Screening a novel Na\(^+\)/H\(^+\) antiporter gene from a metagenomic library of halophiles colonizing in the Dagong Ancient Brine Well in China

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Abstract

Metagenomic DNA libraries constructed from the Dagong Ancient Brine Well were screened for genes with Na\(^+\)/H\(^+\) antiporter activity on the antiporter-deficient Escherichia coli KNabc strain. One clone with a stable Na\(^+\)-resistant phenotype was obtained and its Na\(^+\)/H\(^+\) antiporter gene was sequenced and designated as \(m\)-nha. The deduced amino acid sequence of M-Nha protein consists of 523 residues with a calculated molecular weight of 58 147 Da and a pl of 5.50, which is homologous with NhaH from Halobacillus dabanensis D-8\(^T\) (92%) and Halobacillus aidingensis AD-6\(^T\) (86%), and with Nhe2 from Bacillus sp. NRRL B-14911 (64%).

Introduction

The Na\(^+\)/H\(^+\) antiporter is a ubiquitous integral membrane protein in all biological kingdoms and plays a major role in maintaining cytoplasmic Na\(^+\) homeostasis and pH levels for living cells. In bacteria, the Na\(^+\)/H\(^+\) antiporter has several primary functions, including extrusion of Na\(^+\) or Li\(^+\) in exchange for H\(^+\) to keep the cytoplasm iso-osmotic with the environment and avoid intoxication of living cells (Majernik \et\ al., 2001; Hunte \et\ al., 2005), establishment of an electrochemical potential of Na\(^+\) across the cytoplasmic membrane (Tsuchiya \et\ al., 1977), regulation and maintenance of intracellular pH homeostasis under alkaline conditions (Padan & Shuldiner, 1994), and cell volume regulation (Grinstein \et\ al., 1992).

Several families of Na\(^+\)/H\(^+\) antiporter genes have been identified in microorganisms. Although the primary function of prokaryotic Na\(^+\)/H\(^+\) antiporters in their cells is the tolerance to Na\(^+\), these antiporter proteins belong to different protein families (Hunte \et\ al., 2005). The halo-biont, an ideal organism for screening the salt-tolerance gene, survives as a wild type in naturally or artificially saline environments worldwide; among them, halophilic bacteria are the dominant species. In fact, almost all halophilic microorganisms have potential Na\(^+\) ion transport mechanisms to expel Na\(^+\) ions from the interior of the cells which are based on Na\(^+\)/H\(^+\) antiporters (Oren, 1999).

As the first recorded man-made brine well in the world, the Dagong Ancient Brine Well Zigong, Sichuan in southwestern China, has been producing brine since 250 BC, and the ancient salt-making facilities are still being used (Xiang \et\ al., 2008). However, the construction and facilities of this brine well, which are made of bamboo, wood and stone, have been eroded by halophiles living in the brine. It is proposed that the Na\(^+\) pump with a high Na\(^+\) extrusion activity may be widely distributed among these halophilic microorganisms. In our previous work, it was found that in the Dagong Ancient Brine Well the halophiles constitute a heterogeneous physiological group of microorganisms and some have evolved intriguing changes in basic physiological
materials and methods

extraction of environmental DNA

The halophile genomic DNAs were prepared from the brine in the Dagong Ancient Brine Well using methods originally described by Moon with modifications (Moon et al., 2004). Briefly, 100-mL samples were centrifuged at 14 000 \( g \) and 4 \( ^\circ \)C for 10 min, and the slurry was resuspended with 5 mL phosphate-buffered saline (pH 7.5) centrifuged at 5 g for 2 min at room temperature. The dispersion was again centrifuged at 14 000 g and 4 \( ^\circ \)C for 2 min. The bacterial cell pellets obtained were directly used for extracting environmental DNA using the Ultra-Clean Soil DNA Kit (Mo Bio Laboratories, Solana Beach, CA). Total DNA was subsequently subjected to electrophoresis in 0.8% agarose gels and stored at −20 \( ^\circ \)C.

preparation of electrocompetent E. coli KNabc cells

An overnight culture of E. coli KNabc was inoculated into 100 mL of a modified Luria–Bertani medium (LBK medium) consisting of 1.0% tryptone, 0.5% yeast extract and 87 mM KCl, and then grown at 37 \( ^\circ \)C under aerobic conditions to an OD \( \text{OD}_{600 \text{nm}} \) of 0.4. Cells were harvested by centrifugation at 4000 g for 10 min at 4 \( ^\circ \)C and washed three times in 10 mL of ice-cold sterile 10% glycerol solution before electrocompetent preparation (Yang et al., 2006).

