BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

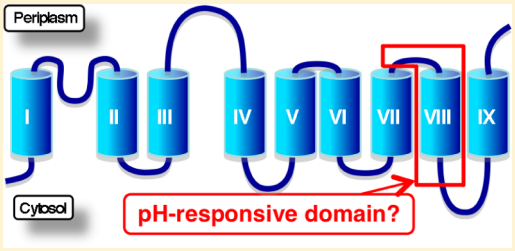
Critical Involvement of the E373−D434 Region in the Acid Sensitivity of a NhaB-Type Na^+/H^+ Antiporter from *Vibrio alginolyticus*

Wakako Kiriyama, Tatsunosuke Nakamura, Masahiro Fukuhara, and Toshio Yamaguchi*

Department of Microbiology, Faculty of Pharmacy, Niigata University of Pharmacy and Applied Life Sciences, Niigata 956-8603, Japan

Supporting Information

ABSTRACT: It has been well established that VaNhaB, a NhaB-type Na^+/H^+ antiporter found in *Vibrio alginolyticus*, exhibits a striking acid sensitivity. However, the molecular basis of the pH-dependent regulatory mechanism of the antiport activity is yet to be investigated. In this study, we generated various chimeric proteins composed of VaNhaB and a pH insensitive ortholog found in *Escherichia coli* (EcNhaB) and analyzed the pH responses of their Na^+/H^+ antiport activities to search for the key residues or domains that are involved in the pH sensitivity of VaNhaB. Our results revealed the significant importance of a stretch of amino acid residues within the loop 8−loop 9 regions (E373−D434) responsible for the acid sensitivity of VaNhaB, along with the possible involvement of other unidentified residues that are widely spread in the primary structure of VaNhaB. Moreover, we demonstrated that the E373−D434 region of VaNhaB was able to confer some degree of acid sensitivity on our pH insensitive chimeric antiporter that is mainly composed of EcNhaB except for seven amino acid substitutions at the N-terminal end. This result strongly suggested the possibility that the E373−D434 region is able to act, at least partially, as machinery that diminishes the activity of the NhaB-type antiporter at an acidic pH.



Monovalent cation/H^+ antiporters are membrane proteins that exist in the cytoplasmic and intracellular membranes of most organisms from bacteria to humans and play important roles in the maintenance of intracellular pH and cation homeostasis by exchanging monovalent cations, such as K^+ or Na^+, for H^+ across the membranes.[1]

In several bacteria, cation/H^+ antiporters are considered to be a determinant factor for their alkaline tolerance, because disruption of certain cation/H^+ antiporter genes often results in hypersensitivity to an alkaline environment.[2−4] Interestingly, these antiporters are fully active in alkaline and inactive in neutral to acidic environments.[5−7] The pH dependency of these antiporters is a key feature that regulates cellular pH homeostasis because it determines the set point of the intracellular pH. Therefore, its molecular mechanism of action has attracted particular attention and has been intensively studied for more than two decades.[8]

Among pH-dependent cation/H^+ antiporters that have been investigated so far, NhaA-type antiporters are the most extensively studied class of antiporters. Biochemical studies, including site-directed and random mutagenesis of EcNhaA from *Escherichia coli*, have identified several important residues that are responsible for its dramatic acid sensitivity[9−11] and have also provided evidence that the shift in environmental pH could induce conformational changes in EcNhaA.[12] Similarly, studies of HPNhaA, a pH insensitive ortholog found in *Helicobacter pylori*, revealed that several amino acid residues in TM4, -5, -10, and -11 are important for the maintenance of its antiport activity at acidic pH.[13] Furthermore, the recent determination of the crystal structure of EcNhaA has greatly advanced our understanding of its pH sensitivity mechanism,[14] and further biochemical studies and computational analysis have provided a model for the molecular mechanisms of the pH-dependent regulation of EcNhaA activity.[8] Additionally, the crystal structure of EcNhaA, in combination with results obtained from biochemical studies, has also allowed a comparative discussion about how the HPNhaA transporter is able to maintain its activity even under acidic conditions.[15]

In contrast to the significant progress of the studies performed on NhaA-type antiporters, however, research studies regarding the mechanisms of the pH-dependent regulation of their activity have been mostly restricted in NhaA-type antiporters to date. Although there have been a number of cation/H^+ antiporters with almost no similarities in sequence to NhaA-type antiporters identified in bacterial genomes[16,17] with some of them showing striking pH sensitivity,[18−21] mechanisms underlying their pH sensitivity are largely unknown.

The NhaB-type antiporters make up a group of antiporters that are widely distributed among Gram-negative bacteria and have almost no similarity to NhaA-type antiporters.[22,23] Interestingly, while their biochemical properties and physiological roles are largely unknown, several pioneering studies have indicated the presence of several subtypes that show distinct pH responses. A NhaB-type antiporter from *Vibrio alginolyticus* (VaNhaB) has been shown to be fully active at

Received: June 5, 2012
Revised: September 6, 2012
Published: September 6, 2012

alkaline pH, while this activity dramatically decreased when the pH was lowered to 7.5 and disappeared at 6.5.[21] On the other hand, a NhaB-type antiporter from *E. coli* (EcNhaB) appeared to be insensitive to pH changes, despite its high degree of amino acid sequence similarity to VaNhaB [79.9% (Figure 1)];

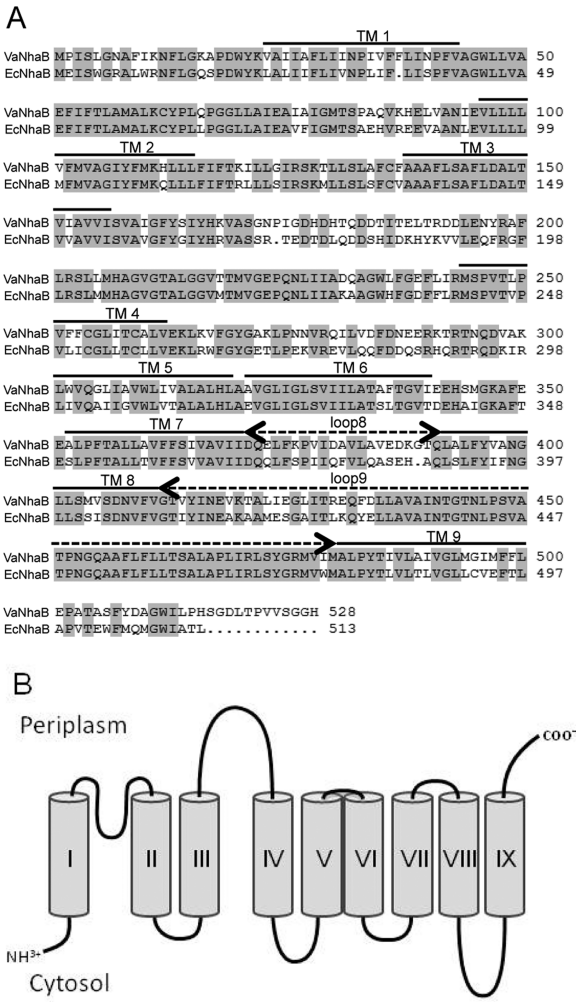


Figure 1. Structures of NhaB proteins. (A) Alignment of amino acid sequences of VaNhaB and EcNhaB. Identical residues are highlighted in gray. Transmembrane domains (TM) of the VaNhaB are indicated according to the nomenclature of Enomoto et al.[25] Note that EcNhaB is smaller than VaNhaB because of the shorter C-terminal tail and several deletions with respect to VaNhaB. (B) Membrane topology model of VaNhaB reproduced based on results of the PhoA fusion analysis by Enomoto et al.[25] Transmembrane domains (I–IX) are shown as cylinders.

