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Full Length Research Paper

A novel NhaD type Na^+/H^+ antiporter gene from a metagenomic library of halophiles colonizing in the Dagong Ancient Brine Well in China

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Metagenomic DNA libraries prepared from the Dagong Ancient Brine Well were screened for the NhaD type Na^+/H^+ antiporter gene by functional complementation with the antiporter-deficient *Escherichia coli* KNabc strain. One NhaD type clone with Na^+ and alkaline pH resistance phenotype was obtained and its inserted Na^+/H^+ antiporter gene was designated as *m-nhaD*. The deduced amino-acid sequence of M-NhaD protein consists of 330 residues with a calculated molecular weight of 34999 Da and a predicted isoelectric point of 7.02, which is homologous to NhaD from *Klebsiella pneumoniae* 342 (93%) and *Yersinia frederiksenii* ATCC 33641 (87%) but with only 77 and 76% sequence coverage, respectively. M-NhaD had a hydropathy profile with 9 putative transmembrane domains and a long carboxyl terminal hydrophilic tail of 31 amino acid residues different from the other NhaD. The *m-nhaD* gene could successfully express in the *E. coli* KNabc and its production rendered the *E. coli* KNabc with both the resistance up to 1.0 M Na^+ and the ability to grow under pH 8.5 - 10 alkaline condition. The difference in structure and function suggested that the *m-nhaD* was a novel NhaD type Na^+/H^+ antiporter gene and it was an attractive candidate gene of salt tolerance genetic breeding.

Key words: Metagenomic library, NhaD antiporter gene, halophiles, Dagong Ancient Brine Well.

INTRODUCTION

The Na^+/H^+ antiporter is a ubiquitous protein inserted in cytoplasmic membranes of cells and in membranes of many organelles in all biological kingdoms and plays a major role in maintaining cytoplasmic Na^+ homeostasis and intracellular pH around neutral for living cells. In the bacteria, most bacteria have multiple genes and operons predicted to encode Na^+/H^+ antiporters except those that live primarily in the cytoplasm of host cells or are sheltered by the homeostatic mechanisms of the host (Krulwich et al., 2009). These bacterial Na^+/H^+ antiporters have key physiological functions, including supporting cytoplasmic pH homeostasis, tolerance to alkali and to fluctuations in osmolarity, concomitantly supporting resistance to toxic levels of their efflux substrates, especially Na^+, and cell volume regulation (Krulwich et al., 2009; Ramos et al., 2011).

The Na^+/H^+ antiport in a bacterial cell is often mediated by different type antiporters working in concert, including a single gene of *nhaA*, *nhaB*, *nhaC*, *nhaD*, *nhaG*, *nhaP*, *nhaH* and *chA*, and multiple subunits Mrp antiporter and MnhABCDEFG system according to the sequence-based Transporter Classification System (Habibian et al., 2005; Ren et al., 2007). The halophlie, an ideal organism for

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screening the Na^+/H^+ antiporter genes to improve organism salt tolerance, wildly survive in naturally or artificially saline environments and among them, halophilic bacteria are dominant species. In fact, almost all halophilic microorganisms have potential transport mechanisms to expel Na^+ ions from the interior of the cells based on monovalent cation/proton antiporters (CPA) (Oren, 1999).

In recent years, Metagenomics provided an access to exhaustively screen valuable genetic resources of the microbes regardless of whether these microbes can be cultured or not (Guazzaroni et al., 2009; Uchiyama and Miyazaki, 2009). And the various target genes have been screened by employing this innovative technology (Schmeisser et al., 2007; Warnecke and Hess, 2009). In our previous works, it was found that in the Dagong Ancient Brine Well located in Zigong of southwestern China, the first recorded man-made brine well in the word, some halophiles have evolved intriguing changes in basic physiological processes to adapt to the special brine environment (Xiang et al., 2008), and a novel NhaP Na^+/H^+ antiporter gene has been directly mined by metagenomic technology (Xiang et al., 2010). Krulwich et al. (2009) thought that the ecologically stressed or versatile bacteria usually have 11-14 gene loci predicted to encode Na^+/H^+ antiporters. Therefore, whether these halophiles colonizing in alkaline and hypersaline brine of Dagong have other special Na^+/H^+ antiporters used to support alkaline pH regulation has not yet been known so far.

In this study, we constructed a metagenomic library from the brine in the Dagong Ancient Brine Well, and the screening of Na^+/H^+ antiporters to participate alkaline pH regulation was performed by Na^+ resistance functional restoration and alkaline pH regulation of the *E. coli* strain KNabc, lacking three major genes *nhaA*, *nhaB* and *chaA* coding Na^+/H^+ antiporters (Nozaki et al., 1996). After the identification of the Na^+/H^+ antiporter genes, the gene expression and the structure and function of the protein it encoded was also analyzed. This is the first report on the identification of a novel NhaD type Na^+/H^+ antiporter gene from the special man-made ancient hypersaline environment.