construction of the metagenomic library and screening the \( \text{Na}^+/\text{H}^+ \) antiporter gene

The halophile genomic DNAs were partially digested with Sau3AI to produce 1.5–6 kbp fragments. These DNA fragments were separated by agarose electrophoresis and ligated into pUC18, which had been digested with BamHI and dephosphorylated with bacterial alkaline phosphatase, using T4 DNA ligase (Mayumi et al., 2008). The ligated recombinant plasmids (20–200 ng) were added to 50 \( \mu \)L of competent cells of E. coli KNabc suspension and mixed thoroughly. Electroporation was carried out at field strength of 16 kV cm \(^{-1}\) in combination with an electric resistance of 300 \( \Omega \) at 25 mF in a 0.1-cm electroporation cuvette. In this way, a metagenomic library was constructed. Recombinants were spread on agar plates containing LBK medium, 5.0 mM LiCl, 1.5% agar, and 100 \( \mu \)g mL \(^{-1}\) ampicillin. The plates were incubated at 37 \( ^\circ \)C for 20 h and salt-tolerant clones were isolated. The clones with the highest level of salt tolerance were further screened on LBK supplemented with a higher concentration of LiCl (7.5 mM), and the resulted clones were screened again on selective plates with higher concentrations of NaCl (0.20, 0.25 M).

molecular analyses of the \( \text{Na}^+/\text{H}^+ \) antiporter gene

The nucleotide sequences of the \( \text{Na}^+/\text{H}^+ \) antiporter gene were determined by the Sanger’s dideoxy-chain termination method. Sequencing was performed using a DNA sequencer (Applied Biosystems, Foster City, CA) with a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Bioscience, Piscataway, NJ). The ORF was searched by ORF FINDER programs from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). The amino acid sequence analysis, database searches and sequence comparisons of protein encoded were performed using a tool from the ExPASy Proteomics Server (http://www.expasy.ch/tools/blast/). Multiple alignments of all amino acid sequences were run using the CLUSTALX program (Thompson et al., 1997). A phylogenetic tree was constructed with the MEGA program version 4.0 using the neighbor-joining method with the Kimura two-parameter model (Kumar et al., 2004).

structure and functional prediction of encoded protein

The amino acid sequence and pI/Mn of primary structure were analyzed, respectively, using the Translate tool and the Compute pI/Mn of the 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 the conserved domains database (CDD) search provided by NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The transmembrane segments
and orientation of the deduced amino acid sequence were identified using the DAS program (Cserzó et al., 1997). The transmembrane helix location and topology of the sequence were predicted using TMHMM and SOSUI from the PREDICTPRO-TEIN Server (http://www.predictprotein.org/). The cellular localization and function of its gene product were defined by INTERPROSCAN (http://www.ebi.ac.uk/Tools/InterProScan/).

Resistance of transformed straits by recombinant plasmid to Na⁺ and pH

The recombinant plasmids, isolated from the stable Li⁺-resistant transformed cells, were retransformed into E. coli KNabc. To test the resistance of transformant E. coli KNabc cells to Na⁺ and pH, the transformant cells were grown, respectively, in the modified LBK liquid medium supplemented with 50 μg mL⁻¹ ampicillin and indicated NaCl concentrations where necessary, and in minimal liquid medium [100 mM Tris-HCl (at indicated pH), 20 mM (NH₄)₂SO₄, 50 mM KCl, 1 mM K₂HPO₄, 0.3 mM MgSO₄, 0.01 mM CaCl₂, 0.2 M NaCl, 40 mM glycerol, a 50 μg mL⁻¹ ampicillin]. Cells were incubated aerobically in 100 mL portions in 250 mL Erlenmeyer flasks in a rotary shaker at 37 °C for 14 h. The cell growth was monitored turbidimetrically at 600 nm.

Nucleotide sequence accession number

The nucleotide sequence reported in this paper has been submitted to GenBank with accession number GU320350.