although its activity gradually decreased to approximately half its maximum when the pH was shifted from alkaline to neutral, the antiport activity was maintained over a wide pH range.[24] Given the high degree of sequence similarity between these two antiporters and the low degree of sequence similarity to NhaA-type antiporters, the apparent difference in pH sensitivity described above would make NhaB-type antiporters an excellent subject for the study of a mechanism of pH sensitivity of the cation/H^+ antiporter, because the residues or domains that determine the significant pH sensitivity of VaNhaB are likely to be present within the regions that are not conserved between VaNhaB and EcNhaB. Furthermore, the preceding topological study indicated that the structure of VaNhaB is quite different from that proposed for NhaA-type antiporters,[25] suggesting the possibility that pH-dependent regulation of VaNhaB might involve a novel mechanism that is distinct from that of EcNhaA.

In this study, we have attempted to identify the regions that are involved in the pH sensitivity of VaNhaB. By analyzing the pH responsiveness of a series of chimeric antiporters, we revealed that a stretch of amino acid residues within the loop 8–loop 9 region plays important roles in the acid sensitivity of VaNhaB. Moreover, our results indicated the involvement of other residues that are widely distributed within the primary sequence of VaNhaB that may be responsible for its acid sensitivity.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Media. *E. coli* strain DH5α was used for routine cloning and grown in LB medium (1.0% Bacto tryptone, 0.5% yeast extract, and 0.5% NaCl) at 37 °C. *E. coli* strain TO114 (*nhaA::Km*^{r}, *nhaB::Em*^{r}, *chaA::Cm*^{r})[26] was used for activity measurement and grown in LBK medium (1.0% Bacto tryptone, 0.5% yeast extract, and 100 mM KCl) at 37 °C. The following antibiotics were added to the medium when required: ampicillin (100 μg/mL), kanamycin (50 μg/mL), erythromycin (160 μg/mL), and chloramphenicol (25 μg/mL).

Cloning of *EcnhaB* and *VanhaB* and Construction of Chimeric *nhaB* Genes. The *EcnhaB* or *VanhaB* gene was amplified by PCR from pHGB2[21] or pTN1[23] using KOD Plus (TOYOBO), respectively. Chimeric NhaB genes were generated by fusion PCR[27] using *EcnhaB* and *VanhaB* as templates and cloned into the pGEM-T easy vector (Promega) by TA cloning. Primers used for fusion PCR are listed in Table 1 of the Supporting Information. After DNA sequences had been confirmed (performed by Macrogen Japan, Inc.), each *nhaB* gene was inserted into *Sal*I and *Xma*I sites of the pHG165 expression vector[28] and introduced into TO114. The intact pHG165 plasmid was also introduced into TO114 as a vector control. For 2× hemeagglutinin (2×HA) epitope tagging of EcNhaB, VaNhaB, and chimeric NhaB antiporters, a pHG165-2HA plasmid was generated by inserting an oligonuculeotide linker that encodes a 2×HA epitope tag between the *Bam*HI and *Pst*I sites of pHG165, and each *nhaB* gene was cloned into the *Xma*I and *Xho*I sites of pHG165-2HA. Oligonucleotides used as a linker are listed in Table 1 of the Supporting Information.

Preparation of Everted Membrane Vesicles from *E. coli*. Everted membrane vesicles were prepared using a French press as described by Rosen.[29] Two milliliters of overnight cultures of TO114 strains was inoculated into 200 mL of fresh LBK medium and grown to an OD_{600} of 0.5–0.7. The following procedures were performed at 4 °C. *E. coli* cells were harvested by centrifugation at 10000*g* for 5 min and washed once with 10 mL of TCDS buffer (10 mM Tris-HCl, 140 mM choline chloride, 250 mM sucrose, and 0.5 mM dithiothreitol). Subsequently, cells were resuspended in 10 mM TCDS buffer containing protease inhibitor cocktail set II (Calbiochem) and passed twice through a French pressure cell (4000 psi) (Thermo). Unbroken cells and cell debris were removed by centrifugation (10000*g* for 10 min), and the supernatant was subjected to ultracentrifugation (100000*g* for 1 h). Pellets were resuspended in 300 μL of TCDS buffer containing protease inhibitor and stored at −80 °C until they were used. The protein concentration was measured with the BCA protein assay kit (Pierce) using BSA as a standard.

dx.doi.org/10.1021/bi300738v | *Biochemistry* 2012, 51, 7766–7774

Measurement of Na^+/H^+ Antiport Activity by the Acridine Orange Quenching Assay. Na^+/H^+ antiport activities were measured by the acridine orange quenching assay as described by Radchenko et al.[30] Vesicles (corresponding to 100 μg of protein) were suspended in 2 mL of assay buffer [10 mM Tris-HCl (pH 6.5–9.0), 5 mM $MgCl_2$, 140 mM choline chloride, and 1.5 μM acridine orange], and 2 mM Tris-DL-lactate (pH 9.0) was added to initiate respiration. Na^+/H^+ antiport activities were measured by monitoring fluorescence dequenching following the subsequent addition of 2.5 mM NaCl. Note that 100 mM NaCl were also used to monitor the activity of VaNhaB and VaS series chimeras when specified. After the dequenching had reached saturation, 25 mM ammonium chloride was added to dissipate the remaining ΔpH for the calculation of the total quenching value. Time-dependent ΔpH changes were monitored by measuring the quenching of acridine orange fluorescence using a Shimadzu-5300PC instrument (Shimadzu) with the excitation wavelength set at 492 ± 1.5 nm and the emission wavelength set at 525 ± 3.0 nm. Antiport activities were expressed as percent dequenching of the total quenching value, as suggested by Swartz et al.[6] Activities of the EcNhaB, VaNhaB, and chimeric NhaB antiporters were calculated by subtracting the values obtained for vesicles of the vector control (TO114 harboring the intact pHG165 plasmid) from those of each strain.

Immunodetection of 2×HA-Tagged Antiporters. Membrane vesicles (corresponding to 20 μg of protein) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (12.5% acrylamide) followed by Western blot analysis. Anti-HA epitope monoclonal antibody 16B12 (Covance) and the anti-mouse IgG antibody–horseradish peroxidase conjugate (Molecular Probes) were used at dilutions of 1/1000 and 1/40000, respectively.

