Biochimica et Biophysica Acta 1828 (2013) 997–1003

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Identification of important charged residues for alkali cation exchange or pH regulation of NhaH, a Na^+/H^+ antiporter of *Halobacillus dabanensis*

Juquan Jiang [a,b,1], Lei Wang [c,1], Yajie Zou [c], Weidong Lu [d], Baisuo Zhao [c], Bo Zhang [c], Susheng Yang [c], Lifu Yang [a,*]

[a] *Rubber Research Institute, Chinese Academy of Tropical Agricultural Sciences, and Key Laboratory of Rubber Biology and Genetic Resource Utilization, Ministry of Agriculture, Hainan Province 571737, PR China*

[b] *Department of Microbiology and Biotechnology, College of Life Sciences, Northeast Agricultural University, Harbin City, Heilongjiang Province 150030, PR China*

[c] *Department of Microbiology and Immunology, College of Biological Sciences, China Agricultural University, and Key Laboratory of Agro-Microbial Resource and Application, Ministry of Agriculture, Beijing 10094, PR China*

[d] *Shandong Provincial Key Laboratory of Applied Mycology, College of Life Sciences, Qingdao Agricultural University, Qingdao 266109, PR China*

ARTICLE INFO

Article history:
Received 2 May 2012
Received in revised form 13 November 2012
Accepted 15 November 2012
Available online 27 November 2012

Keywords:
Halobacillus dabanensis
NhaH
Antiporter
Site-directed mutagenesis
Alkali cation translocation
pH regulation

ABSTRACT

NhaH is a novel Na^+/H^+ antiporter identified from the moderate halophile *Halobacillus dabanensis*. In this study, six conserved charged residues located in the putative *trans*membrane segments (TMS) including TMSV, TMSVI, TMSVIII and TMSXI of NhaH as well as two His residues in Loop III were replaced by site-directed mutagenesis for the identification of their potential roles in the antiport activity and pH regulation. Substitutions D137A, D166A and R325A caused a complete loss of $Na^+(Li^+)/H^+$ antiport activity, revealing that D137, D166 and R325 are indispensable for the antiport activity. Substitution D137E led to a significant increase of the apparent *Km* values for Na^+ and Li^+ without affecting the changes of pH profile, confirming that D137 plays vital roles in alkali cation binding/translocation. Substitution D166E resulted in not only a significant increase of the apparent *Km* values for Na^+ and Li^+ but also an alkaline shift of pH profile, suggesting that D166 is involved in alkali cation binding/translocation as well as H^+ binding or pH regulation. Substitutions E161N, D224A and D224E caused a significant increase of *Km* for Na^+ and Li^+, indicating that E161 and D224 partly contribute to alkali cation binding/translocation. Substitution E229K caused an over 50% elevation of the apparent *Km* for Li^+, without affecting that for Na^+, suggesting that E229 may be mainly responsible for Li^+ binding/translocation. Substitutions H87A and H88A resulted in an acidic shift of pH profile without an effect on *Km* for Na^+ and Li^+, indicating that H87 and H88 are involved in H^+ binding or pH regulation.

1. Introduction

In prokaryotes, Na^+/H^+ antiporters are ubiquitous secondary transporters that catalyze the efflux of intracellular alkali cations in exchange for external protons, which play a vital role in reducing the cytoplasmic concentration of toxic alkali cations and supporting Na^+/K^+-dependent intracellular pH homeostasis under alkaline conditions [1,2]. About ten families of single-gene-encoded Na^+/H^+ antiporters including NhaA [3], NhaB [4], NhaC [5], NhaD [6], NapA [7], NhaP [8], NhaG [9] and NhaH [10] have been identified in many microorganisms. Another kind of Na^+/H^+ antiporter consists of multiple subunits encoded by an operon or a gene cluster such as *mnhABCDEFG* gene cluster from *Staphylococcus aureus* [11], *mrp* operon from *Bacillus subtilis* [1] and *phaA2B2C2D2E2F2G2* gene cluster from *Sinorhizobium fredii* [12,13].

As a model of pH-regulated Na^+/H^+ antiporter, Ec-NhaA, a Na^+/H^+ antiporter of *Escherichia coli* was structurally and functionally studied through the site-directed mutagenesis of conserved charged amino acid residues. D133, D163 and D164 located in the 4th and 5th TMSs have been identified to play a critical role in cation binding and translocation of Ec-NhaA [14,15]. H225 was shown to be closely related to the shift of the pH profile of Ec-NhaA to acidic or alkaline pH and thus thought to play an important role in the pH regulation of Ec-NhaA [16,17]. Similarly, conserved charged/polar amino acid residues such as Ser, Asp, Asn and Thr are essential for the activity of Vc-NhaD, $Na^+(Li^+)/H^+$ antiporter of *Vibrio cholerae* and H93 and H210 for pH regulation [18]. Moreover, Asp residues or His and Leu residues were also shown by random mutagenesis to play an important role in $Na^+(Li^+)/H^+$ antiport activity or pH regulation of Ec-NhaA [19]. Besides the charged/polar residues located in the hydrophobic TMSs, the hydrophilic N terminal and C terminal domains were also identified to be very important for cation exchange activity and specificity [20–22].

In our previous studies, *H. dabanensis* D-8T was identified to be a novel species isolated from Daban Salt Lake in the Xinjiang Province

* Corresponding author. Tel.: +86 898 23300595; fax: +86 898 23300315.
E-mail address: ylfri@126.com (L. Yang).

[1] These authors contributed equally to this work.