MATERIALS AND METHODS

Construction of the metagenomic library

The environmental DNAs were prepared from the brine in the Dagong Ancient Brine Well using methods described by Xiang et al. (2010). The total DNAs were subsequently partially digested with *Sau*3AI to produce 1.5 - 6 kbp fragments. These DNA fragments were ligated with the pUC18, which had been digested with *Bam*HI and dephosphorylated with bacterial alkaline phosphatase. The 20 - 200 ng hybridized plasmids were transformed into the competent cells of *E. coli* KNabc prepared as described previously by electro-poration (Xiang et al., 2010). Recombinants were spread on pH 8.5 agar plates containing modified Luria–Bertani medium (LBK medium) consisting of 1.0% tryptone, 0.5% yeast extract and 87 mM KCl, 0.25 mM NaCl, 1.5% agar and 100 μg/ml of ampicillin. The plates were incubated at 37°C for 20 h and Na^+ and alkaline tolerant clones were isolated.

Molecular analyses of the Na^+/H^+ antiporter gene

The foreign fragment inserted in the pUC18 of Na^+ and alkaline resistance clone was subcloned by Platinum® *Pfx* DNA polymerase (Invitrogen, USA), two primes CP-F (5'-AAGCTTGCATGCCTGCAGGT-3') and CP-R (5'-GAATTCGAGCTCGGTACCC-3'). The PCR products were added 2'-deoxyadenosine 5'-triphosphate by *Taq* DNA polymerase under 60°C for one hour, and then were ligated to pGM-T plasmid vector and cloned into the *E. coli* DH5α. The nucleotide sequences of the inserted fragment in the recombinant pGM-T plasmid were determined by the Sanger's dideoxy-chain termination method. The open reading frame (ORF) was searched by ORF Finder programs from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The database searches and sequence comparisons of protein encoded were performed using tool from the ExPASy Proteomics Server (www.expasy.ch/tools/blast/). Multiple alignments of all amino acid sequences were run by Clustal X 1.8 program (Thompson et al., 1997). A phylogenetic tree was constructed with the MEGA 4.0 program by using the neighbor-joining method with the Kimura two-parameter model (Tamura et al., 2007).

Structure and functional prediction of protein encoded

The amino acid sequence, the molecular weight and the isoelectric point of primary structure were analyzed respectively by ExPASy Proteomics Server (http://www.expasy.ch/tools/). The conserved domain of deduced amino acid sequence was compared with protein sequences in a secondary database using CDD program (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The transmembrane segments and orientation of the deduced amino acid sequence were identified by Dense Alignment Surface (DAS), a special software for prediction of transmembrane regions in prokaryotes (Cserzö et al., 1997). And its transmembrane helix location and topology were predicted using SOSUI and TMHMM (http://www.predictprotein.org/).

SDS-PAGE analysis of protein expression

The recombination *E. coli* KNabc with saline-alkali resistance were incubated for 16 h in the modified LBK liquid medium supplemented with 50 μg/ml ampicillin and 0.25 M NaCl concentrations under pH 8.0 by gentle agitation. The 20 ml cultures were centrifuged for 30 s at 14,000× g at 4ml, washed with 5 ml ice-cold phosphate-buffered saline (PBS), and resuspended in 1 ml PBS. These cells were subsequently lysed with a sonicator and centrifuged, and aliquots of soluble fractions were loaded onto 10% polyacrylamide gels and subjected to sodium dodecyl sulfate polyacrylamide gel electro-phoresis (SDS-PAGE). Proteins were visualized using 0.005% Coomassie Brilliant Blue R-250 in 0.3% v/v acetic acid and gel images were captured with a Fuji LAS-3000 imaging system.

Resistance of recombinant *E. coli* KNabc to Na^+ and pH

The *E. coli* KNabc was respectively grown in the modified LBK liquid medium supplemented with indicated NaCl concentrations or pH value where necessary. Strain was incubated aerobically in 100 ml portions in 250 ml Erlenmeyer flasks in a rotary shaker at 37°C for 12 h, and its growth was monitored turbidimetrically at 600 nm.

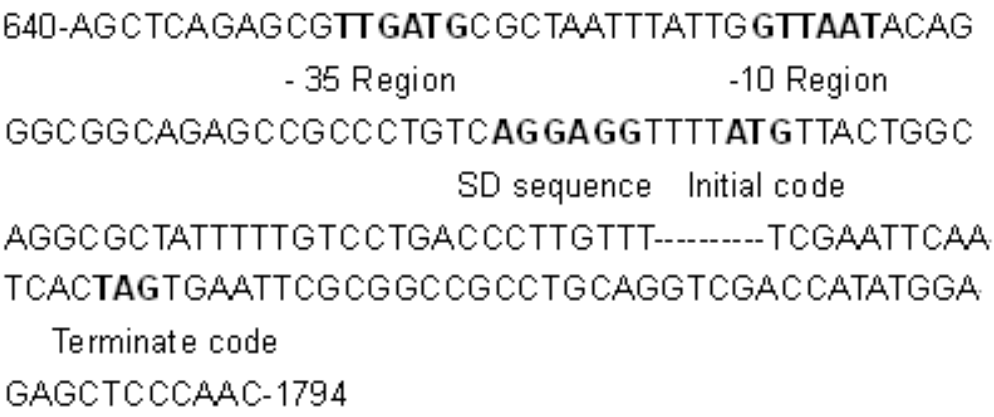

Figure 1. Nucleotide sequence of the gene encoding the M-NhaD antiporter from the metagenomic DNA libraries and its flanking regions. Possible promoter sequences (-35 region and -10 region), a putative Shine-Dalgarno sequence (SD), the putative initiation code ATG and the putative termination code TAG.