RESULTS

pH Dependency of EcNhaB and VaNhaB Activities. Although previous studies indicated that EcNhaB and VaNhaB exhibit distinct pH responses, there was a possibility that observed differences between the two antiporters might be originating from the differences in experimental setup. Therefore, we first attempted to compare pH responses of EcNhaB and VaNhaB using the same experimental conditions, namely, using the same expression system and assay condition. We chose the pHG165 expression vector and the TO114 strain, both of which had been successfully used for measurements of VaNhaB activities, for the expression of NhaB-type antiporters.[21] The TO114 strain had been frequently utilized to study Na^+/H^+ antiportes because it exhibits only marginal Na^+/H^+ antiport activity due to the lack of three major cation/H^+ antiporters, namely, NhaA, NhaB, and ChaA.[26] Activities of NhaB-type antiporters expressed in TO114 strains were assessed by measuring Na^+/H^+ antiport activities of everted membrane vesicles prepared from each strain by the acridine orange quenching assay, which had been used for analyses of various cation/H^+ antiporters.[21] The pH dependency of each antiporter was determined by measuring the antiport activities of everted membrane vesicles expressing either antiporter at 2.5 mM NaCl in buffers adjusted to various pH values. Note that the buffer pH indicated hereafter corresponds to the intracellular pH of intact cells in this assay system.

Na^+/H^+ antiport activities of EcNhaB and VaNhaB between pH 6.5 and 9.0 are shown in Figure 2A. EcNhaB activity appeared to be at its maximum around pH 8.5–9.0 and moderately decreased as the buffer became more acidic, finally reaching the halfway point at pH 6.5 (Figure 2A). These results indicated that EcNhaB shows only a modest pH dependency, which is in agreement with the preceding study with proteoliposomes containing purified EcNhaB protein.[24] In contrast, VaNhaB showed striking pH sensitivity (Figure 2A). While its activity was highest around pH 8.0–9.0 (approximately 90%), a significant decrease was observed at pH 7.5 and the activity almost completely disappeared at pH <7.0 (Figure 2), which is consistent with a previous observation by Nakamura et al.[21]

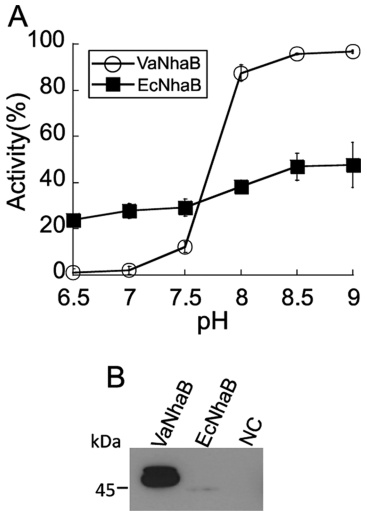


Figure 2. pH profiles of Na^+/H^+ antiport activities of VaNhaB and EcNhaB. (A) pH profiles of the Na^+/H^+ antiport activity of VaNhaB and EcNhaB. Na^+/H^+ antiport activities of everted membrane vesicles harboring VaNhaB (○) and EcNhaB (■) were measured using the acridine orange quenching method at pH 6.5–9.0 as described in Experimental Procedures. Averages of at least three replicates are shown, and standard deviations are indicated as error bars. (B) Western blot analysis. Membrane vesicles (20 μg of protein) from the strains expressing the 2×HA-tagged VaNhaB and EcNhaB were subjected to SDS–PAGE (12.5% acrylamide) and probed with the anti-HA antibody as described in Experimental Procedures. The membrane vesicles expressing native VaNhaB were used as a negative control (NC). A representative blot of several independent experiments is shown.

We next attempted to compare the amounts of VaNhaB and EcNhaB proteins in membrane vesicles by Western blot analysis. Because no antibody that could simultaneously detect both antiporters was available, we introduced the 2×HA epitope to the C-terminus of each VaNhaB and EcNhaB on the same constructs used above to estimate the amount of both proteins in the membrane vesicles. Na^+/H^+ antiport activities of everted membrane vesicles prepared from strains expressing these 2×HA-tagged proteins showed pH profiles similar to those of their native molecules (data not shown). Western blot analysis using the anti-HA antibody demonstrated that the amount of VaNhaB-2HA in the membrane vesicles was significantly higher than that of EcNhaB-2HA (Figure 2B). These results are in agreement with the difference in the maximal activity observed under alkaline conditions (Figure 2A). On the other hand, these results also imply that the observed difference in the pH responses between EcNhaB and VaNhaB is attributed to the property of each antiporter, because the loss of antiport activity under acidic conditions was only observed with VaNhaB despite the fact that its amount in

the membrane vesicles was significantly larger compared to the amount of EcNhaB. It should be noted that apparent molecular masses of proteins were different from the calculated molecular masses of the proteins (i.e., 60.0 and 59.5 kDa for VaNhaB-2HA and EcNhaB-2HA, respectively). Although the reason for these discrepancies is unknown, we concluded that observed bands represent the VaNhaB-2HA and EcNhaB-2HA proteins, respectively, because these bands were not observed in the NC (negative control) lane (Figure 2B) and no other bands were observed in corresponding lanes (data not shown). Furthermore, the apparent molecular mass of EcNhaB-2HA was close to the value that was previously reported for native EcNhaB,[22,24] yet EcNhaB-2HA appeared to be slightly larger than the native EcNhaB, which probably reflects the addition of the 2×HA tag. It should also be noted that such aberrant mobility in SDS−PAGE had been reported for many other membrane proteins.[5,22,31]

Taken together, the results described above confirmed previous observations that VaNhaB but not EcNhaB exhibits a characteristic acid sensitivity, and that VaNhaB has an integrated mechanism for modifying its activity depending on the surrounding pH.

Identification of the Key Domain(s) Responsible for the pH Sensitivity Exhibited by VaNhaB through the Analysis of VE Chimeras. Next, to estimate the location of the regions that are involved in the pH sensitivity within the amino acid sequence of VaNhaB, we generated a series of VE chimeras, which are composed of the N-terminal portion of VaNhaB and the C-terminal portion of EcNhaB, and examined their response to various buffer pHs as described above (Figure 3).

A chimeric antiporter, VE8, which comprises almost the full length of the EcNhaB antiporter, except for the substitution of seven N-terminal amino acids with the corresponding region of VaNhaB, showed an only minor pH response over the tested pH range, although the steepness of the decrease in the antiport activity was slightly larger than that of EcNhaB (Figure 3B). Interestingly, although this chimeric antiporter is composed of mostly EcNhaB, the antiport activity was significantly higher than that of EcNhaB at all the pHs examined (Figure 3B).