0005-2736/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2012.11.015

of China, which can grow and metabolize at pH 5–11 with the optimum pH at 7.5, at a wide range of 0.5–20% (w/v) of NaCl with 10% (w/v) optimal and at the temperature range of 15–50 °C with the optimal at 35 °C [23]. NhaH was cloned from this strain and identified to be a novel single-gene-encoded Na^+/H^+ antiporter that has the highest identity (54%) and similarity (76%) with the NhaG antiporter, but it exhibits a different pH profile with optimal pH at 8.5–9.0 and 8.5 for Na^+/H^+ and Li^+/H^+ antiport activity as compared with the latter [10]. Also, the C terminal hydrophilic domain of NhaH consisting of nine amino acid residues was shown to contribute to alkali cation binding and translocation and pH regulation [24]. As a representative of a Na^+/H^+ antiporter from the moderate halophile, NhaH is worthy of further analysis for the important amino acid residues for the function and pH regulation. Six conserved charged residues located in the four putative TMSs including TMSV, TMSVI, TMSVIII and TMSXI and two His residues in LoopIII were replaced by Ala, Glu, or Lys residues through site-directed mutagenesis in this study. As a result, we found that D137, E161, D166, D224, E229 and R325, to different extents, contribute to alkali cation binding and translocation, and D166, H87 and H88 are involved in H^+ binding and pH regulation.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli strain KNabc, lacking three major Na^+/H^+ antiporters (NhaA, NhaB and ChaA) and its transformant cells were grown in an LBK medium consisting of 1.0% tryptone, 0.5% yeast extract, and 87 mM KCl, to which NaCl or LiCl was added at indicated concentrations when necessary. Ampicillin was added to a final concentration of 50 μg ml^{-1} for the selection of transformant cells.

2.2. Subcloning of the Na^+/H^+ antiporter gene

The plasmid pNAD04 [10] was used for further functional analysis of NhaH, a Na^+/H^+ antiporter of *H. dabanensis* D-8T. To subclone the full-length *nhaH* including its predicted promoter and downstream sequence, two primers HF: 5′-CGGGGATCCAGGAGGTGAATCTTTGGTGGTG-3′ (*Bam*HI site underlined) and HR: 5′-AACTGCAGTCTCAAAGCGCGGTGGCTGCTT-3′ (*Pst*I site underlined) were designed and synthesized, and the PCR reaction was carried out using plasmid pNAD04 as a template. The double-digested PCR fragments were ligated into *Bam*HI- and *Pst*I-digested pUC18, and the resulting recombination plasmid designated as pUCnhaH was re-sequenced to confirm the accuracy of PCR and then used to transform into *E. coli* KNabc, and the corresponding transformant was designated as KNabc/pUCnhaH.

2.3. Site-directed mutagenesis of nhaH

Site-directed mutagenesis was carried out via the QuikChange[R] Site-Directed Mutagenesis Kit according to the protocol provided by the manufacturer (Stratagene Co., Ltd.). Plasmid pUCnhaH was used as the template for site-directed mutagenesis, if not specially indicated. The eleven pairs of oligonucleotide primers corresponding to mutagenic sites were designed and synthesized, as listed in Table 1. All final *nhaH* variants in pUC18 were re-sequenced to confirm the accuracy of mutagenesis, and the corresponding plasmids were, respectively, introduced to *E. coli* KNabc electro-competent cells for growth test and $Na^+(Li^+)/H^+$ antiport activity assays.

2.4. Preparation of everted membrane vesicles

E. coli KNabc cells carrying NhaH or its variants were grown in LBK medium up to the mid-exponential phase of growth and harvested by centrifugation at 5000 *g*, 4 °C for 10 min. Everted membrane vesicles were prepared from transformant cells of *E. coli* KNabc with the empty vector pUC18 (as a negative control), NhaH (as a positive control) or all the NhaH variants by the French press method at 2000 psi and collected by ultracentrifugation at 100,000 *g* for 1 h as described by Rosen [25]. The vesicles were resuspended in a buffer containing 10 mM Hepes–Tris (pH 7.0), 140 mM choline chloride, 0.5 mM dithiothreitol and 250 mM sucrose, and stored at −70 °C before use.

2.5. Assays of $Na^+(Li^+)/H^+$ antiport activity

The $Na^+(Li^+)/H^+$ antiport activity of everted membrane vesicles was estimated according to the extent of the collapse of a performed proton gradient, with acridine orange as a fluorescent probe of the transmembrane pH gradient, as described by Rosen [25]. The assay mixture contained 10 mM Hepes–Tris (at the indicated pH from 6.5 to 9) or 10 mM Ches–KOH (pH 9.5), 140 mM choline chloride, 10 mM $MgCl_2$, 2 μM acridine orange, and 20–40 μg ml^{-1} protein of membrane vesicles. Respiration was initiated by the addition of potassium lactate to a final concentration of 5 mM. Fluorescence was monitored with a Hitachi F-4500 fluorescence spectrophotometer at excitation and emission wavelengths of 495 nm and 530 nm, respectively. After the fluorescence quenching reached a steady state, 5 mM NaCl or LiCl was added and the fluorescence dequenching percentage was recorded as a representative of $Na^+(Li^+)/H^+$ antiport activity. Protein content in everted membrane vesicles was determined by

Table 1
Oligonucleotide primers used for site-directed mutagenesis of NhaH.

