

Identification and Characterization of Functional Residues in a Na^+/H^+ Antiporter (NhaA) from *Escherichia coli* by Random Mutagenesis¹

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Forty-one mutants were isolated by means of random PCR mutagenesis of the *Escherichia coli* Na^+/H^+ antiporter (*nhaA*), which could not support the growth of a *nhaAnhaB* mutant (HIT Δ AB⁻) on plates containing 0.15 M LiCl (pH 7.5) or 0.65 M NaCl (pH 8.0). Most of the mutants were sensitive to both NaCl and LiCl, or to LiCl alone. DNA sequencing revealed that twelve of the mutants had single amino acid substitutions. All the mutations, except for H225P, were of the conserved residues of NhaA homologues and located in the putative transmembrane helices. The Na^+/H^+ and Li^+/H^+ antiporter activities of the mutant NhaA were measured with everted membrane vesicles: eight of the mutants lost both antiporter activities completely under all pH conditions examined. Although both D133A and L138P retained low Li^+/H^+ antiporter activity, D133A lost Na^+/H^+ antiporter activity, while L138P retained normal Na^+/H^+ antiporter activity at pH 7.0 and 8.0. Interestingly, at pH 8.5, L138P no longer showed any Li^+/H^+ antiporter activity. H225P retained relatively high antiporter activities, although their pH dependence was altered. These observations supported the previous indication that His-225 is part of the pH sensor [Gerchman, Y. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90, 1212-1216]. L73R exhibited about 20% each of the activities only at pH 8.0, and showed a similar pH dependence to H225P in both Na^+/H^+ and Li^+/H^+ antiport. Therefore, in addition to His-225, Leu-73, and/or its vicinity may also contribute to the pH sensing.

Key words: cation transport, Li^+ resistance, mutagenesis, Na^+/H^+ antiporter, temperature sensitivity.

The Na^+/H^+ antiporter is distributed ubiquitously in bacterial, plant, and mammalian cells. The antiporter is localized in the cytoplasmic and organellar membranes, and plays pivotal roles not only in establishing $\Delta\mu\text{Na}^+$ necessary for Na^+ -coupled substrate transport, but also in regulation of the intracellular pH, Na^+ concentration, and cell volume (1-3). To date, two distinct Na^+/H^+ antiporters, *nhaA* and *nhaB*, have been cloned from *Escherichia coli* (4, 5), and characterized biochemically, including purification of the antiporters and their reconstitution into liposomes (6, 7). Similar distinct Na^+/H^+ antiporter activities were also reported for other bacteria (8-12). More recently, *chaA*, which is primarily a $\text{Ca}^{2+}/\text{H}^+$ antiporter, was cloned from *E. coli* (13). It is noteworthy that *chaA* exhibits Na^+/H^+ antiport activity only at pH 8.5.

It is well established that NhaA antiporter activity is dependent on the environmental pH such that the activity is greatly enhanced as the pH increases from neutral to alkaline; at pH 7.0, NhaA has negligible antiporter activ-

ity, while at pH 8.5 its activity is enhanced by three orders of magnitude (6). Unlike that of NhaA, NhaB antiporter activity is relatively constitutive, although the affinity for Na^+ increases tenfold as the pH increases from 7.2 to 8.5 (7). Despite these observations, at alkaline pH NhaA alone is not sufficient to maintain the intracellular pH for proper growth of *E. coli* (14). Normally, *E. coli* can survive in a medium containing a high NaCl concentration (0.65 M NaCl, pH 8.0), and tolerates the toxic effect of LiCl (0.15 M LiCl, pH 7.5) by extruding Na^+ and Li^+ through the Na^+/H^+ antiporters. Mutants defective in *nhaA* are not tolerant to an elevated salt concentration in the medium and cannot grow at a high NaCl (0.6 M at pH 7.5) (15) or LiCl (0.3 M) concentration (16). These defects could be overcome by exogenous expression of *nhaA*. Unlike NhaB, the NhaA antiporter exhibits relatively high transporter activity for Li^+ , which may confer tolerance to Li^+ through extrusion of the ion into the medium (15). These observations indicated that the NhaA antiporter may function primarily by regulating the intracellular Na^+ and Li^+ concentrations. Interestingly, the expression of *nhaA* is promoted by an elevated salt concentration in the medium and is further enhanced by alkaline pH (17), which is consistent with the possible role of NhaA in regulation of the intracellular salt concentration.

Recently, *nhaA* homologues were cloned from *Salmonel-*

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la entiriditis (11), *Vibrio arginolyticus* (10), and *Vibrio parahaemolyticus* (8). Their primary sequences are well conserved and the overall amino acid sequence identity is about 60%. They were predicted to have ten to twelve transmembrane helices on hydrophathy plot analyses (6, 8, 10, 11, 18). No significant homology between the primary structures of NhaA and NhaB has been identified. Thus, it is not possible to deduce their functional domains or residues by simply comparing their primary structures. Although a few sequence elements were extracted by comparing their primary sequences with those of other known proteins and were anticipated to be involved in essential functions of the antiporter (8), no details regarding the structure-function relationships have yet been experimentally elucidated except that His-225³ may constitute part of the pH sensor integrated in NhaA, as revealed by site-directed mutagenesis (19). Since the importance of negative charges on cation transporters has been widely observed (20–26), we have addressed the functional involvement of five Asp residues in the NhaA antiporter (four in the transmembrane domains and one in the extra-membrane domain) by site-directed substitution of Asp residues with Asn. From the results of this experiment, we concluded that three conserved Asp residues, Asp-133, Asp-163, and Asp-164, are important for the antiporter function (18). To further identify functional residues throughout the antiporter, we here applied random mutagenesis to the whole *nhaA* gene and isolated novel mutants.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—HIT Δ AB⁻ and HIT Δ AB⁺ (27), defective in both *nhaA* and *nhaB*, and *nhaA* alone, respectively, were cultured aerobically in LB medium with or without ampicillin (50 μ g/ml) and kanamycin (50 μ g/ml) at 37°C. The growth of the mutants was measured in LBK (LB containing 87 mM KCl for NaCl) adjusted to the desired pH with 20 mM Tricine-KOH, and supplemented with appropriate concentrations of LiCl or NaCl. Temperature sensitivity was investigated under the same conditions as above at 30 and 42°C in liquid cultures or on agar plates. For the cloning experiments, pUC18 and pBluescript II KS+ were used as vectors, and JM103 was used as a host strain (28).