Several chimeric NhaB antiporters, namely, VE72, VE244, VE388, and VE426, showed pH responses that seemed to be intermediate between those of EcNhaB and VaNhaB (Figure 3C). The antiport activities of these chimeras decreased rather steeply as the pH was lowered below 8.0 or 7.5, but their steepness was not as significant as that of VaNhaB (Figure 3C), suggesting that the amino acid sequences of VaNhaB included in these chimeras are, at least partially, involved in the pH sensitivity, although not critically involved in the severe acid sensitivity of VaNhaB. On the other hand, VE434 and VE500 exhibited almost the same pH response as VaNhaB; the activities of these chimeras diminished abruptly at pH <7.5 (Figure 3D). These results demonstrated that the residues between positions 427 and 434 of VaNhaB (L427−D434), which are missing in VE426, are indispensable for the characteristic pH response of VaNhaB. Furthermore, Western blot analysis of membrane vesicles bearing 2×HA-tagged chimeric antiporters estimated that the amounts of these proteins in the membrane vesicles were comparable except for the amount of VE244, which showed a significantly weaker signal (Figure 3E). On the basis of this observation, we normalized the pH profiles of these chimeras using the amount and activity of VaNhaB protein as 100% and confirmed that

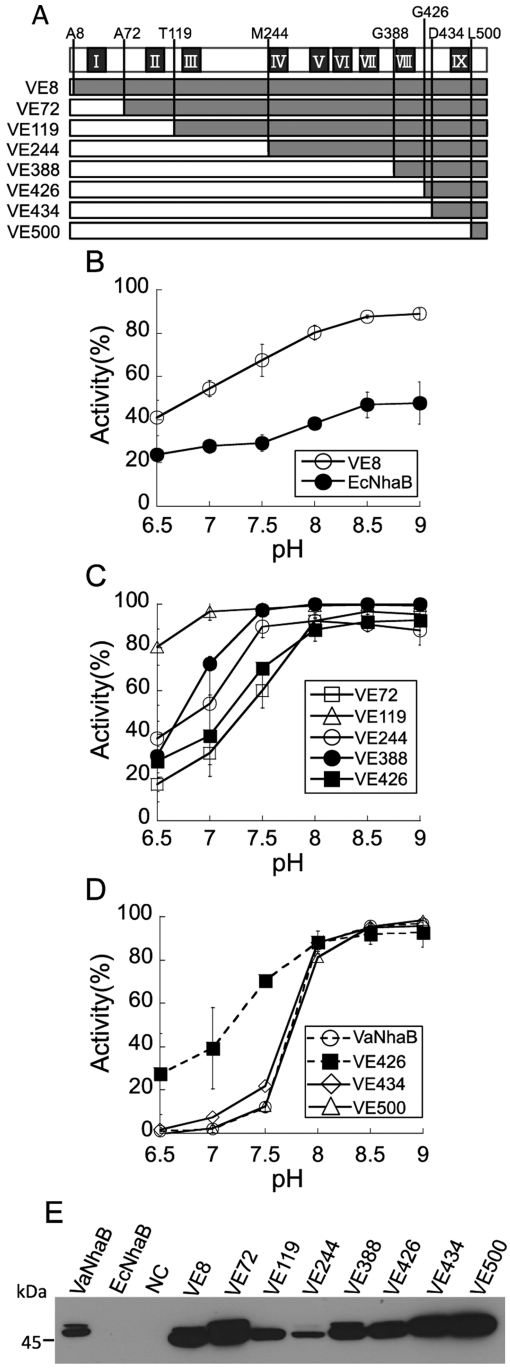


Figure 3. pH profiles of Na^+/H^+ antiport activities of VE chimeras. (A) Schematic representation of VE chimeras. Regions derived from VaNhaB and EcNhaB are colored white and gray, respectively. (B−D) pH profiles of Na^+/H^+ antiport activity of VE chimeras. Na^+/H^+ antiport activities were determined and indicated as in Figure 2. (E) Western blot analysis of 2×HA-tagged VE chimeras. Note that membrane vesicles from the strain expressing 2×HA-tagged VaNhaB and EcNhaB were also included as references (indicated as VaNhaB and EcNhaB, respectively). Membrane vesicles from the strain expressing native VaNhaB were used as a negative control (NC). A representative blot of several independent experiments is shown.

VE72, VE244, VE388, and VE426 show pH profiles distinct from those of VE434 and VE500, whose activities were decreased at pH 7.5 and almost completely disappeared at pH <7.0 (Figure S1 of the Supporting Information).

dx.doi.org/10.1021/bi300738v | *Biochemistry* 2012, 51, 7766−7774

Interestingly, the activity of VE119 did not appear to be largely affected by the buffer pH, maintaining almost 100% activity between pH 9.0 and 7.0 and 80% activity at pH 6.5 (Figure 3C). The pH profile of the VE119 activity strongly suggested that the pH sensitivity is almost lost in this chimera. Nonetheless, given that chimeras VE72 to VE426 showed similar intermediate-type pH responses distinct from that of VE119, it is unlikely that key residues or domains that are indispensable for pH sensitivity are present within the region of residues 72−426 of VaNhaB sequence. In addition, the amount of VE119 protein in the membrane vesicles was estimated to be comparable to amounts of most of the chimeric antiporters described above (Figure 3E). Although we cannot be certain of how exactly the VE119 became virtually insensitive to pH at this point, we assumed that connecting the N-terminus of VaNhaB and the C-terminus of EcNhaB at this position resulted in the formation of an aberrant structure that consequently impaired the pH response of this chimera, probably by hindering a pH-dependent conformational change as observed in the case of the NhaA-type antiporter.[14] Collectively, and because the aim of this study was the determination of the key residue(s) or domain(s) involved in the integrated mechanism of pH sensitivity in VaNhaB, we decided not to investigate VE119 further in this study.

Because the results presented above indicated the importance of the L427−D434 region in the pH sensitivity of VaNhaB, we next attempted to investigate whether the absence of this region in VaNhaB had any impact on the pH response of VaNhaB (Figure 4). In addition, we also tested the effect of the incorporation of the L427−D434 region in EcNhaB by substituting the corresponding region (Figure 4). However, the substitution of L427−D434 of VaNhaB with the corresponding region of EcNhaB or vice versa did not alter the pH response of either antiporter (Figure 4). These results, together with the observations that chimeras VE72 to VE426 exhibited partial pH sensitivity (Figure 3C), suggested the involvement of residues or domains other than the L427−D434 region in the pH sensitivity of VaNhaB.

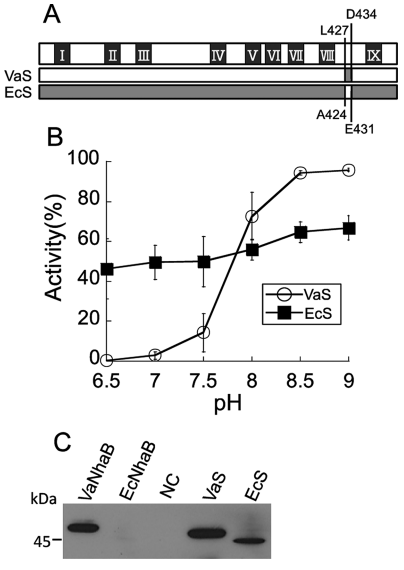


Figure 4. pH profiles of Na^+/H^+ antiport activities of VaS and EcS. (A) Schematic representation of VaS and EcS. Regions derived from VaNhaB and EcNhaB are indicated as in Figure 3. (B) pH profiles of the Na^+/H^+ antiporter of VaS and EcS. Na^+/H^+ antiport activities were determined and are depicted as in Figure 2. (C) Western blot analysis of 2×HA-tagged VaS and EcS. Descriptions of the panels are the same as in Figure 3.