Nucleotide sequence accession number

The nucleotide sequence reported in this paper has been submitted to GenBank with accession number JN168102.

RESULTS

Screening the NhaD type Na^+/H^+ antiporter gene

The growth of antiporter-negative mutant strain *E. coli* KNabc was completely inhibited in selective LBK agar plates containing 0.25 M NaCl and pH 8.5 due to the toxic effect of Na^+ on pyruvate kinase (Majernik et al., 2001). Thus, only those recombinant *E. coli* KNabc strains harboring a gene conferring resistance to Na^+ and alkali pH could grow under the this condition. Two types of candidate genes that enabled the mutant KNabc cells to grow in the presence of conditions employed in current study, judging from the sequences of the hybrid plasmids. One type seemed to be identical to the *nhaP* gene, which had been cloned and sequenced previously in our laboratory. The other type was a novel putative NhaD type Na^+/H^+ antiporter gene designated as *m-nhaD* according to the later analysis.

Sequence and characteristics of the NhaD type Na^+/H^+ antiporter gene

The nucleotide sequences analysis revealed that the length of the DNA inserted into the pUC18 plasmid was 1,974 bp, and it contained one 984 bp intact ORF, a promoter (ATG) and a terminator (TAG) (Figure 1). A Shine-Dalgarno sequence (AGGAGG), -10 region (GTTAAT) and -35 region (TTGATG) were also found in the downstream of ORF (Figure 1). A homology search revealed that the protein encoded by m-mhaD had the highest homology of 93 and 87% similarity with the NhaD from *K. pneumoniae* 342 (accession: YP_002235704) and from *Y. frederiksenii* ATCC 33641 (accession: ZP_004631221), respectively, but with only 77 and 76% amino acid sequence coverage, and had a little lower similarity (16 and 19% identity) to the NhaD type antiporter from *Halomonas elongata* DSM 2581^T (accession: AM167899) and *Vibrio parahaemolyticus* AQ3334 (accession: AB006008).

In phylogenetic relationship between the M-NhaD antiporter protein from current metagenomic library and those from other strains reported, they were clearly divided into two groups, as shown in Figure 2. The M-NhaD was closely related to NhaD from the pathogenic bacteria *K. pneumoniae* 342 and *Y. frederiksenii* ATCC 33641, while it has rather far relationship with those from the pathogenic bacteria *V. parahaemolyticus* AQ3334 and *V. cholerae*, the moderately halophilic bacterium *H. elongata* DSM 2581^T and the alkaliphilic bacterium *Alkalimonas amylolytica* AS1.3430. Their amino acid sequences were aligned in Figure 3.

Structure and function prediction of the NhaD Na^+/H^+ antiporter protein

The amino acid sequence deduced from the *m-nhaD* gene was consisted of 330 amino acid residues with a calculated molecular weight of 34,999 Da and a predicted isoelectric point of 7.02. The most abundant amino-acid residues of this protein was Leu (53/330), followed by Ala (46/330), Val (36/330), Gly (30/330), Ile (38/330) and Phe (20/330). The least abundant residue was Gln (2 residues) and Met (5 residues). Among the 330 amino acid residues, only 34 residues were charged, indicating that M-NhaD is of low polarity. This is consistent with the