Mutation	Mutagenic primer[a]	Codon change
H87A	5′-GAAGCTGCCATTC**GC**TCATCTTTTCTCAC-3′	CAT → GCT
H88A	5′-GAAGCTGCCATTCCAT**GC**TCTTTTCTCACAAAAG-3′	CAT → GCT
D137A	5′-GATGAGTGCGACAG**CG**CCGATCAGTGTAC-3′	GAC → GCG
D137E	5′-GATGAGTGCGACAGA**G**CCGATCAGTGTACTG-3′	GAC → GAG
E161N	5′-CGACCGTCATGGAAGGG**AAT**TCTCTTTTCAATGATGG-3′	GAA → AAT
D166A	5′-GAATCTCTTTTCAATG**CC**GGCATCGCGGTGGTG-3′	GAT → GCC
D166E	5′-GGAATCTCTTTTCAATGA**G**GGCATCGCGGTGGTGC-3′	GAT → GAG
D224A	5′-GTGATTCGGGTATTTG**C**TGATTATCCTCTTGAGG-3′	GAT → GCT
D224E	5′-GTGATTCGGGTATTTGA**G**GATTATCCTCTTGAGGTCG-3′	GAT → GAG
E229K	5′-GGGTATTTGATGATTATCCTCTT**A**AGGATTATCCTCTTGAGGTCGC-3′	GAG → AAG
R325A	5′-CGTTTTAGTGGGA**GC**CACCATAGCTGTG-3′	CGC → GCC

[a] The mutagenic nucleotides are shown in boldface.

the method of Lowry et al. [26] with bovine serum albumin as a standard.

2.6. Calculation of the apparent Km values

No test for the apparent *Km* values for Na^+ and Li^+ was carried out for the variants D137A, D166A and R325A without the detectable $Na^+(Li^+)/H^+$ antiport activity. As for the measurement of the apparent *Km* values of other NhaH variants, pH was adjusted to 8.5 or 9 based on the highest Na^+/H^+ and Li^+/H^+ antiport activity, respectively, and the different Na^+ or Li^+ concentrations were varied from 0.5 mM to 20 mM. The fluorescence dequenching percentages at the corresponding cation concentrations were then recorded as their respective representatives of $Na^+(Li^+)/H^+$ antiport activity. A double-reciprocal plot for each NhaH variant was created by plotting the inverse fluorescence dequenching percentage as a function of the inverse cation concentration and finally the corresponding apparent *Km* value was obtained.

2.7. DNA manipulation and sequence analysis

Preparation and in-gel recycling of plasmid DNA were carried out according to the protocol described in a kit purchased from Promega Corporation. Primers were designed via the software Primer 5.0 and synthesized by China Bioasia Bio-Technology Sequencing Co., Ltd., Beijing. Protein alignment of NhaH from *H. dabanensis* with the representative Na^+/H^+ antiporters from other bacteria downloaded from the NCBI protein database was carried out based on the method described by Wilbur and Lipman [27] via the software DNAman 6.0. Restriction enzyme digestion and ligation were carried out as described by Sambrook et al. [28]. DNA sequencing was conducted by China Bioasia Bio-Technology Sequencing Co. Ltd., Beijing. Topological analysis was performed using the server http://www.sbc.su.se/~erikw/toppred2/ as described by von Heijne [29].

3. Results and discussion

3.1. Subcloning of nhaH

Because of the huge size of the plasmid pNAD04, the complete ORF of *nhaH* plus its predicted promoter was subcloned according to the description in the Materials and methods section. Salt tolerance experiments showed that *E. coli* KNabc/pUCnhaH exhibited the same growth ability in the presence of 0.2 M NaCl as KNabc/pNAD04 (data not shown), revealing that pUCnhaH was suitable for further site-directed mutagenesis.

3.2. Selection of amino acid residues for site-directed mutagenesis

As a representative of Na^+/H^+ antiporter from the moderate halophile *H. dabanensis*, NhaH was identified and characterized in our previous study and shown to be a relatively novel secondary transporter belonging to a large family of bacterial transporters [10]. Therefore, a further mutagenesis and functional study is required to analyze the important functional residues of NhaH and this will be very helpful to positively contribute to the knowledge of this transporter. The protein alignment analysis showed that five negatively charged residues D137, E161, D166, D224 and E229 as well as one positively charged residue R325 are fully conserved between NhaH and other ten representative Na^+/H^+ antiporters (Fig. 1A). The topological analysis showed that D137 is located within hydrophobic TMS V, D166 and E161 within TMS VI, D224 and E229 within TMS VIII, and R325 within TMS XI (Fig. 1B). In addition, two adjacent His residues (Fig. 1A), H87 and H88, are located in hydrophilic Loop III facing the periplasmic side of the membrane (Fig. 1B). Therefore, these eight residues were hypothesized to be importantly functional residues of NhaH and identified via a site-directed mutagenesis method developed by Padan and his colleagues [30,31] for further probing their relevance for cation binding and pH regulation for NhaH. The eleven resulting NhaH variants were designated as H87A, H88A, D137A, D137E, E161N, D166A, D166E, D224A, D224E, E229K and R325A, respectively.

3.3. Effect of NhaH variants by site-directed mutagenesis on bacterial growth

The variants D137A, D166A and R325A failed to complement the Na^+-sensitive growth phenotype of *E. coli* KNabc in the presence of 0.2 M NaCl (Fig. 2), indicating that these three residues play vital roles in $Na^+(Li^+)/H^+$ antiport activity of NhaH. As compared with the wild-type NhaH, *E. coli* KNabc expressing H87A showed a significant growth increase, and *E. coli* KNabc expressing D224A and E229K showed slight growth decreases. There was no growth difference between *E. coli* KNabc expressing wild-type NhaH and its other variants (Fig. 2).