Results

Screening genes conferring Na⁺/H⁺ antiporter activity

For the detection of E. coli clones exhibiting Na⁺/H⁺ antiporter activity, the antiporter-negative mutant strain E. coli KNabc was used as the host for the recombinant plasmids of metagenomic DNA libraries. The resulting recombinant E. coli strains were screened on selective LBK agar plates containing 5.0 mM LiCl. The growth of E. coli KNabc was completely inhibited under this condition due to the toxic effect of Li⁺ on pyruvate kinase in the absence of an antiporter (Majernik et al., 2001). Thus, only recombinant E. coli strains harboring a gene conferring resistance to Li⁺ could grow under the conditions used. By functional complementation tests, 10 clone candidates were obtained out of approximately 300 E. coli clones during the initial screening procedure. To confirm that the Li⁺-resistant phenotype of the clones selected was determined by recombinant plasmids, the plasmids in the clones were isolated and retransformed into E. coli KNabc, and the resulting clones growing in 7.5 mM Li⁺ medium were screened again on selective plates with high concentrations of NaCl (0.20 and 0.25 M). However, only one recombinant plasmid, designated as pM-Nha, conferred a stable Na⁺-resistant phenotype on the resulting recombinant E. coli KNabc strains. The hybrid plasmid pM-Nha was sequenced, and it was revealed that pM-Nha carried a common DNA fragment of a putative Na⁺/H⁺ antiporter gene.

Sequence and characteristics of the Na⁺/H⁺ antiporter gene

The nucleotide sequence analyses of Na⁺/H⁺ antiporter gene revealed that the length of the DNA insert of pM-Nha was 1814 bp, and it contained one intact ORF (1572 bp), a promoter (ATG) and a terminator (TAA) (Fig. 1). A Shine-Dalgarno (AGGAGG), −10 region (TATTAT) and −35 region (TTGACA) in the downstream and a terminator-like sequence (5’-GCAGGCTGT-3’; 5’-ACAGCCTG-3’) in the upstream were also found in the ORF (Fig. 1). A homology search revealed that the protein encoded by ORF had the highest homology of 92%, 86% and 64% identity with the Na⁺-antiporter from Halobacillus aidingensis AD-6 (accession no. ABX57744) and with Nhe2 from Bacillus sp. NRRL B-14911 (accession no. E97305), respectively, and a slightly lower similarity (31% and 33% identity) to the Na⁺ antiporter from Halogeaometricum borinquense DSM 11551 (accession no. EEE17208) or Cyanothece sp. ATCC 29155 (accession no. ACK72385).

Fig. 1. Nucleotide sequence of the gene encoding the Na⁺/H⁺ 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, the putative termination code TAA and the possible terminator following the ORF were indicated by inverted arrows.
In terms of the phylogenetic relationship between the Na\(^+\)/H\(^+\) antiporter protein from the metagenomic library constructed in current study and those from other strains reported, the ORF products of these antiporters were clearly divided into two groups (Fig. 2). The M-Nha was closely related to NhaH from the moderately halophilic strains of *H. dabanensis* D-8\(^T\) and *H. aidingensis* AD-6\(^T\), and also similar to Nhe2 from *Bacillus* sp. NRRL B-14911 and to NhaG from *Bacillus subtilis* ATCC 9372, whereas it has rather distant relationships with those from the halophile *H. borinquense* DSM 11551 and *Aphanothece halophytica* PCC 6803. Their amino acid sequences are aligned in Fig. 3.

**Structure and function prediction of protein encoded by the Na\(^+\)/H\(^+\) antiporter gene**

The amino acid sequence deduced from the ORF, designated as M-Nha (Na\(^+\)/H\(^+\) antiporter from metagenomic library), consisted of 523 amino acid residues with a calculated molecular weight of 58 147 Da and a pI of 5.50. The most abundant amino acid residues of this protein were Leu (75/523), followed by Ile (48/523), Val (46/523), Ala (38/523) and Gly (37/239). The least abundant residue was Cys (two residues) and Trp (five residues). Among the 523 amino acid residues, only 89 residues were charged, indicating that M-Nha is of low polarity. This is consistent with the belief that the Na\(^+\)/H\(^+\) antiporter is an integral membrane protein.