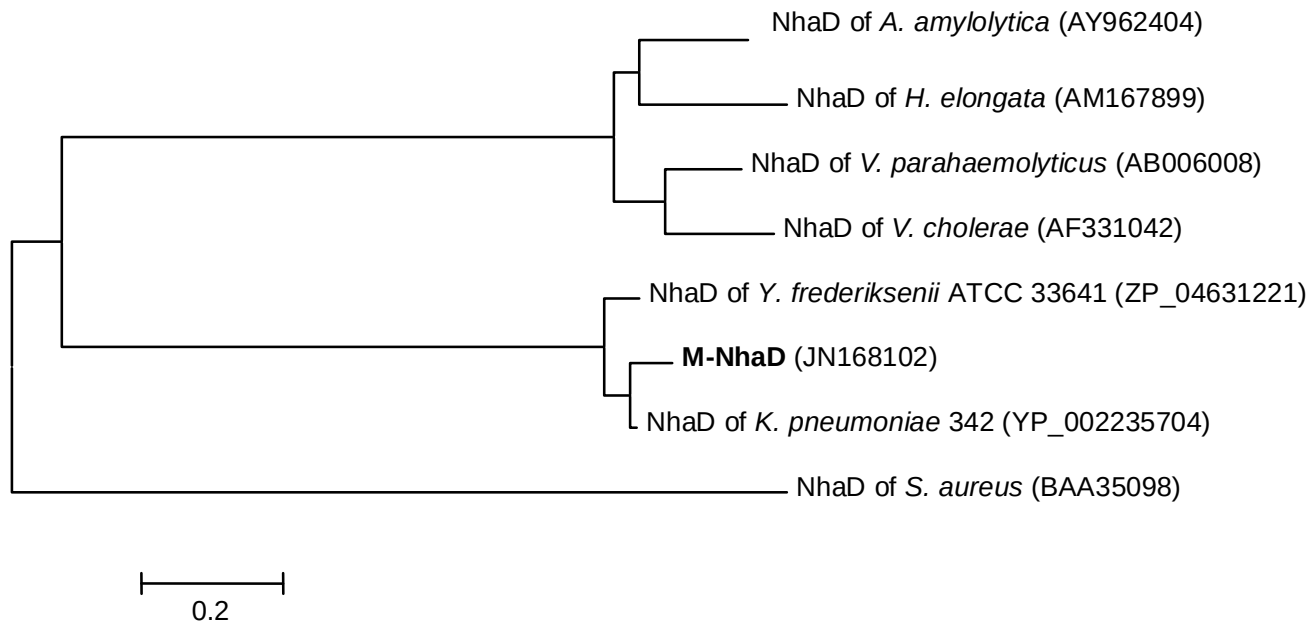

Figure 2. Phylogenetic trees based on the amino acid sequences of the NhaD protein from the metagenomic clone and other reference bacteria by using the neighbor-joining method. The scale bar corresponded to 0.2-estimated amino acid substitution per sequence position. Bootstrap values from 1000 replicates were included. Database accession numbers were shown in brackets after each protein.

belief that the Na^+/H^+ antiporter is an integral membrane protein. DAS revealed that the M-NhaD protein contained nine transmembrane helixes (Figure 4). The SOSUI and TMHMM computer program further confirmed this result of total 9 TMS in M-NhaD released by DAS (Figure 5). The conserved domain analysis against CDD suggested that M-NhaD is a NhaD type Na^+/H^+ antiporter, which belongs to NhaD superfamily of permeases that have been shown to translocate sodium, sulfate, arsenite and organic anions across biological membranes in all three kingdoms of life. Furthermore, CDD also delineated that the NhaD type Na^+/H^+ antiporter contains 8-13 transmembrane helices and can function either independently as a chemiosmotic transporter or as a channel-forming subunit of an ATP-driven anion pump. A similar result was also obtained when analyzed by InterProScan.

Expression of the NhaD Na^+/H^+ antiporter gene

To verify the alkaline pH and Na^+ resistance of recombinant strain endued by the M-NhaD antiporter, the cellular protein expression of the *E. coli* KNabc/M-NhaD, which harbored *m-nhaD* antiporter gene, the *E. coli* KNabc/pUC18, which contained only pUC18 empty vector and the *E. coli* KNabc, were respectively investigated. SDS-PAGE profiles revealed that the protein spectrums of above three strains have distinct difference (Figure 6). A band with a molecular mass of about 34 kDa, which is consistent with the deduced molecular mass of M-NhaD antiporter, was found in the strain *E. coli* KNabc/M-NhaD, while this band was not detected in the *E. coli* KNabc/pUC18 and the *E. coli* KNabc. This result suggested that the *m-nhaD* antiporter gene is successfully expressed in *E. coli* KNabc/M-NhaD and the alkaline pH and Na^+ resistance of *E. coli* KNabc/M-NhaD different from that of *E. coli* KNabc/pUC18 and *E. coli* KNabc is probably caused by this difference protein.

Resistance of the NhaD Na^+/H^+ antiporter to Na^+ and pH

The effects of NaCl concentration on growth of the *E. coli* KNabc/M-NhaD, the *E. coli* KNabc/pUC18 and the *E. coli* KNabc were respectively evaluated in pH 7.0 LBK liquid medium supplemented with indicated NaCl concentrations where necessary. The *E. coli* KNabc/M-NhaD strains could grow well in LBK medium containing 0.8 M NaCl and even survived in the presence of 1.0 M NaCl, while cells of the KNabc/pUC18 and the *E. coli* KNabc did not when Na^+ exceeded 0.05 M (Figure 7B). To test the effect of alkaline pH on cell growth, three strains were respectively grown in LBK liquid medium but at different pH values from 7 to 11. The results were similar to that influenced by NaCl, with growth of the *E. coli* KNabc and the *E. coli* KNabc/pUC18 greatly reduced under pH 8.5-10. However, only a certain growth reduction

