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Cloning and sequencing of an Na^+/H^+ antiporter gene from the marine bacterium *Vibrio alginolyticus*

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Abstract

A gene has been cloned from a DNA library from the marine bacterium *Vibrio alginolyticus* that functionally complements a mutant strain of *Escherichia coli*, NM81, defective in an Na^+/H^+ antiporter (NhaA). The cloned *Vibrio* gene restored NM81 to grow in a medium containing 0.5 M NaCl at pH 7.5 and concomitantly led to an increase in Na^+/H^+ antiport activity. The nucleotide sequence of the cloned fragment revealed an open reading frame, which encodes a protein with a predicted 383 amino acid sequence and molecular mass of 40400 Da. The hydropathy profile is characteristic of a membrane protein with 11 membrane spanning regions. The deduced amino acid sequence is 58% identical with *E. coli* NhaA.

Key words: Sodium ion/proton antiporter; DNA sequence; Marine bacterium; (*V. alginolyticus*)

Na^+/H^+ antiporters have been implicated in a variety of functions including intracellular pH regulation, osmoregulation and the generation of sodium-motive force by extruding cellular Na^+ in exchange for H^+ [1–4]. Recently, *Escherichia coli* was found to have two genes encoding Na^+/H^+ antiporters, designated *nhaA* [5] and *nhaB* [6]. Cloning of a putative Ca^{2+}/H^+ antiporter gene (*chaA*) that also functions as an Na^+/H^+ antiporter has been reported [7]. The *nhaA* gene is regulated by a regulatory protein called NhaR [8], and its transcription is stimulated by high NaCl concentrations and by elevated internal pH values [9]. A mutant strain NM81 carrying a deletion in *nhaA* exhibits residual Na^+/H^+ antiporter activity, but is unable to grow in the presence of 0.5 M NaCl at pH 7.5 [10]. This mutant was used for the cloning of *nha* gene from alkalophilic *Bacillus firmus* OF4 [11] and *Salmonella entiritidis* [12].

In contrast to *E. coli*, the marine bacterium *Vibrio alginolyticus* requires 0.5 M NaCl for optimal growth and has another Na^+ extrusion system, which is an electrogenic primary Na^+ pump driven by an Na^+-translocating NADH-quinone reductase in the respiratory chain [13,14]. This organism also has an Na^+/H^+ antiporter which is driven by a protonmotive force [15]. We have demonstrated that the fine regulation of intracellular pH is performed by the function of a K^+/H^+ antiporter over the external pH range 6.0–9.0 and that the function of Na^+/H^+ antiporter becomes important for the acidification of cell interior especially at alkaline external pH [15,16]. It was necessary to further characterize the respective roles of K^+/H^+ and Na^+/H^+ antiporters in the salt tolerance and pH regulation of this organism. Thus, attempts have been made to clone a gene encoding Na^+/H^+ antiporter from *V. alginolyticus* using *E. coli* NM81.

Genomic DNA was isolated from exponential cultures of *V. alginolyticus* 138–2 grown in a rich medium [17]. DNA fragments obtained by partial digestion with *Sau*3AI were size-fractionated by agarose gel electrophoresis, and the fragments from 10 to 20 kbp were cloned in the *Bam*HI site of the plasmid pACYC184

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The sequence data reported in this paper have been submitted to the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases under the accession number D25214.

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[18]. The plasmids were amplified by transforming into *E. coli* TG1. *E. coli* NM81, defective in Na^+/H^+ antiporter A (NhaA), is unable to grow in LB medium [10] containing 0.5 M NaCl at pH 7.5 (LB-NaCl medium). Therefore, the recombinant plasmids were incorporated into *E. coli* NM81 by electroporation according to the manual of an Electroporator (Invitrogen, San Diego) and then screened for the ability to restore the growth of *E. coli* NM81 in LB-NaCl medium. Twenty two plasmids were selected. When digested with *Eco*RI, all these selected plasmids gave the same pattern of DNA fragments. Among these plasmids, pIN3 was selected for further experiments.