Construction of Plasmids and Random PCR Mutagenesis of *nhaA*—Two primers corresponding to the amino- and carboxyl-termini of *nhaA* were synthesized, and the coding region for *nhaA* was amplified from the chromosomal DNA of wild-type *E. coli* derived from K-12 by PCR. The amplified fragment (1,150 bp) was digested with *EcoRI* and *SphI*, the restriction sites for which were incorporated into the ends of the primers, and ligated into the corresponding sites of pUC18. The resulting plasmid was designated as pUC-GTG. The *nhaA* gene was recloned into pBluescript II KS+ digested with *EcoRI* and *HindIII* for further manipulation (pBlue-GTG). To minimize the toxic effect of overproduction of NhaA, the initiation codon (GTG) for NhaA was substituted with CTG by site-directed mutagenesis as described previously (18), yielding pBlue-CTG. The *nhaA*

gene with the CTG initiation codon was excised by digestion with *SmaI* and *SaII*, and inserted into the *EcoRV* and *SaII* sites of pBR322 (the endogenous *EcoRI* site was eliminated by end-filling) (pBR-CTG). pBR-CTG complemented the defects in *nhaA* and *nhaB* on introduction into HIT Δ AB⁻ or HIT Δ AB⁺. Random mutations were introduced into *nhaA* by PCR as follows. The *nhaA* gene in pBlue-CTG was amplified with Tth polymerase (Toyobo), and the T3 and T7 primers for the pBluescript vector in the presence of a limited concentration of dATP or dTTP (0.4 μ M) by five cycles (denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 70°C for 1 min), followed by 25 cycles with additional dATP or dTTP (200 μ M) as above. One-microliter aliquots of the above sample were taken, and the mutagenized *nhaA* was re-amplified with Pfu polymerase (Stratagene) and primers corresponding to the amino- and carboxyl-termini of the *nhaA* gene, as described above, by eight cycles. The samples thus obtained were digested with *EcoRI*, *BglIII*, and *SphI*. The *EcoRI*-*BglIII* (470 bp) and *BglIII*-*SphI* (710 bp) fragments corresponding to the amino- (amino acid residues 1–153) and carboxyl-terminal (amino acid residues 154–388) regions of NhaA, respectively, were purified and ligated back into the corresponding sites of pBR-CTG. After transformation of HIT Δ AB⁻, ampicillin-resistant transformants were isolated and their growth was examined on LB plates containing 0.15 M LiCl (pH 7.5) or 0.65 M NaCl (pH 8.0).

Preparation of Membrane Vesicles, and Measurement of Na⁺/H⁺ and Li⁺/H⁺ Antiporter Activities—The HIT Δ AB⁻ mutant transformed with *nhaA* plasmids was cultured in 300 ml of LB containing ampicillin (50 μ g/ml) and kanamycin (50 μ g/ml) until the A₆₀₀ reached 0.8. The cells were harvested, washed, and then resuspended in 10 mM Tris-HCl (pH 7.2) containing 140 mM KCl, 5 mM MgSO₄, 1.5 mM β -mercaptoethanol, and 10% glycerol. The cell suspension was passed through a French press, and everted membrane vesicles were collected by ultracentrifugation (100,000 \times *g*, 60 min) after unbroken cells and cell debris had been removed by low speed centrifugation. The membrane vesicles were resuspended in the same buffer as above and glycerol was added to a final concentration of 50%. The protein concentrations of membrane vesicles were adjusted to 10 mg/ml, and the vesicles were stored at -20°C and used for the antiporter assay within a few days. The antiporter activities were measured with 200 μ g of membrane proteins in 2 ml of 10 mM Tricine-KOH (pH 7.0, 8.0, or 8.5) containing 140 mM KCl and 1 μ M quinacrine. The changes in Δ pH were monitored by quenching and dequenching of quinacrine fluorescence (ex = 420 nm, em = 500 nm) as follows. Initially, an internal acidic Δ pH was established by lactate-dependent respiration resulting in fluorescence quenching. Subsequently, the addition of NaCl or LiCl drove the NhaA antiporter and the quenched fluorescence was recovered on resumption of the Δ pH. The antiporter activities are expressed as percent restoration of the initial fluorescence quenching. To preclude endogenous K⁺/H⁺ antiport activity, 140 mM KCl was included in the assay medium throughout the experiments.

Other Procedures and Materials—DNA sequences were determined by the dideoxy method (29) with T7 polymerase (Pharmacia Biotech) and [α -³⁵S]dCTP (NEN/DuPont). For the convenience of sequencing, several primers corresponding to internal regions of *nhaA* were synthesized and

³ The number of His-225 was corrected from the 226 reported by Gerchman *et al.* (see Ref. 18) as they miscounted the residues in NhaA.

used. Protein concentrations were determined by the published method (30). Restriction enzymes and T4 DNA ligase were purchased from BRL. Low-melting point agarose (Sea-Plaque GTG) was from Takara Shuzo. Other reagents used were of the highest grade commercially available.

RESULTS

Isolation of Random Mutants as to *nhaA*—The growth of mutants defective in the Na⁺/H⁺ antiporter, *nhaA*, is sensitive to elevated concentrations of NaCl or LiCl in the medium, and thus the HITΔAB⁻ and HITΔAB⁺ mutants could not grow on plates containing 0.65 M NaCl (pH 8.0) or 0.15 M LiCl (pH 7.5), respectively. When the plasmid expressing wild-type *nhaA* was introduced in the mutants, the defects were complemented and the transformed cells could grow on the selection plates. We constructed several types of *nhaA* expression plasmids and introduced them into the HITΔAB⁻ and HITΔAB⁺ mutants. Although moderate production of NhaA complemented the defect, higher levels caused growth retardation and might be lethal (Noumi *et al.*, unpublished observation). To minimize the toxic effect of overproduction of NhaA, we substituted the initiation codon, GTG, with CTG in the pBR322-derived *nhaA* expression plasmid. pBR-CTG gave better complementation results on the selection plates than pBR-GTG. Thus we utilized the pBR-CTG *nhaA* expression/complementation system in HITΔAB⁻ to isolate random mutants.