Search for the Additional Region(s) Involved in pH Sensitivity within Variable Regions between VaNhaB and EcNhaB. To further search for other regions besides L427−D434 that are involved in the pH sensitivity of VaNhaB, we investigated pH responses of additional sets of chimeric antiporters, which contain substitutions of L427−D434 and additional regions that are not conserved between the VaNhaB and EcNhaB amino acid sequences, using VaS (Figure 4A) as a parental molecule (Figure 5). Among the eight chimeras tested, five, namely, VaS(66−137Ec), VaS(152−206Ec), VaS(219−260Ec), VaS(335−365Ec), and VaS(479−513Ec), showed pH responses similar to that of VaNhaB (Figure 5B). In contrast, VaS(373−426Ec) exhibited an intermediate-type pH response that closely resembled those of VE388 and VE426 (Figures 3C and 5B). The amounts of these chimeric antiporters in the membrane vesicles were estimated to be similar except for the amount of VaS(479−513Ec), which showed a significantly weaker signal compared to the others (Figure 5D) and thus was disregarded hereafter. Nonetheless, the results obtained with the rest of the chimeras clearly indicated that the distinct pH profiles observed for VaS(373−426Ec) were not attributed to the difference in the amount of chimeric proteins in the membrane vesicles. In addition, normalized pH profiles of these chimeras except for that of VaS(479−513Ec) also indicated that VaS(373−426Ec) exhibits a pH response distinctive from those of four other chimeras described above (Figure S2 of the Supporting Information). We concluded then that the E373−D434 region of VaNhaB, which includes the region identified above (L427−D434), contains indispensable components that play important roles in the pH sensitivity of VaNhaB.

We next attempted to investigate whether the increased activity observed in VaS(373−426Ec) was due to the modification of the apparent K_m values for Na^+ or the acidic shift of the "set-point" pH where antiport activities shut off, because both had been shown to cause a shift in the apparent pH profile under nonsaturating conditions in the case of EcNhaA.[32,33] For this purpose, according to Galili et al.,[32] antiport activities of VaS(373−426Ec) and VaNhaB were measured using 100 mM NaCl, a concentration that is supposed to saturate VaNhaB (data not shown). pH profiles of these antiporters at 100 mM NaCl were not significantly different, except for the slight decrease that was observed only in VaNhaB but not in VaS(373−426Ec) at pH 6.5 (Figure 5C). Although this observation might suggest a possibility that the acidic shift of the set-point pH occurred in VaS(373−426Ec), the difference between these two molecules was too small, and therefore, we were unable to properly ascertain whether the increased activity observed in VaS(373−426Ec) at 2.5 mM NaCl compared to that of VaNhaB was mainly due to the acidic shift of the set-point pH, the change in the K_m for Na^+, or a synergistic result of both changes.

Apart from the chimeras mentioned above, two others, VaS(1−40Ec) and VaS(266−321Ec), showed significantly lower activity at 2.5 mM NaCl compared to others under all the conditions tested, although their pH response patterns appeared to be different from each other. VaS(1−40Ec) showed a maximal activity that was only one-quarter of the others, and its activity was modestly decreased when the pH was lowered from 9.0 to 8.0, then disappearing at pH <7.5 (Figure 5B). Antiport activities between pH 8.5 and 7.0

dx.doi.org/10.1021/bi300738v | Biochemistry 2012, 51, 7766−7774

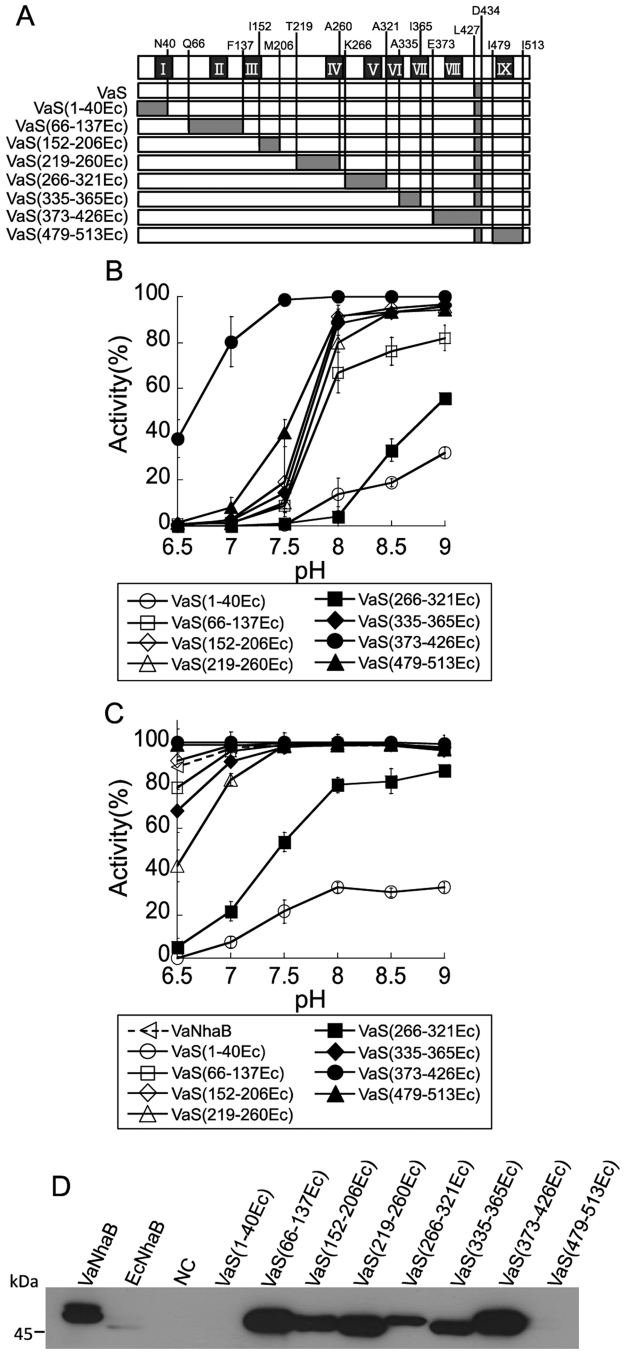


Figure 5. pH profiles of Na^+/H^+ antiport activities of VaS chimeras. (A) Schematic representation of chimeric antiporters generated using VaS as a parental molecule. Regions derived from VaNhaB and EcNhaB are indicated as in Figure 3. (B) pH profiles of Na^+/H^+ antiport activity at 2.5 mM NaCl. (C) pH profiles of Na^+/H^+ antiport activity at 100 mM NaCl. Na^+/H^+ antiport activities were determined and are depicted as described above. (D) Western blot analysis of 2×HA-tagged chimeras used above. Descriptions are as the same as in Figure 3.

