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Short sequence-paper

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

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Abstract

A gene has been cloned from the marine bacterium *Vibrio alginolyticus* that functionally complements a mutant strain of *Escherichia coli*, TO114, defective in three Na^+/H^+ antiport genes (*nhaA, nhaB, chaA*). The nucleotide sequence of the cloned fragment revealed an open reading frame, which encodes a protein with a predicted 528 amino acid sequence and molecular mass of 57212 Da. This gene has 62% identity to *nhaB* gene at the DNA level from *Escherichia coli* and the deduced amino acid sequence is 67% identical with *E. coli* NhaB. This gene is presumably the *V. alginolyticus nhaB* gene and will be named *nhaBv*.

Keywords: Sodium ion/proton antiporter; *nhaB* gene; DNA sequence; Marine bacterium; (*V. alginolyticus*)

Na^+/H^+ antiporters are Na^+ transporters which are widely distributed in eukaryotes and bacteria [1,2]. In bacteria, the Na^+/H^+ antiporter functions for Na^+ extrusion and allows cells to grow in high salinity conditions. *Escherichia coli* has three Na^+/H^+ antiporters, NhaA [3], NhaB [4] and ChaA [5], with no good homologies among them. Why *E. coli* has multiple Na^+ transporters with different structures is not clear. The mechanisms of Na^+ extrusion by these three antiporters also remain unknown. One way to identify amino acid residues essential for Na^+ binding and transport is to compare the amino acid sequences of similar Na^+/H^+ antiporters from different origins.

Selection by functional complementation of mutant strains of *E. coli* has been used to obtain *nhaA* genes from several bacterial species [6–8]. Recently, we cloned and sequenced the *nhaA* gene from *V. alginolyticus* [7]. The deduced amino acid sequence was 58% identical with *E. coli* NhaA. The *nhaA* gene has also been found in *Salmonella entiritidis* [6], *Vibrio parahaemolyticus* [8] and *Haemophilus influenzae* [9]. For the latter, the *nhaA* gene was found by homology search after whole-genome sequencing. In all five NhaA proteins, the predicted fourth and fifth membrane-spanning regions have three conserved aspartic acids. We reported that these negatively charged aspartic acid residues localized in the membrane-spanning regions are essential for translocation of Na^+ by NhaA from *V. alginolyticus* [10]. The potential importance of these aspartic acids for the translocation of H^+ by NhaA from *E. coli* was reported by Inoue et al. [11]. In the NhaA of *E. coli*, His-226 was assigned to be a part of a pH sensor, but none of the NhaA histidine residues were essential for the activity of the carrier [12,13]. NhaA from *E. coli* has eight histidines, but only the His-226 and His-254 were conserved among five NhaA proteins from different origins.

The *nhaB* gene is the second Na^+/H^+ antiporter gene from *E. coli* selected by functionally restoring the growth in high salinity medium of *nhaA* deleted *E. coli* strain, NM81 [4,14]. This gene encodes a membrane protein (NhaB) with predicted 504 amino acids and molecular mass of 55500 Da. Hydropathic analysis of the sequence indicates the presence of 12 putative transmembrane helices [4]. This gene was located at 25.6 min on the *E. coli* map [15] and the neighboring sequence was reported in the accession number M83655. NhaB has been considered to be an extra Na^+ extrusion system when NhaA is not activated [16]. The kinetic properties of NhaA and NhaB are different. The stoichiometry of NhaA exchange is 2 H^+ for each Na^+ [17] and that of NhaB exchange is 3 H^+ for 2 Na^+ [18]. The function of NhaB is not clear; although NhaB was reported to be a major Na^+/H^+

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antiporter [15] and essential for pH regulation at alkaline pH medium [19], other reports were that the activity of NhaB was low in high pH medium [17,20]. Thus the role of NhaB remains obscure and more research is required. Although the whole-genome sequence of *H. influenzae* was published recently [9] and the *nhaB* gene found by homology search, the activity of NhaB in *H. influenzae* has not been investigated.

Comparative studies on ion transport genes from different origins are one way of achieving a better understanding of ion transport functions and mechanisms. We have used the marine bacterium *V. alginolyticus*, which has multiple K^+ uptake systems [21–23] and a K^+/H^+ antiporter that has been suggested to function for internal pH regulation [24,25]. Up to now this organism had two known Na^+ extrusion systems, a Na^+-translocating NADH-quinone reductase (Na^+ pump) [26] and a Na^+/H^+ antiporter [7]. The genes for the Na^+ pump were sequenced recently [27,28]. The existence of two Na^+/H^+ antiporters was previously suggested in *Vibrio parahaemolyticus* [29], and this paper reports the presence of a gene in *V. alginolyticus* that is homologous to *nhaB*.