E. coli NM81/pACYC184 was grown in the LB medium containing 0.5 M KCl at pH 7.5 and *E. coli* NM81/pIN3 was grown in the LB-NaCl medium. Both media contained chloramphenicol (20 μg/ml) and kanamycin (25 μg/ml). Harvested cells were suspended in a medium containing 140 mM choline chloride, 250 mM sucrose, 0.5 mM DTT and 10 mM Tris-HCl (pH 7.5) (medium A), and then inverted membrane vesicles were prepared by a French pressure cell as described in [19]. By the addition of 2 mM Tris DL-lactate, a respiratory substrate, the fluorescence intensity of 1 μM acridine orange was quenched due to the acidification of vesicle interior (Fig. 1). The addition of 2 mM K^+ slightly recovered the fluorescence intensity. Further addition of 10 mM Na^+ greatly recovered the intensity, reflecting a partial collapse of inside acidic ΔpH. The extent of recovery was about twice as large in the vesicles from *E. coli* NM81/pIN3 as that from *E. coli* NM81/pACYC184 (control). Although *E. coli* NM81 is devoid of NhaA, it still has another Na^+/H^+ antiporter NhaB [6,10]. Thus, the dequenching of the fluorescence observed by the addition of Na^+ is explained by the function of NhaB [20]. Since the introduction of pIN3 to *E. coli* NM81 greatly enhanced the dequenching by Na^+, pIN3 is likely to harbor a gene corresponding to Na^+/H^+ antiporter from *V. alginolyticus*.

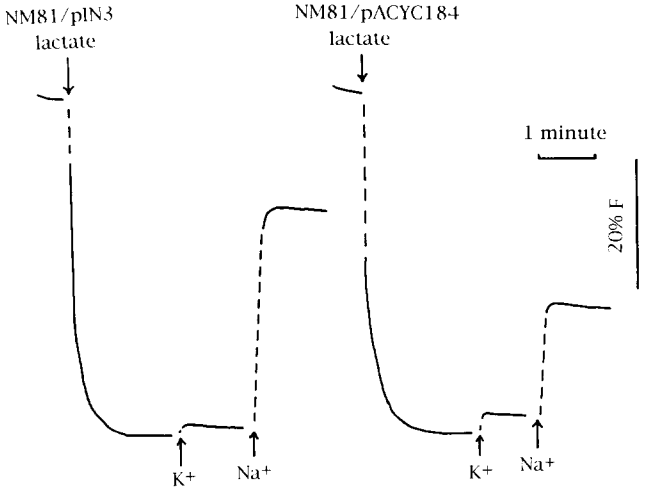

Fig. 1. The Na^+/H^+ antiporter activity of inverted membrane vesicles prepared from *E. coli* NM81/pIN3 and *E. coli* NM81/pACYA184 (control). The reaction mixture contained medium A (described in text), 1 μM Acridine orange and the vesicles (35 mg protein/ml) in a total volume of 2.0 ml. The fluorescence intensity of Acridine orange was monitored with an excitation of 430 nm and emission of 570 nm. At the arrow, 2 mM Tris DL-lactate, 2 mM KCl and 10 mM NaCl, respectively, was added.

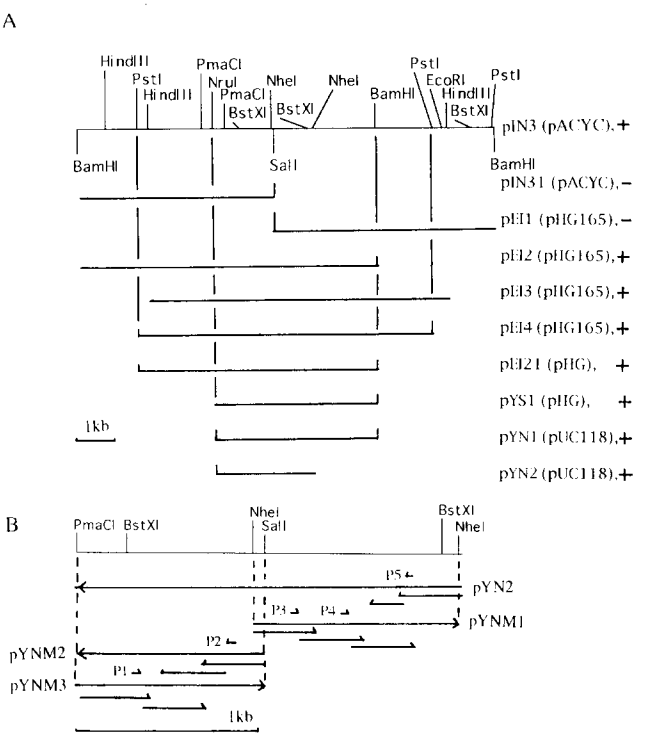

Fig. 2. Subcloning and sequencing of the pIN3 from *V. alginolyticus*. (A) Deletions of the insert of pIN3 and ligations to different vectors were performed. Each of the constructs was screened for the ability to restore the growth of *E. coli* NM81 in LB-NaCl medium, and + sign denotes the positive result. (B) The plasmid pYN2 was subcloned to pYNM1, pYNM2 and pYNM3. Arrows indicate the direction and the extent to which the sequence was determined. Small arrows (P1 to P5) indicate the sites of primers synthesized for sequencing.