Random mutations were introduced into *nhaA* through spontaneous misincorporation of deoxynucleotides by Tth polymerase during the PCR reaction. The amplified DNA fragments were divided into two segments (corresponding to residues 1-153 and 154-388), and ligated back into pBR-CTG, and ampicillin-resistant transformants were isolated. In two independent experiments, 1,610 primary transformants were obtained and their growth abilities on plates containing high NaCl (0.65 M, pH 8.0) or high LiCl (0.15 M, pH 7.5) were examined. *nhaA* mutants were defined as those which could not support the growth of HITΔAB⁻ on the selection plates. According to this criterion, 9 and 32 mutants as to the amino- and carboxyl-terminal regions were isolated, respectively. Most of the mutations resulted in the loss of tolerance to both NaCl and LiCl or LiCl alone, while only two mutants were more sensitive to NaCl than LiCl (Table I).

Mutations in the Na^s and Li^s *nhaA* Mutants—The primary sequences of the 41 mutant *nhaA* genes were determined by sequencing the corresponding regions entirely. In experiment 1, seven single amino acid mutations, one double mutation, one nonsense mutation, and three frameshift mutations were identified (Table I). All mutants, however, had a V279A⁴ mutation, suggesting that

⁴The mutants are designated according to the original residue, the position in NhaA and the substituted residue, respectively. For example, V279A indicates that Val at position 279 of NhaA was substituted with Ala.

TABLE I. Substitution of amino acid residues in *nhaA* mutants, and sensitivity to high concentrations of NaCl and LiCl in agar plates.

Exp. 1 C-terminal region (154-388)				Exp. 2 N-terminal region (1-153)				Exp. 2 C-terminal region (154-388)			
Mutant	Substitution	Na	Li	Mutant	Substitution	Na	Li	Mutant	Substitution	Na	Li
1131	I-171 → T	+/-	+/-	2053	G-14 → R	+/-	-	2074	G-166 → R	-	-
1121	A-188 → V	-	-	2024	L-73 → R	+/-	-	2084	H-225 → P	+/-	-
1111	K-249 → M	+	-	2014	D-133 → A	-	-	2112	F-267 → S	-	-
1151	G-291 → R	-	+/-	2021	L-138 → P	+	-	2071	L-302 → P	-	-
1081	K-300 → E	-	-					2096	G-303 → R	-	-
1132	C-308 → R	+/-	-	2022	G-36 → end	-	-	2083	C-335 → R	-	-
1092	S-369 → P	-	-	2015	G-51 → end	-	-	2075	S-342 → P	-	-
								2072	S-369 → P	-	-
1133	G-291 → R	-	+/-	2023	Initiation-less	-	-	2092	S-369 → P	-	-
	V-382 → A	-	-	2033	Initiation-less	-	-				
				2042	Initiation-less	-	-	2081	Y-175 → C	-	-
1142	W-258 → end	-	-						L-312 → P		
								2114	S-183 → P	+	-
1122	L-173 → fs	-	-						A-268 → V		
1091	R-250 → fs	-	-					2097	N-198 → T	-	-
1101	G-278 → fs	-	-						N-271 → S		
								2093	V-219 → A	+	-
									L-306 → P		
								2102	S-222 → P	+/-	-
									G-291 → R		
								2082	H-243 → R	-	-
									S-342 → P		
								2101	L-266 → P	+	+/-
									L-357 → P		
								2094	L-297 → P	-	-
									L-312 → P		
								2104	L-306 → P	+	-
									F-344 → L		
								2073	L-228 → end	-	-
								2095	L-255 → end	-	-

The tolerance and sensitivity to 0.65 M NaCl (pH 8.0) or 0.15 M LiCl (pH 7.5) are expressed as + and -, respectively, +/- representing partial tolerance. Three mutants have one base deletion resulting in a frameshift (fs) at the indicated residue, and three other mutants have a base substitution within the initiation codon (initiation-less).

V279A was introduced at an early stage of PCR amplification. We isolated V279A as the sole mutation and assessed its effect on Na^+/H^+ antiporter activity. V279A did not alter the antiporter activity and supported the growth of $\text{HIT}\Delta\text{AB}^-$ on the selection plates (data not shown). Therefore, we concluded that V279A was functionally silent, and the phenotype of the mutants isolated in experiment 1 solely represented the effect of a mutation other than V279A. Nevertheless, we did not examine the experiment 1 mutants in the present study to simplify biochemical characterization.

Of the 29 mutants identified in experiment 2, thirteen had single amino acid substitutions, of which two had an identical substitution, S369P (Table I). We also identified nine double mutations, four nonsense mutations and three initiation defective mutations. Obviously, most of the

mutants were more sensitive to LiCl than NaCl, or sensitive to both. We studied the twelve single mutations to facilitate understanding of the relationship between structural alterations and the function of NhaA.

Na^+/H^+ and Li^+/H^+ Antiporter Activities of *nhaA* Mutants—To assess the effects of mutations on the antiporter activities, everted membrane vesicles were prepared from $\text{HIT}\Delta\text{AB}^-$ carrying a vector plasmid (negative control), the wild-type plasmid (positive control) or a mutant *nhaA* expression plasmid⁵. The Na^+/H^+ and Li^+/H^+ antiporter activities were measured as cancellation of ΔpH established through respiratory H^+ pumping. No Na^+/H^+ and Li^+/H^+ antiporter activities were observed under any

⁵ The *nhaA* gene used as the wild-type in this study carries a CTG initiation codon instead of the original GTG codon.