3.4. Vital roles of D137, D166, and R325 residues in the cation transport activity

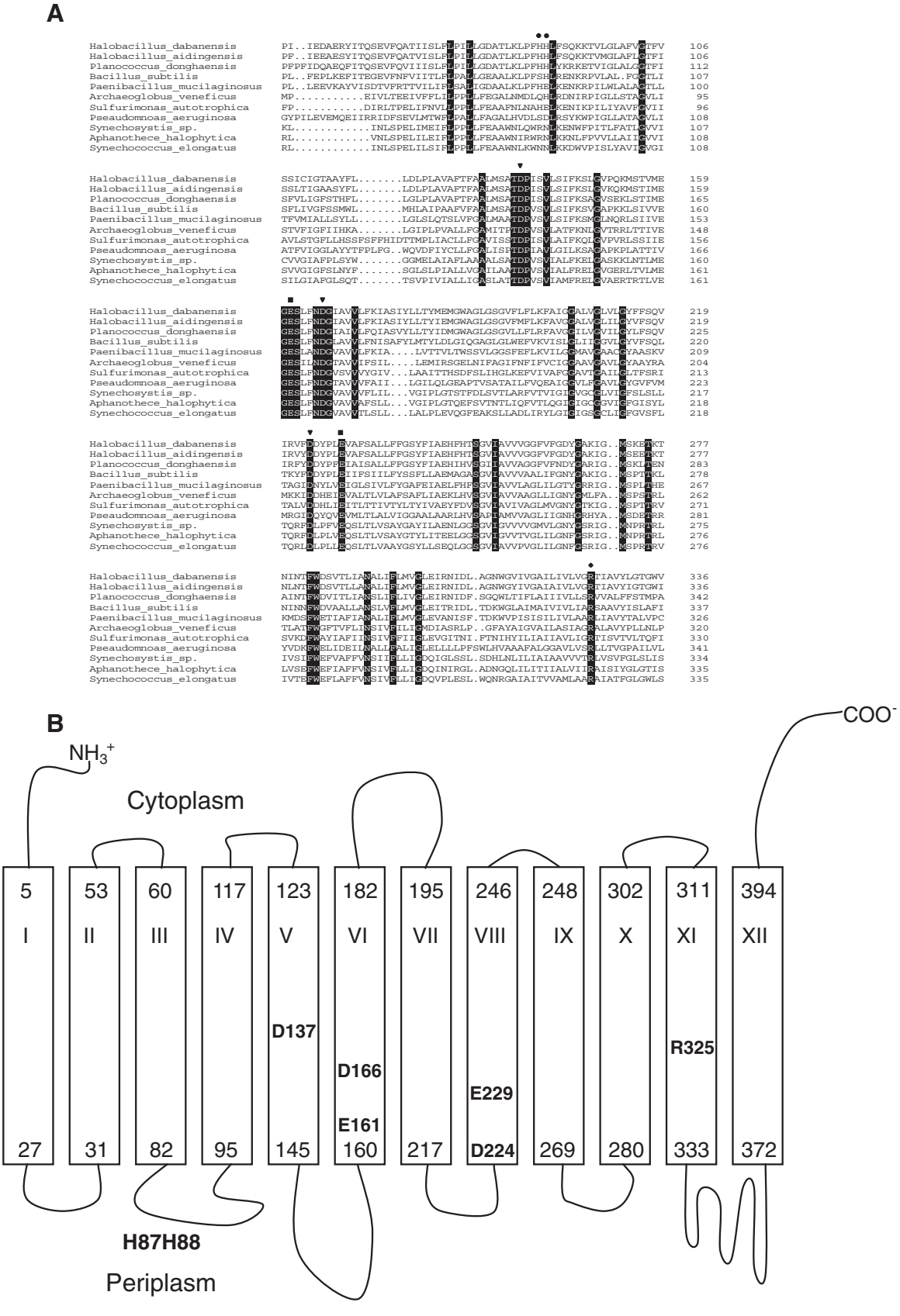
The replacement of D137 or D166 by Ala completely abolished the $Na^+(Li^+)/H^+$ antiport activity and introduction of another acidic residue, Glu, instead of D137 or D166 still could retain a partly active Na^+/H^+ antiporter (Figs. 3B and C and 4B and C), indicating that a carboxyl group in positions 137 and 166 may be critical for the cation transport activity of NhaH and the side-chain size of Asp is more appropriate for NhaH to exhibit its entire cation transport activity than that of Glu at these two active sites. This was supported by the fact that the apparent *Km* values for D137E and D166E also showed an 8 to 9 fold increase, as compared with the wild-type NhaH (Table 2). More importantly, the involvement of R325 in the case of NhaH is intriguing. Arg residue was identified to be indispensible for the activity of MjNhaP1 in *Methanococcus jannaschii*, which is activated at acidic pH [31]. However, positively charged Arg residue was for the first time reported to be critical for the function of Na^+/H^+ antiporter, which is activated at alkaline pH. It is not very clear how this residue affects the function of Na^+/H^+ antiporter, but possibly not due to a positive charge of Arg residue [32].

3.5. Partial roles of D224, E161 and E229 in the cation transport activity

Although the growth test showed almost no difference between *E. coli* KNabc with D224A, D224E and the wild-type NhaH (Fig. 2), the substitutions of D224 by Ala or Glu residues led to a significant decrease of $Na^+(Li^+)/H^+$ antiport activity (Figs. 3D and 4D), indicating that D224 can affect, to some extent, the cation transport activity of NhaH. In bacterial Na^+/H^+ antiporters, the negatively charged Asp residues have been shown to play a vital role in antiport activity, and are possibly related to alkali cation binding and translocation [15,18]. This was confirmed by the result that the apparent *Km* values for Na^+ or Li^+ increased by 5 to 6 fold and 2 to 3 fold (Table 2), respectively, in contrast to the wild-type NhaH. All the above results revealed that D224 can have a partial effect on the alkali cation binding and translocation of NhaH.