V. alginolyticus has an electrogenic Na^+ pump [13,14]. Therefore, pIN3 was incorporated into an Na^+ pump-defective mutant, Nap1 [21], of *V. alginolyticus* by electroporation [22]. *V. alginolyticus* Nap1/pIN3 showed no indication of the recovery of Na^+ pump, implying that pIN3 is not related to Na^+ pump.

The restriction mapping, subcloning and sequencing were performed by the standard methods [23] (Fig. 2). The size of pIN3 was about 15.1 kbp including pACYC184 (4.2 kbp). The genetic information that restores the growth of *E. coli* NM81 in the LB-NaCl medium (+ sign in Fig. 2) was located around the *Sal*I site. The plasmid pYN1, obtained by ligating *Bam*HI-*Nru*I fragment to pUC118, was positive. The shortest positive plasmid pYN2 was used for the nucleotide

sequence. The 1.2-kb *Nhe*I fragment and 1.1-kb *Pma*CI/*Sal*I fragment were inserted into the M13mp18 and 19. The DNA was sequenced by the dideoxy method of Sanger et al. [24] using *Bca*BEST™ Dideoxy Sequencing Kit (Takara Biomedicals, Japan) and Sequenase Ver.2.0 (Toyobo Biochemicals, Japan). The primers used for sequencing were synthesized with a Cyclone Plus DNA Synthesizer (Millipore).

A genome fragment of 2295 nucleotides was sequenced (Fig. 3, the accession number D25214) and an open reading frame, starting with the initiation codon ATG and terminated by the stop codon TAA, extending from 605 to 1753 bp was comprised of 383 amino acid residues with a predicted molecular mass of 40 400 Da. The hydropathy profile of deduced amino acid sequence was characteristic of a membrane protein with 11 membrane spanning regions (see Fig. 4). As compared with *nhaA* from *E. coli* [5], the degree of homology was 58.0% identical within 1164 bp and 57.9% identical within 382 amino acid residues (Fig. 4). Thus, the putative Na^+/H^+ antiporter gene is homologous to *nhaA* from *E. coli*. Very recently, the Na^+/H^+ antiporter gene from a marine bacterium *V. parahaemolyticus* was sequenced (unpublished results), and