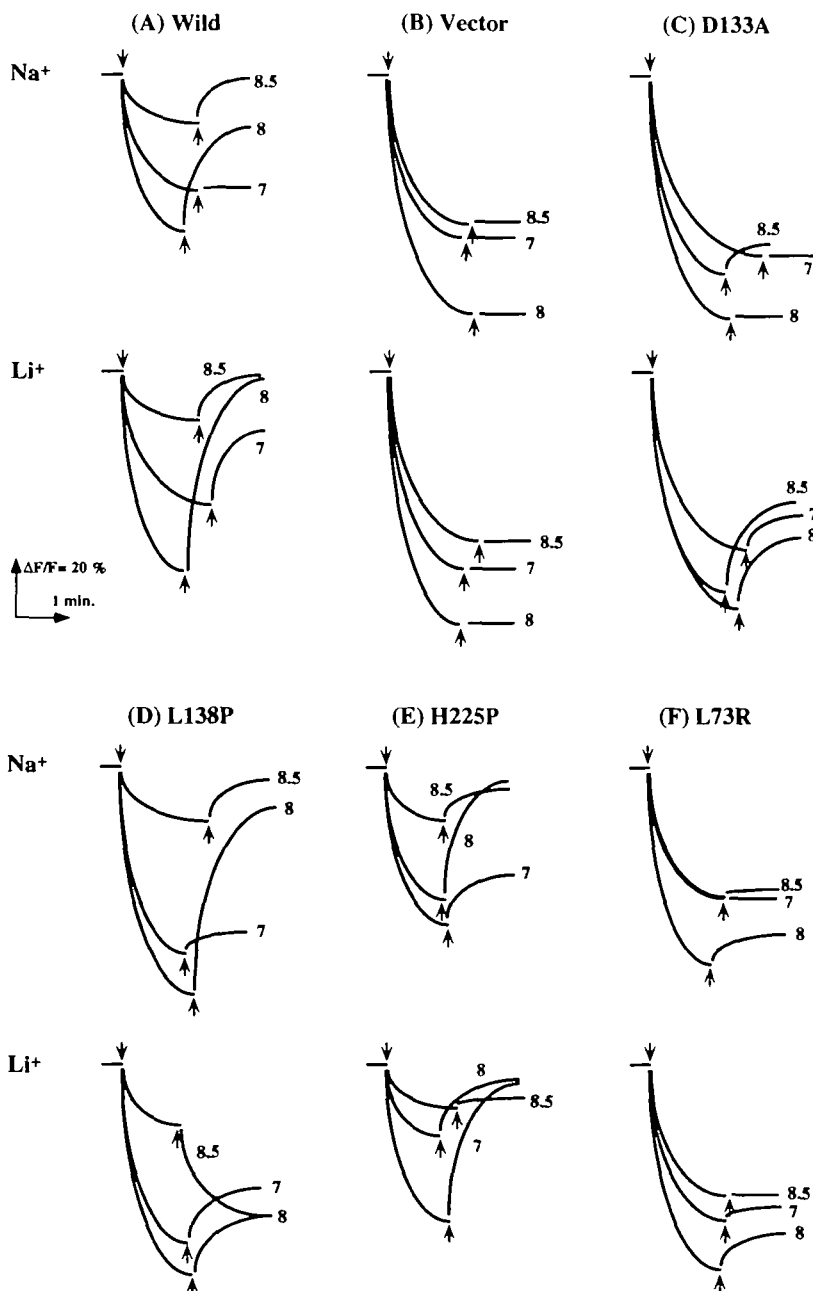


Fig. 1. Na^+/H^+ and Li^+/H^+ antiporter activities in the everted membrane vesicles from NhaA mutants. Everted membrane vesicles were prepared from the $\text{HIT}\Delta\text{AB}^-$ mutant carrying the wild-type (A, positive control) or a mutant (C, D133A; D, L138P; E, H225P; F, L73R) *nhaA* expression plasmid, or the vector alone (B, negative control) as described under "EXPERIMENTAL PROCEDURES." Respiration was initially driven by the addition of K^+ -lactate (5 mM) to the assay mixture (downward arrows). After the fluorescence quenching reached a plateau level, NaCl or LiCl (5 mM) was added (upward arrows) to elicit Na^+/H^+ or Li^+/H^+ antiporter activity of NhaA. The upper and bottom panels show Na^+/H^+ and Li^+/H^+ antiporter activities, respectively.

pH conditions examined when the vector alone was introduced, since the host mutant, HIT Δ AB⁻, was defective in both NhaA and NhaB antiporters (Fig. 1B). Transformation with the wild-type *nhaA* expression plasmid restored both antiporter activities, as revealed by the restoration of fluorescence quenching after the addition of NaCl or LiCl (Fig. 1A). We confirmed the lack of antiporter activities at pH 7.0, while substantial antiporter activities were observed at pH 8.0 and 8.5. Since the antiporter activities were enhanced at pH 8.5, the NaCl contaminating the KCl could sufficiently drive the NhaA antiporter at pH 8.5, so that the initial fluorescence quenching by respiration decreased (Fig. 1A). With lower concentrations of KCl, the initial fluorescence quenching by respiration was increased at pH 8.5 (data not shown).

Eight of the twelve mutants (G14R, G166R, F267S, L302P, G303R, C335R, S342P, and S369P) showed fluorescence patterns similar to those observed with the vector alone, indicating that they lost the antiporter activities under all pH conditions examined (data not shown). Therefore, the mutated residues may be critical for the antiporter activity, stability, and/or assembly of NhaA. While four mutants, L73R, D133A, L138P, and H225P, retained significant levels of antiporter activities. The characteristics of their altered antiporter activities are described in detail in the following sections.

D133A and L138P Mutations Altered the Ion Specificity of the NhaA Antiporter—About 30–40% of the Li⁺/H⁺ antiporter activity was retained by D133A, whereas less than 15% of the Na⁺/H⁺ antiporter activity was observed at any pH examined (Fig. 1C). The responses to the pH of the medium of the two antiporter activities were not altered. In contrast, the L138P mutation had little effect on the Na⁺/H⁺ antiporter activity. The Na⁺/H⁺ antiporter activity of L138P was almost the same as that of the wild-type, and its pH dependence was preserved. The Li⁺/H⁺ antiporter activity of L138P was decreased to 30–40% of that of the wild-type at pH 7.0 and 8.0, and was no longer stimulated by alkaline pH (Fig. 1D). These results are consistent with the observation that the L138P mutant was sensitive to high LiCl but tolerant to high NaCl in the agar plates, as shown in Table I. The growth properties of these two mutants were examined in terms of their sensitivity to NaCl or LiCl in liquid culture. As shown in Fig. 2, the

growth of D133A was highly sensitive to high concentrations of NaCl, whereas L138P was less sensitive to high NaCl in the medium. Despite the retention by these two mutants of about 30% of the Li⁺/H⁺ antiporter activity of the wild-type, they could not survive in medium containing LiCl. Although the residual Li⁺/H⁺ antiporter activity of

