+ as well as Li^+/H^+ antiporter activities. This is based on the finding that an antiporter-deficient mutant of *E. coli* was made salt-tolerant by transformation with this gene, and by direct observation of both antiporter activities in the transformant membrane vesicles by an in vitro assay. Therefore, we concluded that the open reading frame HP1552 is the gene for NhaA of *H. pylori* and named it HNhaA. The most striking functional feature of HNhaA is that its activity was observed even at pH values lower than 7.0, at which ENhaA did not exhibit activity.

This difference in pH range of the antiporter activities of the two bacteria corresponds well with their living environments. While *E. coli* is a typical enterobacterium living at alkaline pH, *H. pylori* lives under acidic conditions in the stomach (pH $\sim$2). Therefore, both antiporters may be evolutionarily selected for their living environment. Based on previous studies [13,21–23], one possible explanation for the survival of *H. pylori* in the gastric mucosa layer (pH $\sim$2) is that the electric potential inside this bacterium is positive, which would prevent the influx of H^+ from the acidic environment. The mechanism responsible for forming the inside positive potential is unclear. From this point of view, the function of HNhaA may be to excrete H^+ from the inside of the cells and to allow the intrusion of Na^+ into the cells from the outside. The antiporter activity observed in this study was obtained with everted membrane vesicles, suggesting that the observed Na^+/H^+ ion exchange was in the opposite direction to that of *H. pylori* in the stomach, which cells are characterized by extrusion of H^+ and intrusion of Na^+.

As shown in Fig. 1, NhaA sequences are well conserved among six different bacterial species except for one additional sequence in *H. pylori*. These approximately 40 extra residues are hydrophilic and are possibly located on the inside of the cell membrane. This is because the one highly hydrophilic domain in ENhaA, which occurs around residue 235, was reported to be inside the cell membrane. The latter conclusion was based on topological analyses using the *phoA* fusion method [12] and the finding that, when ENhaA was incorporated in everted vesicles, this domain was susceptible to protease digestion [24]. These hydrophilic regions in the two NhaAs may be responsible for the altered pH dependence of the antiporter activities. Exchanging these regions between the two antiporters may allow this hypothesis to be tested. Comparison of the hydrophilic regions of the two antiporters may also provide clues to the putative pH sensor mechanisms. It was reported that point mutations at His-225 of ENhaA caused altered responses of the antiporter activity to pH changes [11,26,27], suggesting that this residue is related to the putative pH sensor mechanisms. However, this residue was also conserved in HNhaA, suggesting that, in addition to this residue, other more important residues may be involved in the putative pH sensor mechanisms.

We have introduced an epitope tag sequence at the C-termini of ENhaA and HNhaA, and both of them were active, indicating that the carboxy-terminal region is not essential for transport activity. However, slightly higher activity was observed for tagged ENhaA, suggesting that the tag sequence may be located close to the active site for ion transport.

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