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                                                                  1CTGTGTTTGGCACCTT
GTCCACACGCTCAGGTAAAGTCATCAATCTCTCAGATGATGATATCAGTATTAAACCAATGCAAGTGACCAGTTTATCAAATGG
AAGAAGGCTGCCTTTGCAGTGGATTATTAATATTCCAAAGCATCAAATTAACTTGACCACTCGTATTATCAAATCAGATATGTG
GCTTCCTTTTGTTATACCGTATTGGGAAGGTCCTACCTTAGCTTCTGGCTCCAACGAGGGTTGGGGCTTTATGCAGCTCACCGG
TTATTAAATTCTGATAAAAACCACCCCAATGGGTGGTTTTTTAGTGGATGAAACTTCCTGAATTTTTCTCAAAAGTATGGGGTT
ATCATGACAGTACATGCCATGTTATGCTGATTTTCTCATAATCTTGATAAACATTTGGCGCGATAACCACAAGTACGCGCTGTG
ATCAAAATTGAGTGTTATGTTGGAAAATACGATGTATAGTAAACTTCATATCGGAAAAATCGAAGTTTATACTCTGAAGAAGCG
ATTATTCTAAAGTTTTCGACCGCTATACACTCTTATCATTCTTTGTTAATCGTTTATTAAGATAATAAATATAAGGACGAAACC
605ATGAACGATGTCATTCGTGACTTTTTCAAAATGGAATCAGCTGGTGGTATTCTTCTCGTGATTGCCGCGGCA
  M  N  D  V  I  R  D  F  F  K  M  E  S  A  G  G  I  L  L  V  I  A  A  A
ATTGCGATGACCATTGCTAACTCACCGCTGGGCGAAACTTATCAATCTGTACTGCATACTTATGTATTTGGT
  I  A  M  T  I  A  N  S  P  L  G  E  T  Y  Q  S  V  L  H  T  Y  V  F  G
ATGTCAGTTTCTCACTGGATTAATGACGGCCTAATGGCTGTTTTCTTCTTACTCATTGGCCTTGAAGTTAAG
  M  S  V  S  H  W  I  N  D  G  L  M  A  V  F  F  L  L  I  G  L  E  V  K
CGAGAGCTACTTGAAGGTGCGTTAAAATCAAAAGAAACCGCAATTTTCCCTGCGATTGCAGCGGTTGGCGGT
  R  E  L  L  E  G  A  L  K  S  K  E  T  A  I  F  P  A  I  A  A  V  G  G
ATGTTAGCTCCTGCTCTTATTTACGTAGCCTTTAATGCCAACGATCCTGAGGCGATTTCTGGTTGGGCAATT
  M  L  A  P  A  L  I  Y  V  A  F  N  A  N  D  P  E  A  I  S  G  W  A  I
CCAGCGGCGACAGATATCGCTTTTGCTTTAGGTATCATCGCGTTATTAGGTAAGCGTGTACCAGTAAGCCTA
  P  A  A  T  D  I  A  F  A  L  G  I  I  A  L  L  G  K  R  V  P  V  S  L
AAAGTGTTCTTGCTAGCGCTTGCTATCATCGATGATTTGGGTGTGGTTGTTATTATCGCGCTATTTTACACC
  K  V  F  L  L  A  L  A  I  I  D  D  L  G  V  V  V  I  I  A  L  F  Y  T
GGTGACCTGTCGACCATGGCGTTGCTTGTTGGCTTCATCATGACTGGTGTCCTATTCATGTTGAATGCGAAG
  G  D  L  S  T  M  A  L  L  V  G  F  I  M  T  G  V  L  F  M  L  N  A  K
GAAGTAACAAAACTTACGCCATACATGATCGTCGGTGCGATCTTATGGTTCGCGGTGCTTAAATCTGGTGTT
  E  V  T  K  L  T  P  Y  M  I  V  G  A  I  L  W  F  A  V  L  K  S  G  V
CACGCAACATTGGCAGGTGTAGTGATTGGTTTTGCTATCCCACTAAAGGGAAAGCAGGGCGAACATTCTCCA
  H  A  T  L  A  G  V  V  I  G  F  A  I  P  L  K  G  K  Q  G  E  H  S  P
CTTAAACATATGGAGCACGCGCTTCACCCATACGTGGCATTTGGTATTCTGCCTCTATTCGCATTTGCCAAT
  L  K  H  M  E  H  A  L  H  P  Y  V  A  F  G  I  L  P  L  F  A  F  A  N
GCGGGGATTTCGTTAGAAGGCGTATCGATGTCAGGTCTAACTTCAATGCTGCCACTCGGTATTGCTTTGGGT
  A  G  I  S  L  E  G  V  S  M  S  G  L  T  S  M  L  P  L  G  I  A  L  G
CTACTGGTTGGTAAGCCTCTAGGTATCTTTACCTTTAGCTGGGCAGCGGTAAAAATGGGCGTTGCTAAGTTA
  L  L  V  G  K  P  L  G  I  F  T  F  S  W  A  A  V  K  M  G  V  A  K  L
CCTGAACGCGTTAACTTCAAGCATATCTTTGCGGTATCTGTGTTGTGTGGTATTGGCTTTACAATGTCTATC
  P  E  G  V  N  F  K  H  I  F  A  V  S  V  L  C  G  I  G  F  T  M  S  I
TTCATTTCCTCTTTAGCGTTCGGAAATGTAAGTCCTGAATTTGATACCTATGCTCGACTCGGTATCTTAATG
  F  I  S  S  L  A  F  G  N  V  S  P  E  F  D  T  Y  A  R  L  G  I  L  M
GGTTCGACGACAGCAGCATTACTTGGCTATGCCTTGCTGCACTTTTCTTTGCCTAAGAAAGCGCAAGCGTAA
  G  S  T  T  A  A  L  L  G  Y  A  L  L  H  F  S  L  P  K  K  A  Q  A  *
GATAAAAATGCGCTATTTTGTAAAGCCTCCCACTCGGGAGGCTTTTTTATACCCATAGTTCAGTACATCAGCGACTTAATAGCG
ACACTTTCCGTATTGAGCGGATGTCTAGACCATGGAGATGAACGGAATCATCGAAAAAGGTAGGTTGGTAGATCTGCGGCGAAAA
AAAGCCCAGCCGAAAATTTGACTGGGCGGATACAAACATAGGAGTTAATAAGAAAAAAGCAGCAACGTAACAATTAGATGGAAA
CAAGTTTGACGGCCAGTGAAACTTAAAATGCCCTAGTTGTTACATTTATTCAGACCGAGCCTTTTCCAAACAGTTCCATTTTCT
TTCAAAAAAATTCGATTTAATTCTTTCTGCCCGCTTGTATCGTAAAGAAGTAAGAGAAGAGGCGAACAGCGTAACCAACTTAAT
GGCGGTTTGAAACTCGATAGCCCTCTTTTCTATAGTAGCTATAAACTTAAATTGCATTTCAAACCCGCACCTCCTGCTCCGACC
GCCCAACGACATTGCTAGCGTTTCAAATACGATGG2295
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Fig. 3. Nucleotide sequence and the deduced amino acid sequence of Na^+/H^+ antiporter from *V. alginolyticus*.