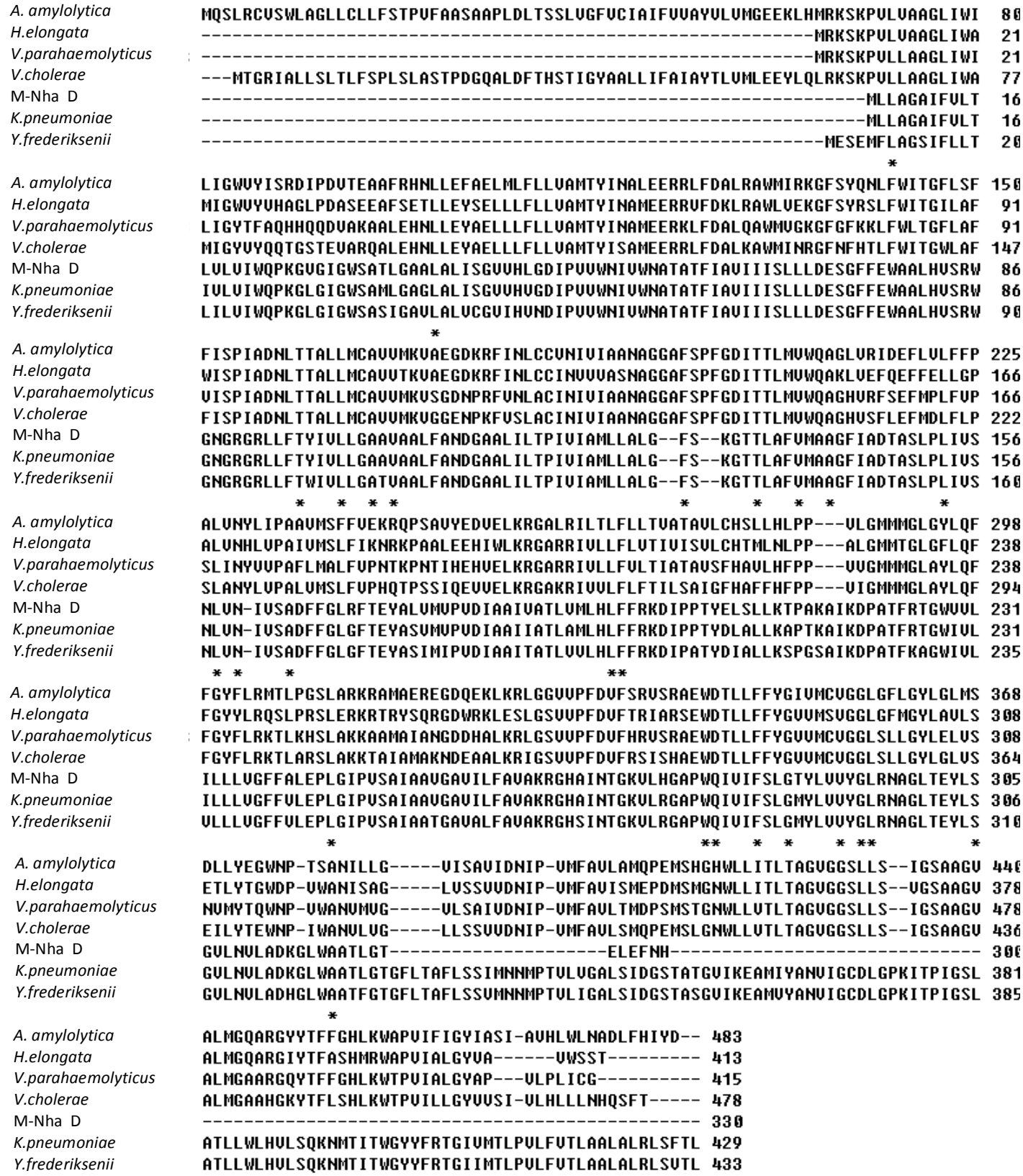

Figure 3. Alignment of the predicted amino acid sequence of M-NhaD type Na^+/H^+ antiporter protein from the metagenomic DNA libraries from the Dagong Ancient Brine Well with those from *A. amylolytica* AS1.3430 (AY962404), *H. elongata* DSM 2581^T (AM167899), *V. parahaemolyticus* AQ3334 (AB006008), *V. cholerae* (AF331042), *K. pneumoniae* 342 (YP_002235704) and *Y. frederiksenii* ATCC 33641 (ZP_04631221). The amino acid residues conserved in all sequences were marked with star.