Although the dense alignment surface approach revealed that the M-NhaP contained 11 peaks (Fig. 4), the probability for the 10th peak was only around 20% when its transmembrane segment (TMS) was analyzed using TMHMM computer program (data not shown). The SOSUI analysis further confirmed this result of total 10 peaks in M-NhaP released by TMHMM (Fig. 5). Thus it was likely that the M-Nhap only contained 10, not 11, transmembrane domains. The conserved domain analysis against CDD suggested that M-NhaP is a cpa1 Na\(^+\)/H\(^+\) antiporter from bacteria, which

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**Fig. 2.** Phylogenetic trees based on the amino acid sequences of the Na\(^+\)/H\(^+\) antiporter protein from the metagenomic clone and other reference bacteria using the neighbor-joining method. The scale bar corresponded to 0.1 – estimated amino acid substitution per sequence position. Bootstrap values from 1000 replicates were included. Database accession numbers were shown in parentheses after each protein. The underline emphasizes that M-Nha is from the metagenomic DNA libraries of Dagong Ancient Brine Well in our study.
was classified as a model that may span more than one domain and had not been assigned to any domain superfamily yet. Furthermore, CDD also showed that M-Nha had significant similarity to NhaP type Na\(^{+}\)/H\(^{+}\) antiporter with a unique C-terminal domain in the Na\(^{+}\)/H\(^{+}\) exchanger family. A similar result was also obtained when it was analyzed by INTERPROSCAN. Gene ontology delineation indicated that M-Nha was integrated to membrane (GO: 0016021) and exchanged Na\(^{+}\) for H\(^{+}\) in an electroneutral manner.

**Resistance of Na\(^{+}\)/H\(^{+}\) antiporter protein to Na\(^{+}\) and pH**

The effects of NaCl concentration on the growth of transformant cell *E. coli* KNabc/pM-Nha, which harbored the recombinant Na\(^{+}\)-resistant plasmid pM-Nha, and *E. coli* KNabc/pUC18, which contained only empty pUC18 vector, were evaluated. The *E. coli* KNabc/pM-Nha strains can grow well in LBK medium containing 0.2 M NaCl and can even survive in the presence of 0.25 M NaCl, whereas cells of other strains were inhibited. The effects of pH on growth were also investigated. The transformant cell *E. coli* KNabc/pM-Nha can grow well at pH 7.0, whereas cells of other strains were inhibited. The results suggest that M-Nha has high resistance to Na\(^{+}\) and pH.
E. coli KNabc/pUC18 do not (Fig. 6). To test the effect of pH on cell growth, E. coli KNabc/pUC18 and KNabc/pM-Nha were grown in minimal medium as described above but at different pH values from 7 to 8.5. The results were similar to that influenced by NaCl, with a greatly reduced growth of E. coli KNabc/pUC18 under alkaline conditions, especially at pH above 8.0, compared with that below neutral pH. However, only a certain growth reduction range was observed for E. coli KNabc/pM-Nha harboring nha gene in alkaline medium (Fig. 6). This result indicated that the protein encoded by m-nha gene offered the antiporter-negative mutant E. coli KNabc cells not only resistance to Na\(^+\), but also the ability to grow under alkaline conditions.

**Discussion**

Modern biotechnology has a steadily increasing demand for novel genes for application in various industrial processes and the development of genetically modified organisms. Identification, isolation and cloning for novel genes at a reasonable pace is the main driving force behind the development of unprecedented experimental approaches (Vakhlu et al., 2008). Furthermore, 99.9% of the microbial species represented in any biotope are not culturable at the moment (Streit & Schmitz, 2004; Tringe et al., 2005), which highlights the limitation of any gene discovery protocol dependent on culturing (Vakhlu et al., 2008). Thus, the diversity of enzymes with special fundamental functions, such as Na\(^+\)/H\(^+\) antiporters that usually require purification from pure culture of a specific organism before analysis, is only partially understood at present. Correspondingly, a large fraction of genes in the environment cannot be disclosed due to difficulties in enriching and isolating microorganisms in pure culture. Metagenomics, a culture-independent strategy, provides an access to valuable genetic resources of the microorganisms regardless of whether they can be cultured (Cowan et al., 2005; Guazzaroni et al., 2009). The various target genes have been screened by using a metagenomic library (Schmeisser et al., 2007). In this study, we applied this methodology for the direct cloning of genes encoding Na\(^+\)/H\(^+\) antiporters from the Dagong Ancient Brine Well by functional complementation of antiporter-negative mutant strain. Our results demonstrated that metagenomic DNA libraries are also suitable for direct cloning of functional genes.
genes encoding integral membrane proteins from a brine environment.