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  1'  MKHLHRFFSSDASGGILLIAAILAMIMANSGATSGWYHDFLETPVQLRVGSLEINKNM
      ..  **.  ...***.*.***  .**..***    ..  *.  *.*            .....
  1"  MNDVIRDFFKMESAGGILLVIAAAIAMTIANSP-LGETYQSVLHT--------YVFGMSV

 60'  LLWINDALMAVFFLLVGLEVKRELMQGSLASLRQAAFPVIAAIGGMIVPALLYLAFNYAD
      ****.********.**********.*.* *    * **.***.***..***.*.*** .*
 52"  SHWINDGLMAVFFLLIGLEVKRELLEGALKSKETAIFPAIAAVGGMLAPALIYVAFNAND

120'  PITREGWAIPAATDIAFALGVLALLGSRVPLALKIFLMALAIIDDLGAIIIIALFYTNDL
      *  .*****************..****.***..**.**.**********...*******.**
112"  PEALSGWAIPAATDIAFALGILALLGKRVPVSLKVFLLALAIIDDLGVVVIIALFYTGDL

180'  SMASLGVAAVAIAVLAVLNLCGARRTGVYILVGVVLWTAVIKSGVHATLAGVIVGFFIPL
      *  .*.*.  . . .** .**  .. . . *..**.** ***************..** ***
172"  STMALLVGFIMTGVLFMLNAKEVTKLTPYMIVGAILWFAVLKSGVHATLAGVVIGFAIPL

240'  KEKHG-RSPAKRLEHVLHPWVAYLILPLFAFANAGVSLQGVTLDGLTSILPLGIIAGLLI
      *.*.*  .** *..**.***.**.  ***********.**.**...****.*****  ***.
232"  KGKQGEHSPLKHMEHALHPYVAFGILPLFAFANAGISLEGVSMSGLTSMLPLGIALGLLV

299'  GKPLGISLFCWLALRLKLAHLPEGTTYQQLMVVGILCGIGFTMSIFIASLAFGSVDPELI
      ******  *.* *... .*.****.....*..*..************.*****.*.**.
292"  GKPLGIFIFSWAAVKMGVAKLPEGVNFKHIFAVSVLCGIGFTMSIFISSLAFGNVSPEFD

359'  NWAKLGILVGSISSAVIGYSWLRVRLRPSV
      ..*.****.**...*..**.  *. .*
352"  TYARLGILMGSITAALLGYALLHFSLPKKAQA
```

Fig. 4. Alignment of the deduced amino acid sequence of NhaA from *E. coli* with that from *V. alginolyticus*. Residues identical to both sequences are indicated with *. Predicted transmembrane segments in NhaA from *V. alginolyticus* are underlined.

the deduced amino acid sequence was found to be 97.4% identical to that from *V. alginolyticus*, implying the presence of the same type of Na^+/H^+ antiporter in marine bacteria.

The NhaA from *E. coli* is electrogenic and has a stoichiometry of 2 H^+ exchanged for each Na^+ [25]. Since the Na^+/H^+ antiport activity of *V. alginolyticus* observed at alkaline external pH is also electrogenic [15], the antiporter of *V. alginolyticus* is functionally similar to *E. coli* NhaA. The expression of *nhaA* gene in *E. coli* has been shown to be regulated by a regulatory protein NhaR [8, 9]. The structural gene corresponding to *nhaR* gene, however, could not be found in the upstream and downstream regions of *nhaA* gene from *V. alginolyticus*. In contrast to the case of *V. alginolyticus*, *E. coli* NhaA starts from GTG [26]. Since *V. alginolyticus* is halophilic and requires 0.5 M NaCl for optimal growth, the Na^+/H^+ antiporter is likely to be constitutively expressed for the extrusion of cellular Na^+ in marine bacteria.

The deduced amino acid sequence of Na^+/H^+ antiporter also has a partial similarity with NhaB [6] and ChaA [7] from *E. coli*, NhaC from *Bacillus firmus* [11], Na^+/H^+ antiporter protein from *Enterococcus hirae* [27], and K^+/H^+ antiporter protein (KefC) from *E. coli* [28]. These informations will help to assign common amino acid residues in the function of Na^+/H^+ antiport activity.

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