increased when they were measured with 100 mM NaCl, while the degree of maximal activity (i.e., activity at pH 9.0) was unchanged. In addition, the activity of VaS(1−40Ec) disappeared at pH 6.5 in the presence of 100 mM NaCl, suggesting the possibility that the alkaline shift of the set-point pH might have occurred in this molecule, because the disappearance of the activity was not observed in VaNhaB under the same condition (Figure 5C). Nonetheless, the amount of VaS(1−40Ec) protein in membrane vesicles, as estimated by Western blot analysis with the 2×HA-tagged chimera, appeared to be extremely smaller than others (Figure 5D), suggesting that the lower net activity observed above was due to the smaller amount of this chimeric protein in the membrane vesicles compared to the amounts of the others, and that the apparent alkaline shift of the pH profile was probably caused by the diminished net activity at each pH as compared to others. On the other hand, the activity of VaS(266−321Ec) at 2.5 mM NaCl steeply decreased as the pH was lowered from 9.0 and almost disappeared at pH ≤8.0 (Figure 5B). The antiport activity of this chimera in the presence of 100 mM NaCl increased at all pHs tested except for pH 6.5 as compared to that measured with 2.5 mM NaCl, while the maximal activity (at pH 9.0) was smaller than that of VaNhaB. In addition, the activity steeply decreased as the pH shifted from pH 8.0 to 6.5 (Figure 5C). These results indicated that the lower net activity of VaS(266−321Ec) observed at 2.5 mM NaCl was probably due to both the alkaline shift of the set-point pH and the increase in the apparent K_m for Na^+ compared to that of VaNhaB (Figure 5C). The amount of VaS(266−321Ec) proteins in the membrane vesicles was estimated to be comparable to the amounts of others (Figure 5D), suggesting that the differences in the pH profile observed for VaS(266−321Ec) from the others were attributed to the change in the properties of the antiporter rather than its amount in the membrane vesicles.

In addition, measurements of the activities of other VaS-based chimeras at 100 mM NaCl also suggested that several other chimeras, the VaS(219−260Ec) and VaS(335−365Ec) chimeras in particular, might exhibit alkaline shifts of the set-point pH to some extent (Figure 5C). These results might suggest intriguing possibilities that regions of VaNhaB substituted in these chimeras could be related to the determination of the set-point pH and/or the apparent K_m for Na^+. However, it is highly unlikely that those changes in the pH profile reflect losses of integrated components necessary for the acid sensitivity of VaNhaB, the determination of which is the focus of this study, because substitutions made on these molecules did not result in the reduction of the acid sensitivity compared to that of VaNhaB. Therefore, we did not further investigate these molecules in this study.

Evaluation of the VaNhaB E373−D434 Region as the Potential "pH-Responsive Machinery" in VE8. Our results demonstrate that the replacement of the E373−D434 region with the corresponding region from EcNhaB partially impaired the pH sensitivity of VaNhaB, which implies that the E373−D434 region of VaNhaB has the potential to, at least partially, act as the molecular machinery involved in diminishing the antiport activity of VaNhaB depending on the cytoplasmic pH. To investigate this possibility, we attempted to test the pH response of another chimeric antiporter, Ec(371−431Va), which is composed mostly of EcNhaB except for the replacement of the Q371−E431 region with the corresponding E373−D434 region of VaNhaB. However, our attempt was unsuccessful because of the very low activity of this chimeric antiporter (data not shown). We therefore utilized VE8 (Figure 3) as a parental molecule, because it showed an only marginal pH response, and modified it accordingly to see whether the incorporation of the E373−D434 region had any impact on its response to pH. Strikingly, the activity of the resultant chimeric antiporter, VE8(371−431Va), exhibited a pH profile distinct

dx.doi.org/10.1021/bi300738v | Biochemistry 2012, 51, 7766−7774

from that of its parental molecule, VE8, when measured with 2.5 mM NaCl, being relatively constant at pH 7.5−9.0 and significantly dropping at pH <7.0 (Figure 6B). In addition, this

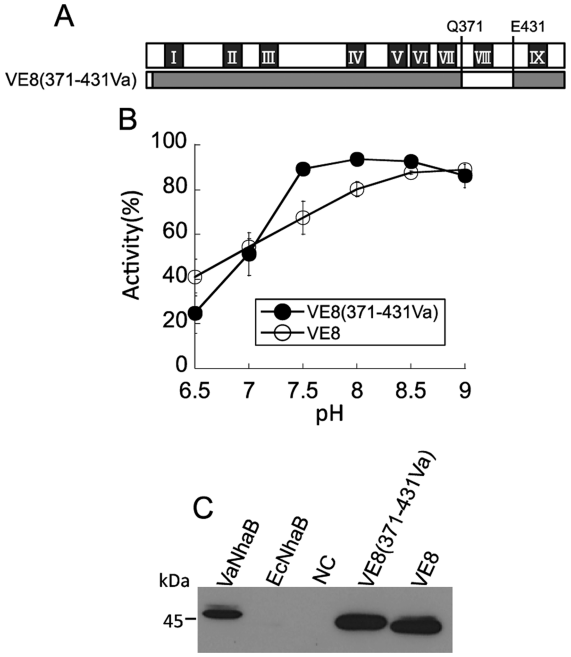


Figure 6. pH profiles of Na^+/H^+ antiport activity of VE8(371−431Va). (A) Schematic representation of VE8(371−431Va). Regions derived from VaNhaB and EcNhaB are indicated as in Figure 3. (B) pH profiles of the Na^+/H^+ antiporter of VE8(371−431Va) at 2.5 mM NaCl. (C) pH profiles of the Na^+/H^+ antiporter of VE8(371−431Va) at 100 mM NaCl. Na^+/H^+ antiport activities were determined and are depicted as described above. (D) Western blot analysis of 2×HA-tagged VE8(371−431Va). Descriptions are the same as in Figure 3.

difference in the pH response was not observed when activities were measured at 100 mM NaCl (Figure 6C). The amount of the VE8(371−431Va) protein in the membrane vesicles was estimated to be similar to that of VE8 (Figure 6D), indicating that the alteration of the pH profile was not attributed to the difference in the amount of the two chimeric proteins in the membrane vesicles. These results demonstrated that the E373−D434 region of VaNhaB confers pH sensitivity to VE8 to some extent, which strongly suggests that this region is able to function as a part of the pH-dependent regulatory machinery that could modulate antiport activity depending on the environmental pH, even in the pH insensitive chimeric antiporter VE8.

DISCUSSION

In this study, we have conducted a search for key residues or domains that are responsible for the significant acidic pH sensitivity of VaNhaB. Taking advantage of the high degree of amino acid sequence similarity with EcNhaB, we have generated various chimeric NhaB antiporters and analyzed their activities at different pHs. Our results revealed that residues E373−D434, which reside within the periplasmic loop 8−cytoplasmic loop 9 region of VaNhaB, are involved in the intrinsic mechanism of acidic pH sensitivity, although the exact function of this region is yet to be determined. Moreover, the incorporation of this particular region into VE8 successfully conferred some degree of acid sensitivity to the resultant molecule (Figure 6B), indicating that this region is able to act, at least partially, as the machinery that diminishes the antiport activity under acidic conditions, even in a pH insensitive chimeric antiporter. Taken together, these results strongly indicated that the E373−D434 region might be a part of the pH-dependent regulatory mechanisms of VaNhaB activity.