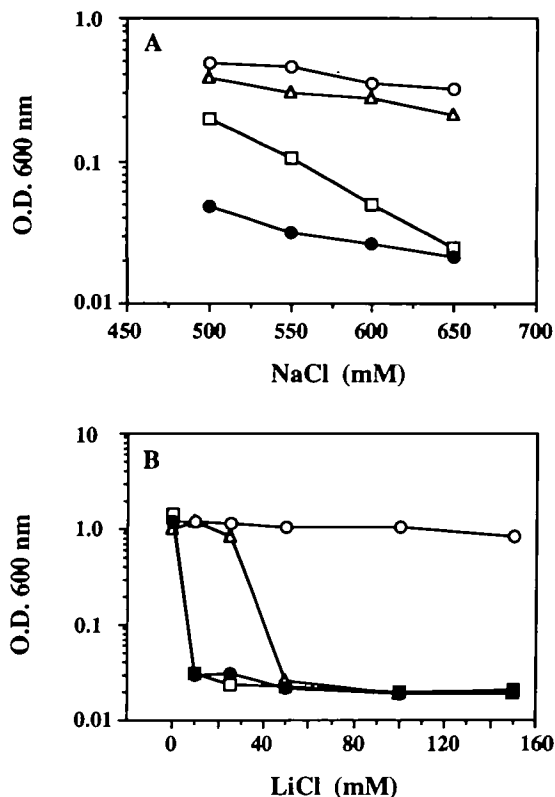


Fig. 2. Growth properties of the D133A and L138P mutants in high-NaCl- or high-LiCl-containing medium. The HIT Δ AB⁻ mutant carrying the vector only (●, negative control), or the wild-type (○, positive control) or a mutant *nhaA* (□, D133A; △, L138P) expression plasmid was grown aerobically in LBK (pH 8.5) containing the indicated concentrations of NaCl (A) or LiCl (B) at 37°C. The growth yields were taken as the plateau level of A_{600} and plotted as a function of the concentration of NaCl or LiCl in the medium.

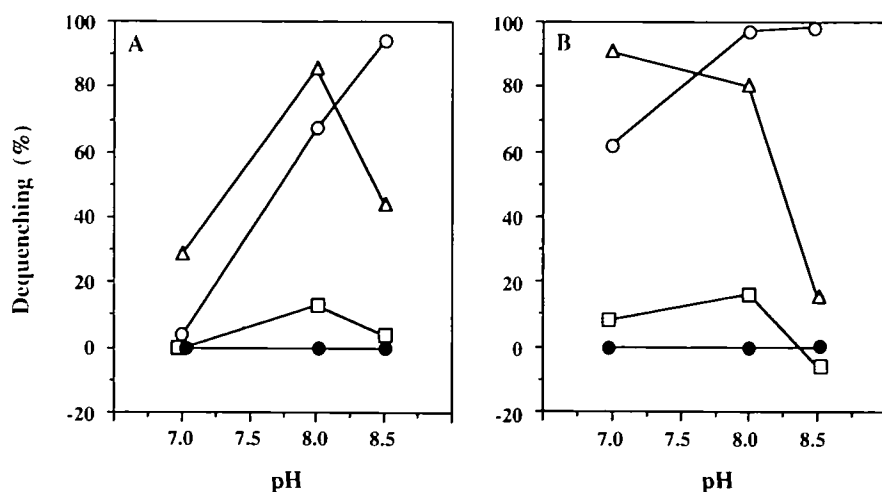


Fig. 3. pH dependence of the Na⁺/H⁺ and Li⁺/H⁺ antiporter activities of H225P, L73R, and wild-type NhaA. The Na⁺/H⁺ and Li⁺/H⁺ antiporter activities of the mutant H225P (△), L73R (□), and wild-type (○) NhaA, and the vector alone (●) expressed as percent dequenching of the initial fluorescence quenching were taken from Fig. 1 and plotted as a function of the pH of the assay mixture.

these mutants appeared to be similar on fluorescence dequenching measurements, L138P showed slight tolerance to low concentrations of LiCl (Fig. 2). The most marked alteration in the Li^+/H^+ antiporter activity of L138P was observed at pH 8.5, where the fluorescence quenching by respiration was further enhanced on the addition of LiCl, rather than the restoration seen with normal antiporter activity (Fig. 1D). Although the exact mechanism is not known, the mutation might have abolished the Li^+/H^+ antiporter activity specifically at pH 8.5 (for details see "DISCUSSION").

L73R or H225P Mutations Altered the pH Response of the NhaA Antiporter—Although the H225P mutant was isolated as being sensitive to high NaCl and LiCl, it retained relatively high antiporter activities (Fig. 1E). As discussed for H225R (19), the pH dependence of H225P as to activation of the antiporter was altered; the highest antiporter activity was observed at pH 8.0 with H225P, but at pH 8.5 or higher with the wild-type antiporter (Fig. 1E). As shown in Fig. 3, the activation of H225P showed marked pH dependence. More marked alterations of its pH-dependent activation were observed in Li^+/H^+ antiporter activity. With the wild-type antiporter, the maximal Li^+/H^+ antiporter activity was attained at around pH 8.5, while H225P was most active at pH 7.0, where it showed equivalent activity to the wild-type, but exhibited only 15% of the

activity of the wild-type at pH 8.5 (Fig. 3). These results supported the previous indication that His-225 constitutes part of the pH sensor in the NhaA antiporter.

L73R showed severely impaired antiporter activities but retained significant residual activities at pH 8.0: about 20% each of the Na^+/H^+ and Li^+/H^+ antiporter activities of wild-type were observed (Fig. 1F). No selective impairment of cation recognition or antiport was observed, and the Na^+/H^+ and Li^+/H^+ antiporter activities of L73R were both maximal at pH 8.0 and abolished at pH 8.5 (Fig. 3). These results suggested that Leu-73 and/or its vicinity may also contribute to the pH sensing.