Besides the above three Asp residues, there are two conserved charged Glu residues, E161 and E229 located in the TMS VI and TMS VIII, respectively (Fig. 1). Both E161A and E229K supported the growth of *E. coli* KNabc in the presence of 0.2 M NaCl (Fig. 2). However, E161A resulted in a significant decrease of $Na^+(Li^+)/H^+$ antiport activity of NhaH (Figs. 3D and 4D) and a significant increase of the apparent *Km* values for Na^+ and Li^+ (Table 2), revealing that E161 play a partial role in alkali cation binding and translocation. It should be noted that the substitution of E229K displayed the elevated Li^+/H^+

antiport activity (Fig. 4D), but did not affect the Na^+/H^+ antiport activity (Fig. 3D). E229K showed an over 50% decrease of *Km* value for Li^+ but no change for Na^+, as compared with wild-type NhaH (Table 2). These results suggested that E229 is mainly responsible for Li^+ binding and translocation of NhaH. Based on differences in nonhydrated ionic radii (0.68 Å for Li^+ and 0.95 Å for Na^+) and the suggestions by Kaim et al. [33] that Li^+ requires fewer coordinating residues than Na^+, Schushan et al speculated that the ion-binding



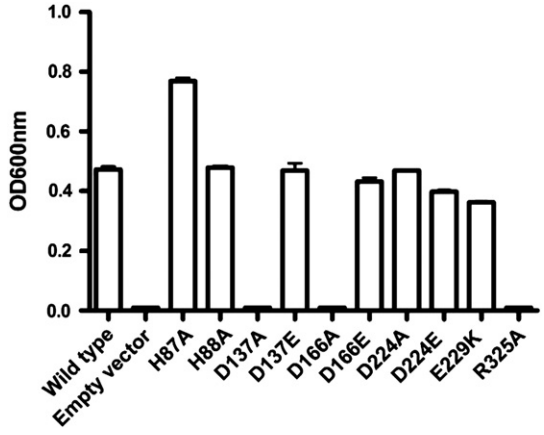


Fig. 2. Growth of *E. coli* KNabc with the wild-type NhaH and its variants in the presence of 0.2 M NaCl. *E. coli* KNabc cells with NhaH and its variants were grown at 37 °C for 24 h and then OD600nm for optical density in each culture was measured.

sites overlap only partially and observed that mutations D278E or E215D of a human CPA2-family Na^+/H^+ antiporter NHA2 led to the deficiency in Na^+ tolerance phenotype or Li^+ tolerance phenotype, respectively [34]. Therefore, it seems very possible that Na^+ and Li^+ may target the different sites of Na^+/H^+ antiporters, though the relative mechanism is still not very clear. This will be very helpful to understand why such bacterial secondary transporters as LeuT can be specifically coupled to Na^+, but not Li^+ or K^+ [35].

3.6. Involvement of H87, H88 and D166 residues on the pH regulation

In addition to the above conserved charged residues, there are two special His residues located in the hydrophilic Loop III (Fig. 1). H87 and H88 are adjacent to each other in the amino acid sequence of NhaH and they are the sole His residues located in the hydrophilic Loop III exposed to the periplasm. His residues involved in the H^+ binding and pH regulation were identified to be mainly located in the hydrophilic loops in the Ec-NhaA [16,17] and Vc-NhaD [18]. It seems to be possible that these two His residues act as the pH sensors of NhaH. Therefore, these two His residues were replaced by Ala residue, respectively. H87A significantly enhanced the growth of *E. coli* KNabc in the presence of 0.2 M NaCl (Fig. 2), and it is reported for the first time that a mutated residue can increase the growth of *E. coli* KNabc. In contrast, H88A did not change the growth of *E. coli* KNabc in the presence of 0.2 M NaCl (Fig. 2). Mutation in H87 and H88 both shifted pH profiles for Na^+ and Li^+ to acidic pH by 0.5 (Figs. 3A and 4A), but not change