Several of our chimeric antiporters exhibited unexpected stronger or weaker net activities at 2.5 mM NaCl. For example, the activity of VE8 was significantly higher than that of EcNhaB at all the pHs tested, even though the only difference in the primary structure between these two antiporters is just seven amino acids from the N-terminal end (Figures 2 and 3). In contrast, VaS(1−40Ec), which comprises mostly VaNhaB except for 40 N-terminal amino acids and eight amino acids in loop 9 (corresponding to L427−D434 of VaNhaB) that were derived from EcNhaB, showed markedly reduced activity compared to that of VaNhaB (Figure 5). The Western blot analysis using 2×HA-tagged constructs indicated that the amounts of these proteins in the membrane vesicles were significantly higher and lower than those of EcNhaB and VaNhaB, respectively (Figures 2B, 3E, and 5C). Although the exact mechanisms are uncertain at this point, it seems highly likely that the amino acid sequence of the N-terminal end, probably the first seven amino acids or the nucleotide sequence encoding the corresponding region, has paramount importance in the determination of the amount of the NhaB proteins in the membrane. On the other hand, there are several cases where the amounts of chimeric proteins in the membrane fraction do not correspond to their net activity. For example, the VaS(266−321Ec) chimera showed significantly lower activity than VaNhaB, although the amount of tagged protein in the membrane vesicles was comparable to others (Figure 5). In contrast, while VaS(479−513Ec) exhibited activities comparable to those of other chimeras, its amount in the membrane vesicles was estimated to be very small (Figure 5). Although highly speculative, one possible cause of these controversial results might be that the substitution made on VaS(479−513Ec) somehow increased the specific activity of this chimera and simultaneously decreased the stability of the proteins. In the case of VaS(266−321Ec), however, only the decrease in specific activity might have occurred, probably because of the alkaline shift of the set-point pH and the alteration in the apparent K_m for Na^+. However, other possibilities, such as aberrant degradation of the tagged chimeric proteins as in the case of VaS(479−513Ec), cannot be ruled out at this point. Further studies, including measurements of the activities of purified chimeric proteins, would be needed to confirm this possibility.

The exact role of the E373−D434 region in the acid sensitivity of VaNhaB is currently unclear. According to the topology model proposed by Enomoto et al., this region contains most of periplasmic loop 8, the full length of TM9, and a part of cytoplasmic loop 9.[25] Although it is structurally unrelated, it is noteworthy that a similar membrane-spanning segment related to pH regulation is well-documented in EcNhaA, which has a "pH sensor" domain in cytoplasmic loop 9 followed by a "signal transducer" domain at the adjacent TM9, which is shown to be capable of changing its conformation according to pH.[14] These observations might imply a possibility that the E373−D434 region of VaNhaB also functions as a "pH sensor/transducer" domain, probably receiving the signal at cytoplasmic loop 9 and transmitting it through TM9, although the consequence of the conformational

dx.doi.org/10.1021/bi300738v | Biochemistry 2012, 51, 7766−7774

change might be different from that for EcNhaA because the shut-off of the activity did not seem to occur in the case of VaNhaB between pH 9.0 and 6.5 (Figure 5C). In addition, the suggested mechanism of pH-dependent regulation for EcNhaA involves the complex interactions of several functional domains that are separated in the primary structure. According to the proposed model for EcNhaA, the signal of pH change is transmitted from the pH sensor/transducer domain via the conformational change in the TM9−TM11 region, which consequently alters the assembly of TM4 and TM11 that consists of an essential part of the cation exchange machinery.[14] Interestingly, our results also suggested that additional residues or domains other than the E373−D434 region are responsible for the acid sensitivity of VaNhaB. For example, the chimeric antiporters VE72, VE388, and VE426 exhibited intermediate-type pH sensitivities that are distinct from that of VE8, despite the absence of the whole E373−D434 region. Moreover, the observation that the incorporation of the E373−D434 region into the VE8 was able to achieve an only partial acid sensitivity (Figure 6) suggested that the presence of the E373−D434 region alone is not sufficient to exhibit a VaNhaB-like acid sensitivity. On the other hand, no partial substitutions other than the E373−D427 region on VaS resulted in the loss of acid sensitivity (Figure 5), suggesting that some of those putative residues or domains might be expendable if other necessary components, including residues in the E373−D434 region, are present. Taken together, it seems highly likely that the mechanism of acid sensitivity in VaNhaB comprises multiple components, including the E373−D434 region as one component and other parts that might be distributed within the primary structure of VaNhaB. Although it is highly hypothetical at this point, it would be possible that these components, including the E373−D434 region, might be present in the vicinity and might associate or disassociate according to the change in the surrounding pH, which may consequently elicit conformational changes that would result in the modulation of the activity of VaNhaB. Clearly, identification of residues besides the E373−D434 region that are involved in the acid sensitivity of VaNhaB is required to assess this possibility, and such investigation is currently in progress. In addition, subsequent studies of their spatial configuration under acidic or alkaline conditions will also be needed to improve our understanding of the molecular mechanism involved in the integrated acid sensitivity of VaNhaB.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Oligo-DNA primers used for fusion PCR and oligonuculeotide linkers used for the construction of pHG165-2HA (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Address: 265-1 Higashijima, Akiha-ku, Niigata-shi, Niigata-ken 956-8603, Japan. Phone and fax: +81-250-25-5263. E-mail: yamaguchi@nupals.ac.jp.

Funding

This research was supported by Grant-in-Aid for Young Scientists (B) 22770132.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Prof. Toru Shigematsu and Prof. Eduardo Blumwald for helpful discussions. We also thank Mario Fon for proofreading and editing the manuscript.

■ ABBREVIATIONS

Nha, Na^+/H^+ antiporter; VaNhaA, *V. alginolyticus* NhaA; EcNhaB, *E. coli* NhaB; HpNhaA, *H. pylori* NhaA; TM, transmembrane region; PCR, polymerase chain reaction; BSA, bovine serum albumin; ChaA, $Na^+(K^+,Ca^{2+})/H^+$ antiporter.

■ REFERENCES

(1) Brett, C. L., Donowitz, M., and Rao, R. (2005) Evolutionary origins of eukaryotic sodium/proton exchangers. *Am. J. Physiol.: Cell Physiol. 288*, C223−C239.

(2) Padan, E., Bibi, E., Ito, M., and Krulwich, T. A. (2005) Alkaline pH homeostasis in bacteria: New insights. *Biochim. Biophys. Acta 1717*, 67−88.

(3) Padan, E., Maisler, N., Taglicht, D., Karpel, R., and Schuldiner, S. (1989) Deletion of *ant* in *Escherichia coli* reveals its function in adaptation to high salinity and an alternative Na^+/H^+ antiporter system(s). *J. Biol. Chem. 264*, 20297−20302.

(4) Putnoky, P., Kereszt, A., Nakamura, T., Endre, G., Grosskopf, E., Kiss, P., and Kondorosi, A. (1998) The *pha* gene cluster of *Rhizobium meliloti* involved in pH adaptation and symbiosis encodes a novel type of K^+ efflux system. *Mol. Microbiol. 28*, 1091−1101.

(5) Taglicht, D., Padan, E., and Schuldiner, S. (1991) Overproduction and purification of a functional Na^+/H^+ antiporter coded by *nhaA* (*ant*) from *Escherichia coli*. *J. Biol. Chem. 266*, 11289−11294.

(6) Swartz, T. H., Ito, M., Ohira, T., Natsui, S., Hicks, D. B., and Krulwich, T. A. (2007) Catalytic properties of *Staphylococcus aureus* and *Bacillus* members of the secondary cation/proton antiporter-3 (Mrp) family are revealed by an optimized assay in an *Escherichia coli* host. *J. Bacteriol. 189*, 3081−3090.