Temperature-Sensitive Phenotype of nhaA Mutants—The $\text{HIT}\Delta\text{AB}^-$ mutants carrying the vector alone (negative control), the wild-type (positive control) plasmid, or one of the twelve single mutation expression plasmids was replicated on high-NaCl- or high-LiCl-containing plates, and then incubated at 30 and 42°C. After 2 days incubation, the growth abilities at the high and low temperatures were examined. The transformants with the wild-type *nhaA* plasmid grew normally on high-NaCl and high-LiCl plates at all temperatures examined, and negative controls did not grow under any selection conditions. Mutants other than L73R and H225P did not show any temperature-dependent growth alteration on the selective plates. As shown in Table I and Fig. 2, L73R and H225P were more or less viable on

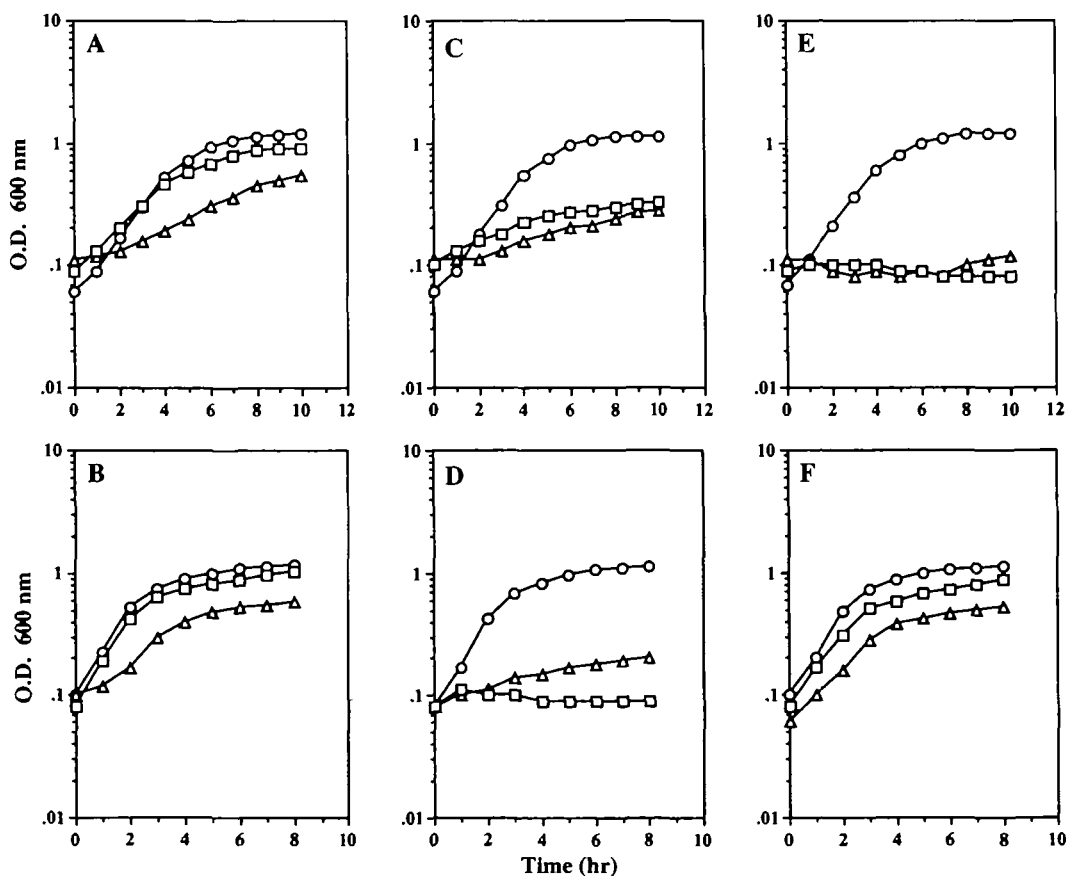


Fig. 4. Temperature sensitivity of the growth of the H225P and L73R mutants in high-NaCl- or high-LiCl-containing medium. The $\text{HIT}\Delta\text{AB}^-$ mutant carrying the wild-type (A and B) or a mutant *nhaA* (L73R, C and D; H225P, E and F) expression plasmid was grown

aerobically in LBK (pH 7.0, \circ), or LBK containing 0.65 M NaCl (pH 8.0, \triangle) or 0.15 M LiCl (pH 7.5, \square). Cell growth was measured turbidimetrically at 30°C (A, C, E) and 42°C (B, D, F).

the high-NaCl plates, while they were not on high-LiCl plates. Interestingly, L73R could grow at 30°C but not at 42°C on both high-NaCl and high-LiCl plates, and thus this mutation was high temperature-sensitive. In contrast, H225P did not grow at 30°C but did at 42°C, and thus was low temperature-sensitive. We investigated these growth phenotypes in more detail by measuring the growth of these mutants in liquid culture with high NaCl or high LiCl concentrations. As shown in Fig. 4, H225P could not grow at 30°C, but survived and grew normally at 42°C in both high-NaCl and high-LiCl media. In contrast to H225P, L73R showed distinct temperature-sensitive growth in high-NaCl and high-LiCl media. L73R could not grow at all in the high-LiCl medium at 42°C but grew slowly at 30°C. In the high-NaCl medium, L73R grew slowly at both 30 and 42°C, although growth was retarded at 42°C in comparison with in the case of the wild-type. These results suggested that these NhaA mutants were sensitive to temperature, including their transport activity, assembly, and stability.

DISCUSSION

To date, only a limited number of functional residues have been identified in the NhaA antiporter. Gerchman *et al.* identified His-225 as part of the pH sensor based on the results of a site-directed mutagenesis study (19). Similarly, by site-directed mutagenesis we recently identified three Asp residues, Asp-133, -163, and -164, important for antiporter activity (18). In the present study, we isolated twelve independent single mutants with loss of antiporter

activities. It is noteworthy that all twelve mutated residues found in this study were conserved in the sequences of four *nhaA* homologues, suggesting that the residues identified in our random mutagenesis may have essential roles in the antiporter. In addition, most of these mutations (the exception being H225P) are located in the putative transmembrane region (Fig. 5). These results, similar to other reports (31, 32), indicated that residues important for both transport and assembly are widely distributed in the transmembrane region.