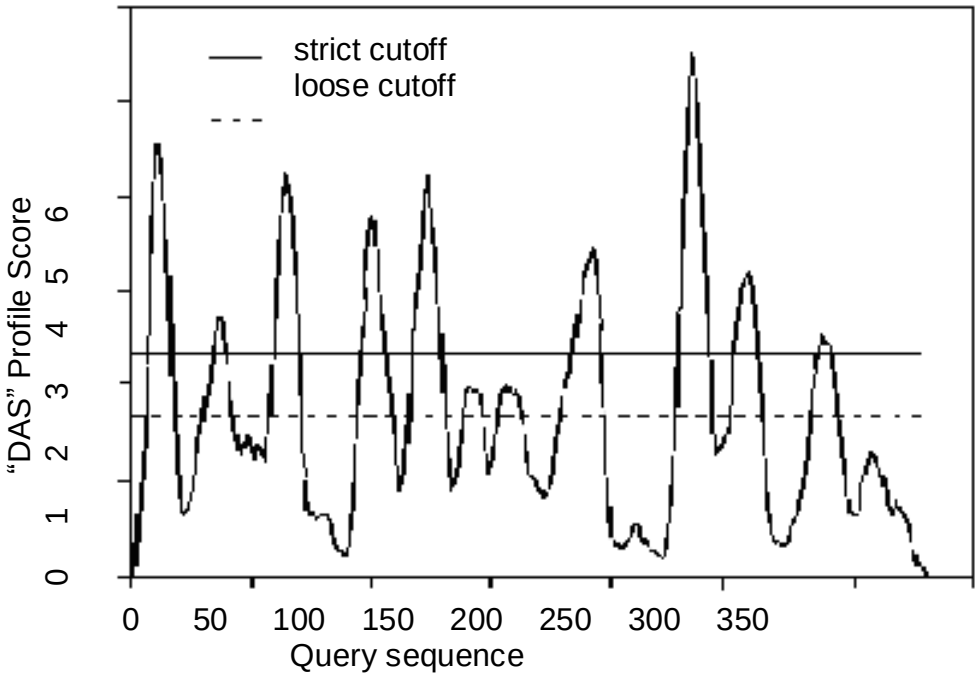

Figure 4. Transmembrane regions of the M-Nha Na^+/H^+ antiporter predicted by DAS program according to the Dense Alignment Surface method. The DAS profile scores were plotted from the N terminus to the C terminus.

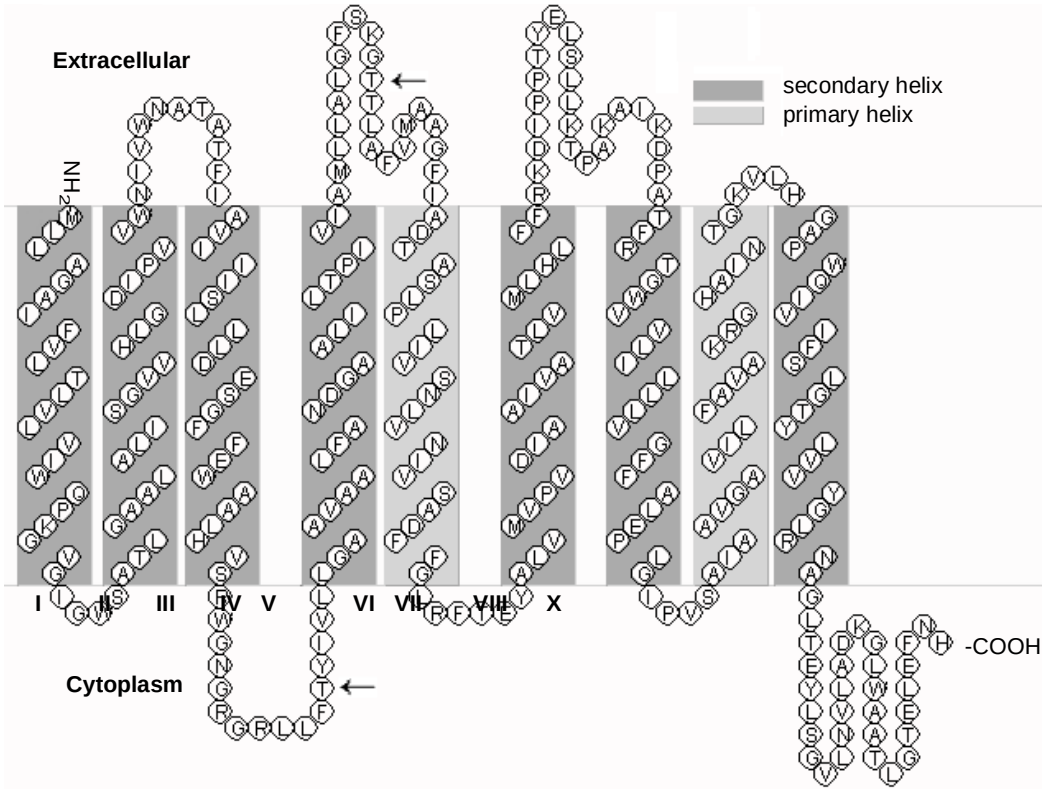

Figure 5. Membrane topology of the M-NhaD antiporter based on those predicted by the SOSUI program. Transmembrane segments I-X were shown under the box. Two conserved Thr residues in the TMS were designated by arrows.