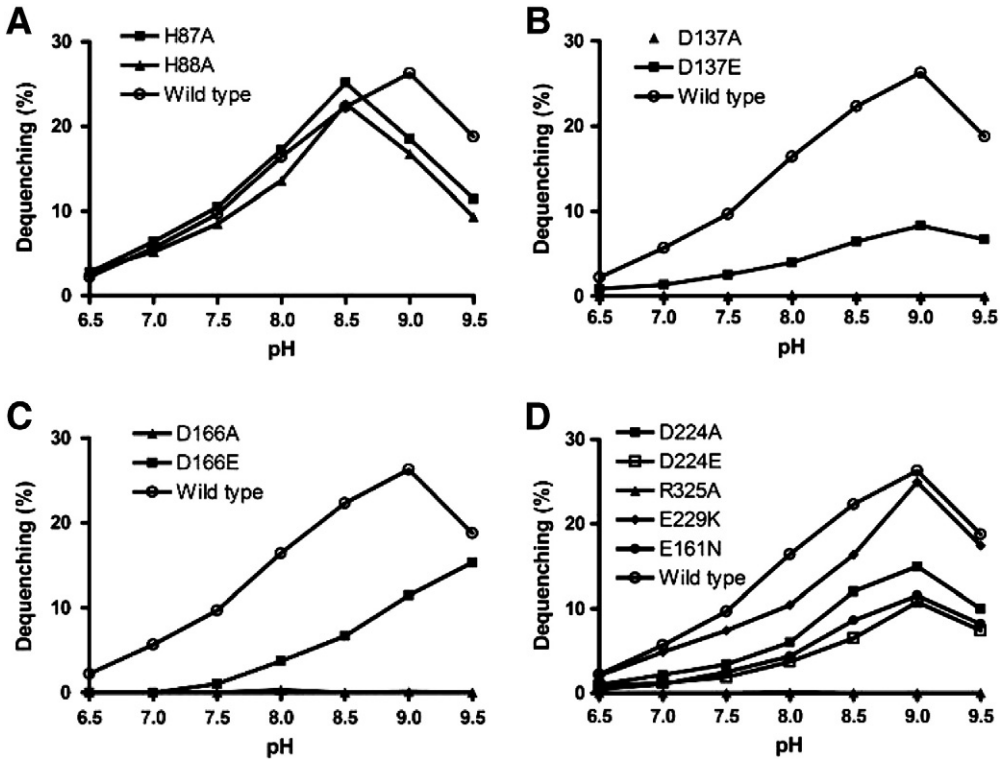


Fig. 3. Na^+/H^+ antiport activity of NhaH and its variants at different pH values. Each data point represents the average of three independent determinations.

Fig. 1. Site-directed mutagenesis of NhaH, a Na^+/H^+ antiporter identified from *H. dabanensis*. A. Sequence alignment of NhaH from *H. dabanensis* and the representative Na^+/H^+ antiporters from other bacteria. The sequences used for alignment were collected from *Halobacillus aidingensis* (accession no. ABX57744), *Planococcus donghaensis* (accession no. ZP_08095407), *Bacillus subtilis* (accession no. BAA89487), *Paenibacillus mucilaginosus* (accession no. YP_0046449080, *Archaeoglobus veneficus* (accession no. YP_004341038), *Sulfurimonas autotrophica* (accession no. YP_003893082), *Pseudomonas aeruginosa* (accession no. ABJ13160), *Synechocystis* sp. (accession no. NP_441245), *Aphanothece halophytica* (accession no. BAB69459), and *Synechococcus elongatus* (accession no. YP_399830). Three conserved Asp residues (D137, D166 and D224, marked with ▼), two conserved Glu residues (E161 and E229, marked with ■), one conserved Arg residue (R325, marked with ♦) and two His residues (H87 and H88, marked with ●) were selected for site-directed mutagenesis. B. Putative membrane topology of NhaH from *H. dabanensis*. The putative 12 transmembrane segments were predicted by TopPred II program. The mutated amino acid residues in bold were shown in their corresponding TMS (marked in Roman numerals from I to XII). Asp residues were substituted by Ala and Glu residues, respectively. Glu residues were substituted by Asn (for E161) or Lys (for E229) residue. Arg or His residues were substituted by Ala residue.

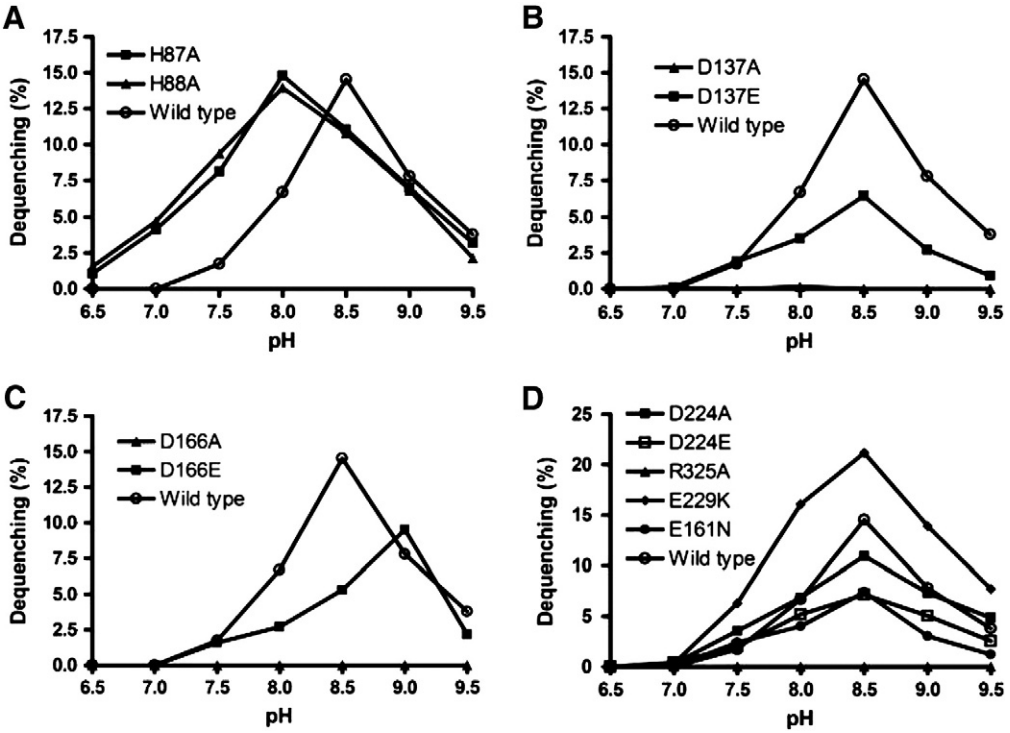


Fig. 4. Li^+/H^+ antiport activity of NhaH and its variants at different pH values. Each data point represents the average of three independent determinations.