Although less conservation and homology were observed between NhaA and other Na⁺/H⁺ antiporters and Na⁺-transporting proteins, Kuroda *et al.* (8) extensively searched for and reported novel sequence elements homologous to those in other proteins: (i) residues 67-83 comprise a possible amiloride binding region, (ii) residues 218-235 are homologous to those in other transporters, and (iii) residues 335-353 are homologous to those in other Na⁺-related proteins. Pinner *et al.* also reported diffuse and restricted homology around residues 303-312, which is conserved in NhaA and NhaB (5), and partially overlaps the "sodium consensus box" previously identified in other Na⁺-translocating systems (33). Surprisingly, five of our mutants, L73R, H225P, G303R, C335R, and S342P, were mapped within the above four sequence elements. These results strongly indicated that the residues mutated randomly in this study were important for the antiporter function, and, *vice versa*, the elements identified in the homology search may have critical functions.

Most of the mutants isolated in the present study are

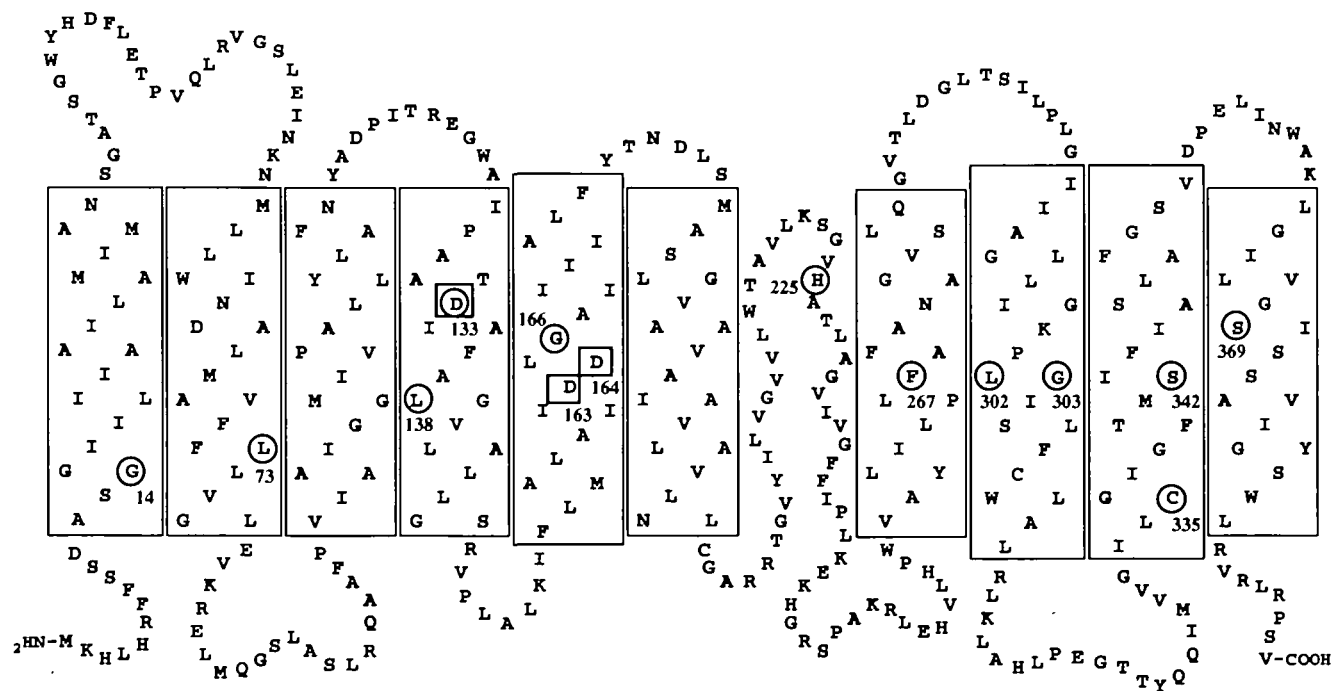


Fig. 5. Locations of mutated residues in the putative transmembrane region of *E. coli* NhaA. The possible transmembrane structure of the *E. coli* NhaA is depicted. We re-examined the locations of transmembrane domains based on hydropathy analysis (44), amino acid conservation and charge distributions (45, 46), and moved the putative sixth transmembrane helix slightly toward the amino-terminus, maintaining the total number of transmembrane

domains at ten compared with our previous model (18). Although the hydrophobicity of the central domain (residues 200-258) is sufficiently high, its topological location remains ambiguous. We tentatively placed the central domain in the membrane. The mutated residues identified in the present study are circled and numbered. The open square boxes indicate the three important Asp residues identified on site-directed mutagenesis in our previous study (18).

sensitive to both Li^+ and Na^+ , or Li^+ alone. The selective sensitivity of mutants to Li^+ may be explained by the higher toxicity of Li^+ to cells than Na^+ (34, 35). Therefore, even partial loss of antiporter activity may result in sensitivity to Li^+ , whereas only a marked decrease in antiporter activity would result in sensitivity to Na^+ . Consistent with this idea, the growth of D133A, L138P, and H225P on high-LiCl plates was fully abolished, although they retained substantial Li^+/H^+ antiporter activity at pH 7.0 and 8.0. Whereas L73R survived on high-NaCl plates, despite that the mutant lost 90% of its Na^+/H^+ antiporter activity at pH 8.0. Susceptibility to high NaCl was observed only when mutants had lost more than 95% of the Na^+/H^+ antiporter activity, as seen with D133A and S369P. We only isolated two mutants, G291R and G291R/V382A, which were more sensitive to Na^+ than Li^+ , although we did not study them in detail because both mutants also had a V279A mutation. As these two mutants have G291R in common, it may be interesting to study further the G291R mutation as a single mutation. It is noteworthy that Glu-291 is located close to the conserved element for Na^+ -translocating proteins, as discussed above.