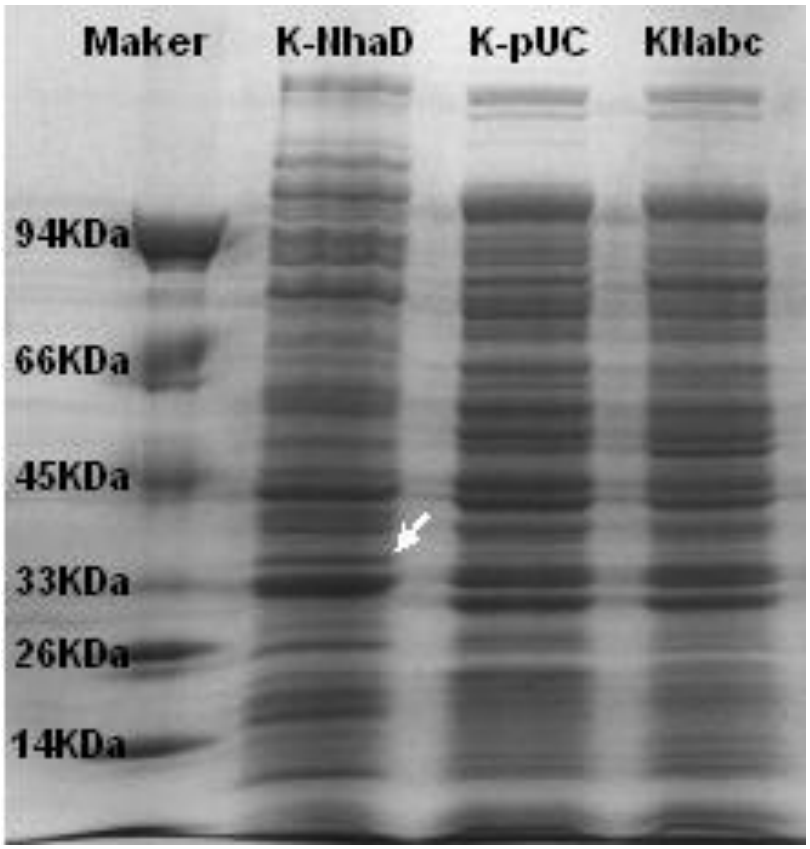

Figure 6. SDS-PAGE profiles of cellular protein expression of the *E. coli* KNabc/M-NhaD that harbored *m-nhaD* gene, the *E. coli* KNabc/pUC18 that contained pUC18 empty vector and the antiporter-deficient *E. coli* KNabc. Maker represents molecular mass of standard proteins. K-NhaD, K-pUC and KNabc represent expression proteins of the *E. coli* KNabc/M-NhaD, the *E. coli* KNabc/pUC18 and the *E. coli* KNabc, respectively. The blank arrow indicates the protein encoded by *m-nhaD* gene.

range was observed for the *E. coli* KNabc/M-NhaD harboring *m-nhaD* gene in such high alkaline medium (Figure 7A). This result indicated that the protein encoded by *m-nhaD* gene offered the antiporter-negative mutant *E. coli* KNabc cells not only the resistance to Na^+, but also the ability to grow under alkaline conditions.

DISCUSSION

The NhaD type antiporters, assigned in an ion transporter superfamily, are widely distributed in nature, and are found in genomes of pathogenic vibrios, nitrogen-fixing symbionts, magnetotactic cocci and photosynthetic bacteria as well as in higher plants and obligate intracellular parasites of *Chlamydia* genus (Habibian et al., 2005). In the microbial, several NhaD homologues from the Haloalkaliphile *A. amylolytica* AS1.343 (Aa-NhaD), *V. parahaemolyticus* AQ3334 (Vp-NhaD) and *V. cholerae* (Vc-NhaD) have thus far been intensively studied (Habibian et al., 2005). They all exhibited Na^+/H^+ and alkaline pH optima in the same type of membrane vesicle assay used, but the pH and Na^+ resistance profile of M-NhaD was significantly different from Vp-NhaD and Vc-NhaD, while similar with those of Aa-NhaD in some degree, exhibited hyperalkaline and hypersaline resistance (Figure 7). M-NhaD was highly active between pH 7.0 - 10 and the optimal pH 8.5 (Figure 7A). In contrast, Aa-NhaD was only active above pH 8.5 and the optimal pH might well be greater than 9.5. Vp-NhaD exhibited alkali-stimulated activity from pH 8 to 9 but reduced activity at pH 9.5 (Dibrov et al., 2004), whereas Vc- NhaD was active only in a pH range from pH 7.25 to 8.5 with an optimum at pH 8.0 (Goldberg et al., 1987). The different pH responses of the NhaD antiporters probably reflect the dependence of the antiporters activity on cytoplasmic pH in their different hosts, as is found for the major NhaA antiporter of *E. coli* and its homologues in different bacteria (Liu et al., 2005). Thus, for the halophiles colonizing in alkaline and hypersaline brine of