Genomic DNA was isolated from *V. alginolyticus* 138-2, DNA fragments obtained by partial digestion with Sau3AI were size-fractionated by agarose gel electrophoresis, and the fragments from 2 to 4 kbp were ligated in the *Bam*HI site of the plasmid pHG165 [30]. The plasmids were transformed first into *E. coli* NovaBlue (Novagen, USA) by electroporation according to the manual of the Electroporator (Invitrogen, USA). The DNA library was prepared from about 20000 colonies. *E. coli* TO114, defective in 3 Na^+/H^+ antiporters (Δ*nhaA*, Δ*nhaB*, Δ*chaA*) [20], grows in LBK100 medium (1% tryptone, 0.5% yeast extract, 100 mM KCl, 10 mM Tricine and 10 mM Bis-Tris which was adjusted to pH 7.0 with KOH) but is unable to grow in LBNa200 medium (1% tryptone, 0.5% yeast extract, 200 mM NaCl, 10 mM Tricine and 10 mM Bis-Tris (pH 7.6 by KOH)). Therefore, the recombinant plasmids were incorporated into *E. coli* TO114 by electroporation and then screened for the ability to restore growth in LBNa200 medium. After 24 h growth, 9 colonies appeared. The plasmids from these were purified and digested with restriction enzymes. Eight of the plasmids contained the *nhaAv* gene, but the other one, pTN1, did not contain *nhaAv* gene but restored the growth of TO114 in media containing 200 mM NaCl.

The size of pTN1 was about 6.5 kbp including pHG165 (3.3 kbp). The DNA of both strands of the inserted DNA fragment of 3188 bp was sequenced by the dideoxy chain termination method [31] using a taq cycle sequencing kit (Takara, Japan), PCR (model PTC-150, MJ Research, USA) and automated fluorescent DNA sequencer (model DSQ-1000, Shimazu, Japan). Two truncated open reading frames and one complete open reading frame were identified (Fig. 1, accession number D83728). The central complete open frame, starting with the initiation codon ATG

