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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*

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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 *s*egments (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.

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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.

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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.

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