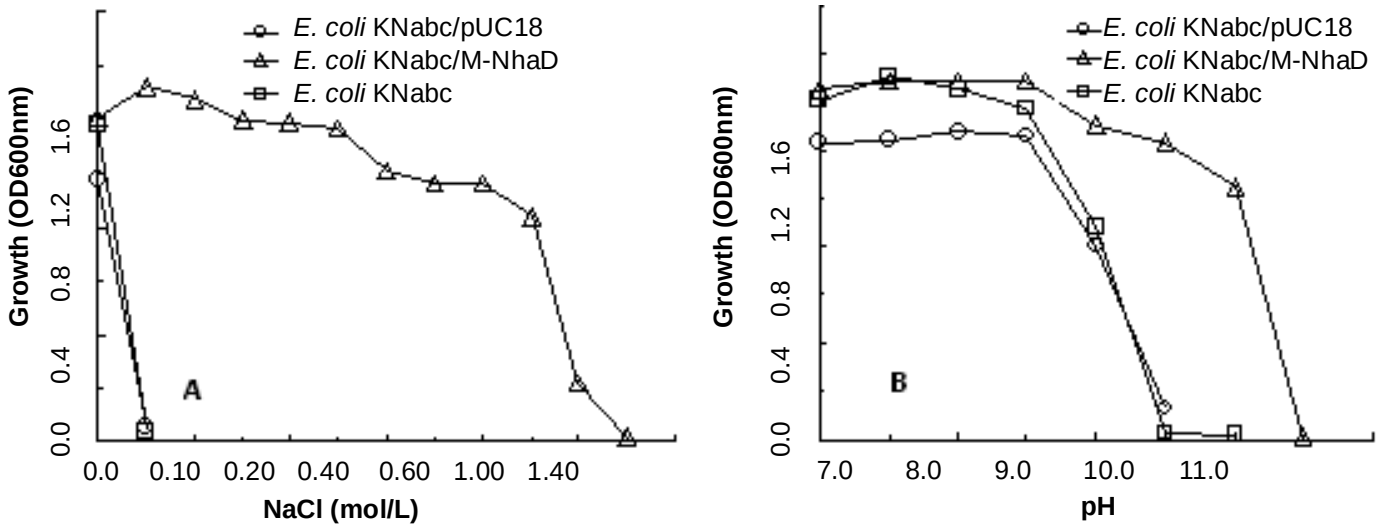

Figure 7. Growth of the recombinant strian *E.coli* KNabc/M-NhaD harbored *m-nhaD* gene, the *E. coli* KNabc/pUC18 that contained pUC18 empty vector and the antiporter-deficient *E. coli* KNabc in (A) LBK medium with 0 - 1.4 M NaCl under the pH 7.0, or (B) LBK medium with pH 7.0 - 10.5.

Dagong Well, the M-NhaD pH profile could play a role in cytoplasmic pH homeostasis at and above the optimal pH for growth. In NhaD type Na^+/H^+ transporter, it is also shown that the polar or charged amino acid residue, localized within hydrophobic segments or at the membrane-water interface, plays an important role for binding and transporting cations like H^+ and Na^+ (Liu et al., 2005). The Ser-150, Asp-154, Asn-155, Thr-157, Asn-189, Asp-199, Thr-201, Thr-202, Asp-344, Thr-345, Ser-389, Asp-393, Asn-394, Ser-425, and Ser-431 are involved either directly or indirectly in ion translocation in Vc-NhaD from the *V. cholerae*. And mutation of any of them arrests the overall reaction of cation-proton antiport over a pH range from 6.0 to 9.0 (Liu et al., 2005). However, the only Thr-157 and Thr-201 of Vc-NhaD are evolutionarily conserved in the M-NhaD, positioned in Thr-96 and Thr-135 (Figures 3 and 5). The other above residues are substituted by some neutral amino acid or deleted. Interestingly, these substitutions or deletions that happened in M-NhaD have not affected either the pH profile of activity or resistance for alkali cations mediated by it. The Na^+-binding sites of the Na^+ motive ATPases are thought to be formed by closely positioned conserved Asp, Thr, and Asn residues. In the Ec-NhaA from the *E. coli*, Asp-163, Asp-164, and Thr-132 are parts of the catalytic site in bacterial Na^+/H^+ antiporter (Hunte et al., 2005). Therefore, it seems highly likely that Thr-96 and Thr-135 residues, located in short periplasmic loops flanking between TMS III to TMS V (Figure 5), are involved in the alkali cation binding and/or translocation in the M-NhaD. This result has been confirmed by site-directed mutagenesis in *V. cholerae* (Habibian et al., 2005).

The protein encoded by *m-nhaD* gene showed high similarity of 93 and 87% to NhaD from *K. pneumoniae* 342 and *Y. frederiksenii* ATCC 33641 based on the 77 and 76% amino acid sequence coverage, respectively. Interestingly, M-NhaD has a long carboxyl terminal hydrophilic tail (31 amino acid residues) similar to NhaP, and NhaG type Na^+/H^+ antiporters (Figure 5), while the other NhaD from the *K. pneumoniae* 342, *Y. frederiksenii* ATCC 33641, *V. parahaemolyticus* AQ3334, *V. cholerae*, *A. amylolytica* AS1.3430 and *H. elongata* DSM 2581^T does not have such one. The activities of NhaG decreased when 26 residues in the C-terminal of NhaG type Na^+/H^+ antiporter from *B. subtilis* were lost (Gouda et al., 2001), and 56 residues in the C-terminal region of SynNhaP were necessary for antiporter activity in *Synechocystis* sp (Hamada et al., 2001). It was reported that both the ion specificity and activity of a Na^+/H^+ antiporter were partially determined by the structural properties of the C-terminal hydrophilic tail (Hamada et al., 2001; Waditee et al., 2001). Consequently, the mechanism of ion transport mediated by M-NhaD from the Dagong Ancient Brine Well should be different from that of the other NhaD, though they all had a certain similarity of amino acid sequence. With the differences of amino acid sequence and the putative secondary structure of the protein encoded by *m-nhaD* from those NhaD type Na^+/H^+ antiporter genes reported previously, it is admissible to believe that *m-nhaD* is a novel NhaD type Na^+/H^+ antiporter gene.

This study was significant in helping us understand the necessity of the existence of Na^+/H^+ antiporter in the Dagong Ancient Brine Well to maintain the intracellular environment homeostasis for halophiles, but also the result suggested that *m-nhaD* gene was very potentially attractive in salt tolerance genetic breeding.

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