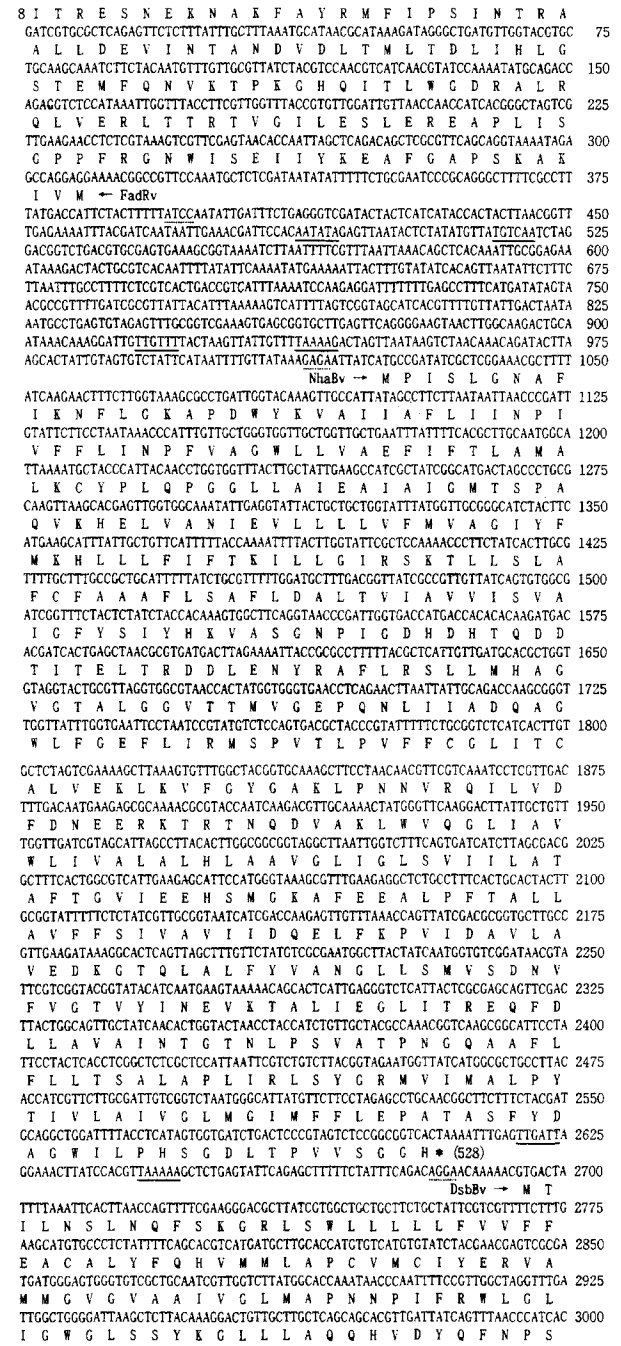

Fig. 1. Nucleotide sequence of the 3188 bp DNA fragment in pTN1. The sequences of *fadRv*, *nhaBv* and *dsbBv* and the deduced amino acid sequences of FadRv (part; complementary strand), NhaBv (complete) and DsbBv (part) are shown. Putative −10 and −30 consensus sequences for *fadRv*, *nhaBv* and *dsbBv* are underlined. Putative ribosome binding sites are dotted underlined. The amino acid sequences are written below the nucleotide sequences for *nhaBv* and *dsbBv* but above them for *fadRv*.

(starts #1024) and terminated by the stop codon CAC extending to 1587 bp, coded for 528 amino acid residues with a molecular mass of 57212 Da. From the homology

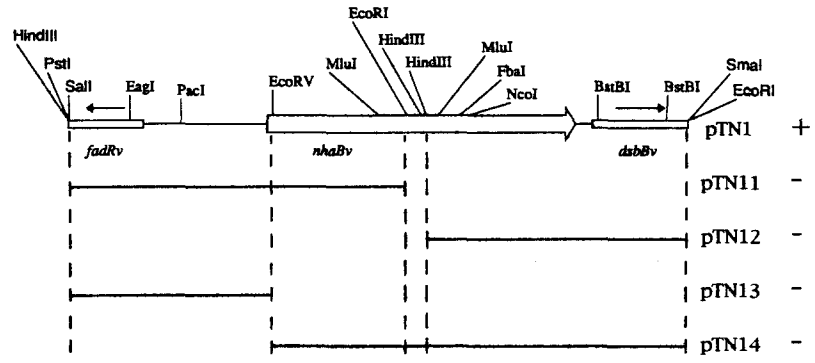

Fig. 2. Subcloning of pTN1. Left and right end restriction sites are in multi-cloning sites of pHG165. Deletion plasmids, pTN11 (*Eco*RI cut and ligation), pTN12 (*Hin*dIII cut and ligation), pTN13 (*Eco*RV and *Sma*I cut and ligation) and pTN14 (*Eco*RV and *Pst*I cut and blunt ligation) were performed. Each of the constructs was screened for the ability to restore the growth of *E. coli* TO114 in LBNa200 medium, and + sign denotes a positive result.

search program, tfasta, at GenomeNet (Kyoto center), this gene is homologous to *nhaB* from *E. coli*. The 5′ side gene has homology to *fadR* [32] and the 3′ side gene has homology to *dsbB* [33] from *E. coli*.

Restriction mapping and subcloning were performed by standard methods [34] (Fig. 2). None of the fragments produced by cutting with *Eco*RV, *Eco*RI or *Hin*dIII could restore the growth of *E. coli* TO114 in LBNa200 medium. This includes pTN14 which lacked only three of the N-terminal amino acids in the structural gene, but contained none of the upstream promoter region. These data showed that the entire *nhaB* homologous gene from *V.*

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NhaBv:MPISLGN--AFIKNFLGKAPDWYKVAIIAFLIINPIVFFLINPFVAGWLLVAEFIFTLAMALKCYPLQPGGLLAI
NhaBh:----MT.IQ..M.....AS.E...L..VV.....F.S..I.....
NhaBe:----------MA....QS.....L.L.I...V..LI ...S.....L.....
                ****  * *** *   *** **   * * ** ********************** *******

:EAIAIGMTSPAQVKHELVANIEVLLLLVFMVAGIYFMKHLLLFIFTKILLGIRSKTLLSLAFCFAAAFLSAFLD
:...I....NAKH..A.IM..F..I...I......F...Q...YV....L.VK....IA..I....S.....
:..VF.....AEH.RE.VA..L.....M.....Q.....RL..S....M....S..V.....
 **  ****    *  *  ** ** *** ******* *** ***  **  *  ****  **  **  *********

:ALTVIAVVISVAIGFYSIYHKVASGNPI-GDHDHTQDDTITELTRDDLENYRAFLRSLLMHAGVGTALGGVTTM
:....V..I....M...GV.....NLIDAV.ISD.RK.I.QQHEI..KF.....M.....V...
:....V.....V...G...R...SR-TE.T.LQD.SH.DKHYKVV..QF.G.....M.....
 **** ** **** ***  ** ***        *   *  *       **  * ***** ********** ***