$Na^+(Li^+)/H^+$ antiport activity of NhaH and the apparent *Km* values for Na^+ and Li^+, which indicates that H87 and H88 residues are associated with H^+ binding and pH regulation. Though a significant effect of H87A on the growth capability cannot be well explained, it is possible that H87A may enhance the growth capacity of *E. coli* KNabc by changing the mechanism of H^+ binding and pH regulation and that H87 should be more important for the binding of NhaH with H^+ and the regulation by pH than H88. Also, the substitution of D166 by Glu residue led to an alkaline shift of the pH profile of the antiporter by 0.5 pH unit (Figs. 3C and 4C), suggesting that D166 is involved in H^+ binding and pH regulation.

Acknowledgements

We are so grateful to Dr. Terry A. Krulwich (Department of Biochemistry, Mount Sinai School of Medicine of the City University, New York) for friendly donation of *E. coli* strain KNabc and good advice on this study. This work was supported by the Chinese National Program for High Technology Research and Development (grant no. 2003AA241150).

Table 2
Apparent *Km* values of the various versions of NhaH to Na^+ and Li^+.

Mutation	Apparent *Km* (mM)	
	Na^+	Li^+
Wild type	0.81 ± 0.01	2.42 ± 0.02
H87A	0.83 ± 0.03	2.11 ± 0.01
H88A	1.00 ± 0.07	2.41 ± 0.03
D137E	7.21 ± 0.17	20.4 ± 0.33
E161N	5.62 ± 0.11	15.9 ± 0.23
D166E	6.40 ± 0.15	6.84 ± 0.51
D224A	4.30 ± 0.05	4.63 ± 0.21
D224E	5.12 ± 0.01	7.70 ± 0.33
E229K	0.90 ± 0.15	1.12 ± 0.02

Km values are averages with three standard errors for three independent determinations.

References

[1] M. Ito, A.A. Guffanti, B. Oudega, T.A. Krulwich, *mrp*, a multigene, multifunctional locus in *Bacillus subtilis* with roles in resistance to cholate and to Na^+ and in pH homeostasis, J. Bacteriol. 181 (1999) 2394–2402.
[2] E. Padan, E. Bibi, M. Ito, T.A. Krulwich, Alkaline pH homeostasis in bacteria: new insights, Biochim. Biophys. Acta 177 (2005) 67–88.
[3] R. Karpel, Y. Olami, D. Taglicht, S. Schuldiner, E. Padan, Sequencing of the gene *ant* which affects the Na^+/H^+ antiporter activity in *Escherichia coli*, J. Biol. Chem. 263 (1988) 10408–10414.
[4] E. Pinner, E. Padan, S. Schuldiner, Cloning, sequencing and expression of the *nhaB* gene, encoding a Na^+/H^+ antiporter in *Escherichia coli*, J. Biol. Chem. 267 (1992) 11064–11068.
[5] T. Nakamura, H. Enomoto, T. Unemoto, Cloning and sequencing of the *nhaB* gene encoding an Na^+/H^+ antiporter from *Vibrio alginolyticus*, Biochim. Biophys. Acta 1275 (1996) 157–160.
[6] M. Ito, A.A. Guffanti, J. Aemsky, D. Mackivey, T.A. Krulwich, Role of the *nhaC*-encoded Na^+/H^+ antiporter of alkaliphilic *Bacillus firmus* OF4, J. Bacteriol. 63 (1997) 3851–3857.
[7] M. Waser, B.D. Hess, K. Davies, M. Solioz, Cloning and disruption of a putative Na^+/H^+ antiporter gene of *Enterococcus hirae*, J. Biol. Chem. 267 (1992) 5396–5400.
[8] J. Utsugi, K. Inaba, T. Kuroda, M. Tsuda, T. Tsuchiya, Cloning and sequencing of a novel Na^+/H^+ antiporter gene from *Pseudomonas aeruginosa*, Biochim. Biophys. Acta 1398 (1998) 330–334.
[9] T. Gouda, M. Kuroda, T. Hiramatsu, K. Nozaki, T. Kuroda, T. Mizushima, T. Tsuchiya, *nhaG* Na^+/H^+ antiporter gene of *Bacillus subtilis* ATCC, which is missing in the complete genome sequence of strain, and properties of the antiporter, J. Biochem. 130 (2001) 711–717.
[10] L. Yang, J. Jiang, B. Zhao, B. Zhang, D. Feng, W. Lu, L. Wang, S. Yang, A Na^+/H^+ antiporter gene of the moderately halophilic bacterium *Halobacillus dabanensis* D-8T: cloning and molecular characterization, FEMS Microbiol. Lett. 255 (2006) 89–95.
[11] T. Hiramatsu, K. Kodama, T. Kuroda, T. Mizushima, T. Tsuchiya, A putative multisubunit Na^+/H^+ antiporter from *Staphylococcus aureus*, J. Bacteriol. 180 (1998) 6642–6648.
[12] J. Jiang, W. Wei, B. Du, X. Li, L. Wang, S. Yang, Salt-tolerance genes involved in cation efflux and osmoregulation of *Sinorhizobium fredii* RT19 detected by isolation and characterization of Tn5 mutants, FEMS Microbiol. Lett. 239 (2004) 139–146.
[13] L. Yang, J. Jiang, W. Wei, B. Zhang, L. Wang, S. Yang, The *pha2* gene cluster involved in Na^+ resistance and adaption to alkaline pH in *Sinorhizobium fredii* RT19 encodes a monovalent cation/proton antiporter, FEMS Microbiol. Lett. 262 (2006) 172–177.
[14] C. Hunte, E. Screpanti, M. Venturi, A. Rimon, E. Padan, H. Michel, Structure of a Na^+/H^+ antiporter and insights into mechanism of action and regulation by pH, Nature 435 (2005) 1197–1202.