About 10 families of Na\(^+\)/H\(^+\) antiporter genes have been identified in microorganisms in the past, including a single gene of nhaA, nhaB, nhaC, nhaD, nhaG, nhaP, nhaH and chaA, and multiple subunits of Mrp antiporter and MnhABCDEFG system (Hunte et al., 2005; Yang et al., 2006). In these genes, only nhaH comes from the halophilic bacteria H. dabanensis D-8T and H. aidingensis AD-6T. Although the gene m-nha cloned in the current study also comes from the halophiles, to our knowledge it was the first Na\(^+\)/H\(^+\) antiporter gene directly mined by metagenomic technology from the halophiles colonizing a high-salt environment. In single subunit Na\(^+\)/H\(^+\) transporter, it is shown that the negatively charged amino acid residue Asp, localized in the membrane-spanning regions, plays an important role in the binding and transporting of cations such as H\(^+\) and Na\(^+\) in several antiporter proteins (Majernik et al., 2001). Asp-133, Asp-163 and Asp-164 were proposed to be involved in binding sodium ions in NhaA from E. coli (Inoue et al., 1995). Asp-137 of Nha from H. dabanensis D-8T and H. aidingensis AD-6T (Yang et al., 2006; Zou et al., 2008), Asp-138 of SynnhaP from Synechocystis sp., and Asp-139 of AphaP from A. halophytica were also believed to be necessary for Na\(^+\)/H\(^+\) antiporter activity (Hamada et al., 2001; Waditee et al., 2001), and replacement of these residues could inactivate the Na\(^+\)/H\(^+\) antiporters. In M-Nha, most Asp residues (14/19) were predicted to be in the hydrophobic region, while the alignment of M-Nha with Na\(^+\)/H\(^+\) antiporters of another six microorganisms indicated that three aspartates, including Asp-138, Asp-167 and Asp-224, were conserved in M-Nha (Fig. 3).

The protein encoded by m-nha gene showed a high similarity of 92%, 86% and 62% to NhaH from H. dabanensis D-8T, H. aidingensis AD-6T and B. subtilis, respectively. Interestingly, M-Nha has a long carboxyl terminal hydrophilic tail (140 amino acid residues), similar to Nhap and NhaG type Na\(^+\)/H\(^+\) antiporters, whereas NhaH does not. It was reported that both the ion specificity and activity of an Na\(^+\)/H\(^+\) antiporter were partially determined by the structural properties of the C-terminal hydrophilic tail (Hamada et al., 2001; Waditee et al., 2001). NhaG from B. subtilis possesses a hydrophilic segment with > 100 amino acid residues at the carboxyl terminal region (Gouda et al., 2001), and such a long hydrophilic domain is not present in any other microbial Na\(^+\)/H\(^+\) antiporter except SynNhaP (NhaS1) in Synechocystis sp. (Hamada et al., 2001) and AphaP in A. halophytica (Waditee et al., 2001). The activities of NhaG decreased when 26 residues in the C-terminal of the protein were lost (Gouda et al., 2001), and 56 residues in the C-terminal region of SynNhaP were necessary for antiporter activity (Hamada et al., 2001). Hydropathy analysis usually showed that the Na\(^+\)/H\(^+\) antiporter had 10–12 hydrophobic and also probably membrane-spanning regions (Majernik et al., 2001; Yang et al., 2006). Our results also revealed that m-nha gene product fits well into this model. The NhaH and NhaG had 12 TMS, but M-Nha had only 10 TMS, although they all had high similarity of amino acid sequence. Consequently, the mechanism of ion transport by M-Nha from the Dagong Ancient Brine Well should be different from that of NhaH, NhaG and SynNhaP. With the differences of amino acid sequence and the putative secondary structure of the protein encoded by m-nha from those Na\(^+\)/H\(^+\) antiporter genes reported previously, it can be proposed that m-nha is a novel Na\(^+\)/H\(^+\) antiporter gene. This study was significant in not only 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 enriches our knowledge about the different mechanisms of Na\(^+\)/H\(^+\) antiporter in halophiles in such an extreme environment.
Na\textsuperscript{+}/H\textsuperscript{+} antiporter gene from Dagong Ancient Brine Well

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References


Gouda T, Kuroda M, Hiramatsu T, Nozaki K, Kuroda T, Mizushima T & Tsuchiya T (2001) nhaG Na\textsuperscript{+}/H\textsuperscript{+} antiporter gene of Bacillus subtilis ATCC, which is missing in the complete genome sequence of strain, and properties of the antiporter. J Biochem 130: 711–717.


Padan E & Schuldiner S (1994) Molecular physiology of Na\textsuperscript{+}/H\textsuperscript{+} antiporters, key transporters in circulation of Na\textsuperscript{+} and H\textsuperscript{+} in cells. Biochim Biophys Acta 1185: 129–151.