Although both D133A and L138P retained low Li^+/H^+ antiporter activity, D133A lost Na^+/H^+ antiporter activity, while L138P retained normal Na^+/H^+ antiporter activity at pH 7.0 and 8.0. These two mutated residues are located in the putative fourth transmembrane helix. These results together with those of our previous study (18) suggested that the fourth and fifth transmembrane helices may constitute an ion channel and thus are important for ion selectivity. We have demonstrated the importance of three Asp residues, Asp-133, -163, and -164, for antiporter activity by site-directed mutagenesis of Asp residues to Asn (18). None of the Asn for Asp mutants retained any Na^+/H^+ and Li^+/H^+ antiporter activities. The D133A mutant in the present study lost the Na^+/H^+ antiporter activity almost completely but retained about 20–40% of the Li^+/H^+ antiporter activity. These results indicated that the negative charge at position 133 is not essential for antiporter activity, particularly for Li^+/H^+ antiporter activity, although Asp appeared to play a critical role. Asn at position 133 might be more detrimental to antiporter activity than Ala because of the structural hindrance. Experiments involving the substitution of three Asp residues with several other amino acids are currently in progress.

The L138P mutant showed a novel phenotype as to Li^+/H^+ antiporter activity, while its Na^+/H^+ antiporter was as active as the wild-type NhaA. At pH 7.0 and 8.0, the Li^+/H^+ antiporter activity of L138P was about 30% of that of the wild-type NhaA. At pH 8.5, L138P showed the opposite response in fluorescence quenching on the addition of LiCl (Fig. 1D). A simple explanation for this may be that H^+ was transported inward to the everted membrane vesicles together with Li^+ . However, this was not the case because the same H^+ movement was not observed if the initial quenching was not driven by respiration (data not shown). That is, the inward movement of H^+ caused by Li^+ was observed only when the initial quenching was driven by respiration. A plausible alternative explanation is that the mutant could bind Li^+ but not antiport H^+ at pH 8.5. As described under "RESULTS," the initial quenching by respiration-driven H^+ influx was apparently decreased at

pH 8.5 due to enhanced Na^+/H^+ antiporter activity driven by Na^+ contaminating the KCl (140 mM) in the medium. Supposing that the L138P antiporter could bind Li^+ and selectively lost its Li^+/H^+ antiporter activity, Li^+ replaced Na^+ on the mutant antiporter and eventually blocked the Na^+/H^+ antiporter on the addition of LiCl. If this is the case, the respiration-driven H^+ influx would be resumed, resulting in fluorescence quenching. This explanation was further supported by the observation that the same H^+ movement was not observed with a lower concentration of KCl (40 mM) in the medium (data not shown). These results indicated that L138P could bind Li^+ but lost Li^+/H^+ antiporter activity at pH 8.5. Zhang and Fillingame reported a similar mutant of the DCCD-binding subunit of F_1F_0 -ATPase (36). They introduced four successive substitutions into the subunit, which rendered the subunit capable of binding Li^+ as well as H^+ , although Li^+ could not be transported. Interestingly, Li^+ binding was observed only at pH 8.5. They suggested that a X-Glu-Ser-Y or X-Glu-Thr-Y sequence may constitute a general structural motif for monovalent cation binding, and that the flexibility provided by residues X and Y will be crucial for this structure. However, no corresponding sequences were observed in the transmembrane domains of NhaA. Leu-138 and its vicinity consisting of Asp-133 and Asp-163 and Asp-164 in NhaA may substitute for the above sequence, and play essential roles in ion selectivity and transport reaction.

Gerchman *et al.* substituted Arg for His-225 by site-directed mutagenesis (19). The H225R mutant exhibited similar antiporter activity to that of the wild-type, although its pH dependence was altered. They indicated that H225 is part of the pH sensor of the NhaA antiporter. Recently, they further substituted H225 with Asp, Cys, Ser, or Ala, and suggested that the polarity and/or hydrogen-bonding are essential at position 225 for pH regulation of NhaA (37). In the present study, we isolated the H225P mutant on random mutagenesis. This mutant retained relatively high antiporter activities, similarly to H225R, H225D, H225C, and H225S, but the pH-dependent modulation of the antiporter activities was altered as in the case of H225R. The pH shift was more prominent in the Li^+/H^+ antiporter activity, which was not examined in the previous studies. These observations supported the previous indication that H225 constitutes part of the pH sensor in NhaA and indicated that the polarity of the side chain at position 225 may not be essential. We also found that the L73R mutant exhibited altered pH responsiveness. Furthermore, as described above, in the case of the L138P mutant the pH-dependency was also shifted only for Li^+/H^+ antiporter activity. These results suggested that these residues in the second and fourth transmembrane helices affected the pH sensor mainly formed from a domain surrounding His-225.

The L73R mutant showed high temperature-sensitive growth in high- Na^+ and high- Li^+ media. The temperature sensitivity was more prominent in the high- Li^+ medium. Certain mutants were reported previously to show this kind of temperature sensitivity. If the mutated residue is involved in the salt-bridge interaction with the counter-residue, a lower temperature may prevent abolition of the interaction (38–41). However, this was not the case with the present mutation because the mutated residue, Leu-73, is not involved in the salt-bridge interaction. The replaced

residue, Arg, might disturb the proper conformation or interactions with other residues surrounding Leu-73 due to the insertion of a positive charge in the second helix. Interestingly, H225P showed the opposite temperature sensitivity to L73R; *i.e.* very clear low temperature-sensitive growth in both high-Na⁺ and high-Li⁺ media. Low temperatures have been shown to destroy the assembly of some oligomeric proteins (42, 43). However, this does not apply to NhaA because this molecule functions as a single polypeptide. Although the precise reason for this low temperature-sensitivity of H225P is not known at present, it will be interesting to examine whether or not this effect is reversible, and we should determine whether or not the effect of a low temperature was exerted on the integration of the antiporter into the membrane or simply on its conformational state.

The random mutagenesis method presented here will provide a fundamental basis for the analysis of molecular structure-function relationships. The residues identified in this study are good initial candidates for detailed site-directed mutagenesis. To date, only three Asn for Asp mutants which have lost their antiporter activities have been reported (18). The mutants presented here would also be good tools for isolating second site suppressors. The topological model presented here is of course not definite, and further experiments involving such as PhoA fusion and site-specific antibody binding will provide more precise details regarding the membrane topology of the NhaA antiporter. Such studies are currently in progress in our laboratory to facilitate better understanding of the relationship between the function and molecular structure of NhaA.

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