[15] H. Inoue, T. Noumi, T. Tsuchiya, H. Kanazawa, Essential aspartic acid residues, Asp-133, Asp-163 and Asp-164, in the transmembrane helices of a Na^+/H^+ antiporter (NhaA) from *Escherichia coli*, FEBS Lett. 363 (1995) 264–268.
[16] Y. Olam, A. Rimon, Y. Gerchman, A. Rothman, E. Padan, Histidine 225, a residue of the NhaA-Na^+/H^+ antiporter of *Escherichia coli* is exposed and faces the cell exterior, J. Biol. Chem. 272 (1997) 1761–1768.
[17] A. Rimon, Y. Gerchman, Y. Olami, S. Schuldiner, E. Padan, Replacements of histidine 226 of NhaA-Na^+/H^+ antiporter of *Escherichia coli*, J. Biol. Chem. 270 (1995) 26813–26817.
[18] R. Habibian, J. Dzioba, J. Barrett, M.Y. Galperin, P.C. Loewen, P. Dibrov, Functional analysis of conserved polar residues in Vc-NhaD, Na^+/H^+ antiporter of *Vibrio cholerae*, J. Biol. Chem. 280 (2005) 39637–39643.
[19] T. Noumi, H. Inoue, T. Sakurai, T. Tsuchiya, H. Kanazawa, Identification and characterization of functional residues in a Na^+/H^+ antiporter (NhaA) from *Escherichia coli* by random mutagenesis, J. Biochem. 121 (1997) 661–670.
[20] A. Rothman, E. Padan, S. Schuldiner, Topological analysis of NhaA, a Na^+/H^+ antiporter from *Escherichia coli*, J. Biol. Chem. 271 (1996) 32288–32292.
[21] A. Hamada, T. Hibino, T. Nakamura, T. Takabe, Na^+/H^+ antiporter from *Synechocystis* species PCC 6803, homologous to SOS1, contains an aspartic residue and long C-terminal tail important for the carrier activity, Plant Physiol. 125 (2001) 437–446.
[22] R. Waditee, T. Hibino, Y. Tanaka, T. Nakamura, A. Incharoensakdi, T. Takabe, Halotolerant cyanobacterium *Aphanothece halophytica* contains an Na^+/H^+ antiporter, homologous to eukaryotic ones, with novel ion specificity affected by C-terminal tail, J. Biol. Chem. 276 (2001) 36931–36938.
[23] W. Liu, J. Zeng, L. Wang, Y. Dou, S. Yang, *Halobacillus dabanensis* sp. nov. and *Halobacillus aidingensis* sp. nov., isolated from salt lakes in Xinjiang, China, Int. J. Syst. Evol. Microbiol. 55 (2005) 1991–1996.
[24] L. Yang, B. Zhang, L. Wang, S. Yang, The salt tolerance and pH regulation of NhaH, a Na^+/H^+ antiporter mediated by C terminal hydrophilic domain, Chin. Sci. Bull. 53 (2008) 2313–2317.
[25] B.P. Rosen, Ion extrusion system in *Escherichia coli*, Methods Enzymol. 125 (1986) 328–336.
[26] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[27] W.J. Wilbur, D.J. Lipman, Rapid similarity searches of nucleic acid and protein data banks, Proc. Natl. Acad. Sci. U. S. A. 80 (1983) 726–730.
[28] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, in: 3rd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 2001.
[29] G. von Heijne, Membrane protein structure prediction, hydrophobicity analysis and the positive-inside rule, J. Mol. Biol. 225 (1992) 487–494.
[30] L. Galili, A. Rothman, L. Kozachkov, A. Rimon, E. Padan, Trans membrane domain IV is involved in ion transport activity and pH regulation of the NhaA-Na(+)/H(+) antiporter of *Escherichia coli*, Biochemistry 41 (2002) 609–617.
[31] E. Padan, T. Tzubery, K. Herz, L. Kozachkov, A. Rimon, L. Galili, NhaA of *Escherichia coli*, as a model of a pH-regulated Na^+/H^+ antiporter, Biochim. Biophys. Acta 1658 (2004) 2–13.
[32] J. Hellmer, A. Teubner, C. Zeilinger, Conserved arginine and aspartate residues are critical for function of MjNhaP1, a Na^+/H^+ antiporter of *M. jannaschii*, FEBS Lett. 542 (2003) 32–36.
[33] G. Kaim, F. Wehrle, U. Gerike, P. Dimroth, Molecular basis for the coupling ion selectivity of F1F0 ATP synthase: probing the liganding groups for Na^+ and Li^+ in the c subunit of the ATP synthase from *Propioigenium modestum*, Biochemistry 36 (1997) 9185–9194.
[34] M. Schushan, M. Xiang, P. Bogomiakov, E. Padan, R. Rao, N. Ben-Tal, Model-guided mutagenesis drives functional studies of human NHA2, implicated in hypertension, J. Mol. Biol. 396 (2010) 1161–1196.
[35] J.R. Thomas, P.C. Gedeon, B.J. Grant, J.D. Madura, LeuT conformational sampling utilizing accelerated molecular dynamics and principal component analysis, Biophys. J. 103 (2012) L1–L3.