:VGEPQNLIIADQAGWLFGEFLIRMSPVTLPVFFCGLITCALVEKLKVFGYGAKLPNNVRQILVDFDNEERKTRT
:.....E...M.K.N.M..FL..A...I...I...L..F....F.L....E...DE.WK..S.L.RTNSEKMS
:.....KA..M...H..D.FL.....V..LI...L..L.....RW....ET..EK..EV.QQ..DQS.HQ..
 ******* ** * * *  *  ** *** **  *** ** ****   ****  **  *   *   *

:NQDVAKLWVQGLIAVWLIVALALHLAAVGLIGLSVIILATAFTGVIEEHSMGKAFEEALPFTALLAVFFSIVAV
:K..KI..GM.A...I....G..F.....I..F..S....TD..TI....Q.S.....V....V...
:R..KIR.I..AI.G...VT.....E.....SL...TD..AI....T.S.....T....V...
  **   *  *  *  **   ** *** ******* ** **  ***  **  **** * ******* **** ***


:IIDQELFKPVIDAVLAVEDKGTQLALFYVANGLLSMVSDNVFVGTVYINEVKTALIEGLITREQFDLLAVAINT
:....K..S.I.HF..SA.E-N.....LF.....SI.....A.....A.A..TN.V.APH..E..S.....
:....Q..S.I.QF..QASE-HA..S...IF.....SI.....I....A.A.MES.A..LK.YE.....
 **** ** * *  **       ** ***  *****  ****** * **** * *   * *   *  ** *****

:GTNLPSVATPNGQAAFLFLLTSALAPLIRLSYGRMVIMALPYTIVLAIVGLMGIMFFLEPATASFYDAGWILPH
:.....SIS.....Y.....S.I..LA.E.I.PA..IWLASL.L...I
:.....W.....L..TL...LCVE.T.A.V.EW.MQM...ATL
 ********************  ********** ****** **   **   * *   *     * *

:SGDLTPVVSGGH  NhaBv
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Fig. 3. Comparison of the deduced amino acid sequences of NhaB from *V. alginolyticus* (NhaBv), *H. infuenzae* (NhaBh) and *E. coli* (NhaBe). Each residue identical with NhaBv is indicated by a dot (.) and the gaps inserted are indicated by hyphens (–). The conserved residues are shown by asterisks (*), and the conserved Asp (D) and Glu (E) are additionally underlined.

alginolyticus (which we will call *nhaBv*) is required to restore the salt tolerance.

Upstream of *nhaBv*, there is a *fadR* (fatty acid metabolism gene) homologous gene as in *E. coli* [4,32] and *H. influenzae* [9]. The predicted amino acid sequence of *fadR* gene fragment from *V. alginolyticus* is 72% and 55% identical with that of *E. coli* and *H. influenzae*, respectively.

Downstream from *nhaBv*, is *dsbB* (oxido-reductase gene) as is found in *E. coli* [33] and *H. influenzae* [9]. The predicted amino acid sequence of the DsbB from *V. alginolyticus* is 49%, 47%, and 43% identical with those from *E. coli*, *Shigella flexneri* [35], and *H. influenzae*, respectively. It is interesting to note that *fadR*, *nhaB* and *dsbB* genes line up in the same order in the chromosome of *E. coli*, *H. influenzae*, and *V. alginolyticus*. In *S. flexneri*, part of the *nhaB* gene was also reported to exist upstream of *dsbB* (accession number D38254).

Fig. 3 shows the alignment of the deduced amino acid sequences of NhaB from *V. alginolyticus* (NhaBv), *H. influenzae* (NhaBh) and *E. coli* (NhaBe). Although the numbers of amino acid residues for NhaBv (528), NhaBh (522) and NhaBe (504) were slightly different with each other, NhaBv was 67% and 68% identical with NhaBh and NhaBe, respectively. The number of conserved amino acid residues among the three strains was 313 (about 60%).

It is reasonable to assume that negatively charged amino acid residues localized in the membrane-spanning regions are important for the binding and transport of cations like H^+ and Na^+ [10]. There are 19 aspartic acids and 21 glutamic acids in the NhaBv protein. Of these 40 negatively charged residues, 18 are conserved in the two NhaB proteins from *E. coli* (NhaBe) and *H. influenzae* (NhaBh). Therefore, these negative charges are candidates for site-directed mutagenesis experiments to identify which of these residues are important for the function of NhaB.

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