(7) Yamaguchi, T., Tsutsumi, F., Putnoky, P., Fukuhara, M., and Nakamura, T. (2009) pH-dependent regulation of the multi-subunit cation/proton antiporter Pha1 system from *Sinorhizobium meliloti*. *Microbiology 155*, 2750−2756.

(8) Padan, E. (2008) The enlightening encounter between structure and function in the NhaA Na^+/H^+ antiporter. *Trends Biochem. Sci. 33*, 435−443.

(9) Noumi, T., Inoue, H., Sakurai, T., Tsuchiya, T., and Kanazawa, H. (1997) Identification and characterization of functional residues in a Na^+/H^+ antiporter (NhaA) from *Escherichia coli* by random mutagenesis. *J. Biochem. 121*, 661−670.

(10) Gerchman, Y., Olami, Y., Rimon, A., Taglicht, D., Schuldiner, S., and Padan, E. (1993) Histidine-226 is part of the pH sensor of NhaA, a Na^+/H^+ antiporter in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A. 90*, 1212−1216.

(11) Rimon, A., Gerchman, Y., Kariv, Z., and Padan, E. (1998) A point mutation (G338S) and its suppressor mutations affect both the pH response of the NhaA-Na^+/H^+ antiporter as well as the growth phenotype of *Escherichia coli*. *J. Biol. Chem. 273*, 26470−26476.

(12) Rothman, A., Gerchman, Y., Padan, E., and Schuldiner, S. (1997) Probing the conformation of NhaA, a Na^+/H^+ antiporter from *Escherichia coli*, with trypsin. *Biochemistry 36*, 14572−14576.

(13) Tsuboi, Y., Inoue, H., Nakamura, N., and Kanazawa, H. (2003) Identification of membrane domains of the Na^+/H^+ antiporter (NhaA) protein from *Helicobacter pylory* required for ion transport and pH sensing. *J. Biol. Chem. 278*, 21467−21473.

(14) Hunte, C., Screpanti, E., Venturi, M., Rimon, A., Padan, E., and Michel, H. (2005) Structure of a Na^+/H^+ antiporter and insights into mechanism of action and regulation by pH. *Nature 435*, 1197−1202.

(15) Kuwabara, N., Inoue, H., Tsuboi, Y., Mitsui, K., Matsushita, M., and Kanazawa, H. (2006) Structure-function relationship of the fifth transmembrane domain in the Na^+/H^+ antiporter of *Helicobacter pylori*: Topology and function of the residues, including two consecutive essential aspartate residues. *Biochemistry 45*, 14834−14842.
(16) Saier, M. H., Jr., and Paulsen, I. T. (2000) Whole genome analysis of transporters in spirochetes: *Borrelia burgdorferi* and *Treponema pallidum*. *J. Mol. Microbiol. Biotechnol. 2*, 393−399.
(17) Krulwich, T. A., Hicks, D. B., and Ito, M. (2009) Cation/proton antiporter complements of bacteria: Why so large and diverse? *Mol. Microbiol. 74*, 257−260.
(18) Kuroda, T., Fujita, N., Utsugi, J., Kuroda, M., Mizushima, T., and Tsuchiya, T. (2004) A major Li^+ extrusion system NhaB of *Pseudomonas aeruginosa*: Comparison with the major Na^+ extrusion system NhaP. *Microbiol. Immunol. 48*, 243−250.
(19) Utsugi, J., Inaba, K., Kuroda, T., Tsuda, M., and Tsuchiya, T. (1998) Cloning and sequencing of a novel Na^+/H^+ antiporter gene from *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta 1398*, 330−334.
(20) Nozaki, K., Inada, K., Kuroda, T., Tsuda, M., and Tsuchiya, T. (1996) Cloning and sequencing of the gene for Na^+/H^+ antiporter of *Vibrio parahaemolyticus*. *Biochem. Biophys. Res. Commun. 222*, 774−779.
(21) Nakamura, T., Fujisaki, Y., Enomoto, H., Nakayama, Y., Takabe, T., Yamaguchi, N., and Uozumi, N. (2001) Residue aspartate-147 from the third transmembrane region of Na^+/H^+ antiporter NhaB of *Vibrio alginolyticus* plays a role in its activity. *J. Bacteriol. 183*, 5762−5767.
(22) Pinner, E., Padan, E., and Schuldiner, S. (1992) Cloning, sequencing, and expression of the *nhaB* gene, encoding a Na^+/H^+ antiporter in *Escherichia coli*. *J. Biol. Chem. 267*, 11064−11068.
(23) Nakamura, T., Enomoto, H., and Unemoto, T. (1996) Cloning and sequencing of *nhaB* gene encoding an Na^+/H^+ antiporter from *Vibrio alginolyticus*. *Biochim. Biophys. Acta 1275*, 157−160.
(24) Pinner, E., Padan, E., and Schuldiner, S. (1994) Kinetic properties of NhaB, a Na^+/H^+ antiporter from *Escherichia coli*. *J. Biol. Chem. 269*, 26274−26279.
(25) Enomoto, H., Unemoto, T., Nishibuchi, M., Padan, E., and Nakamura, T. (1998) Topological study of *Vibrio alginolyticus* NhaB Na^+/H^+ antiporter using gene fusions in *Escherichia coli* cells. *Biochim. Biophys. Acta 1370*, 77−86.
(26) Ohyama, T., Igarashi, K., and Kobayashi, H. (1994) Physiological role of the *chaA* gene in sodium and calcium circulations at a high pH in *Escherichia coli*. *J. Bacteriol. 176*, 4311−4315.
(27) Yamaguchi, T., Apse, M. P., Shi, H., and Blumwald, E. (2003) Topological analysis of a plant vacuolar Na^+/H^+ antiporter reveals a luminal C terminus that regulates antiporter cation selectivity. *Proc. Natl. Acad. Sci. U.S.A. 100*, 12510−12515.
(28) Messing, J., and Vieira, J. (1982) A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. *Gene 19*, 269−276.
(29) Rosen, B. P. (1986) Ion extrusion system in *Escherichia coli*. *Methods Enzymol. 125*, 328−336.
(30) Radchenko, M. V., Tanaka, K., Waditee, R., Oshimi, S., Matsuzaki, Y., Fukuhara, M., Kobayashi, H., Takabe, T., and Nakamura, T. (2006) Potassium/proton antiport system of *Escherichia coli*. *J. Biol. Chem. 281*, 19822−19829.
(31) Rath, A., Glibowicka, M., Nadeau, V. G., Chen, G., and Deber, C. M. (2009) Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Proc. Natl. Acad. Sci. U.S.A. 106*, 1760−1765.
(32) Galili, L., Rothman, A., Kozachkov, L., Rimon, A., and Padan, E. (2002) Trans membrane domain IV is involved in ion transport activity and pH regulation of NhaA-Na^+/H^+ antiporter of *Escherichia coli*. *Biochemistry 41*, 609−617.
(33) Galili, L., Herz, O., and Padan, E. (2004) Unraveling functional and structural interactions between transmembrane domains IV and XI of NhaA Na^+/H^+ antiporter of *Escherichia coli*. *J. Biol. Chem. 279*, 23104−23113.