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A new Na^+/H^+ antiporter, NhaD, of *Vibrio parahaemolyticus*

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Abstract

A gene encoding an Na^+/H^+ antiporter was cloned from chromosomal DNA of *Vibrio parahaemolyticus*, a slightly halophilic bacterium, and expressed in *Escherichia coli* cells. The gene enabled mutant *E. coli* cells, which were unable to grow in the presence of 10 mM LiCl (or 0.2 M NaCl) because of the lack of major $Na^+(Li^+)/H^+$ antiporters, to grow under such conditions. We detected Na^+/H^+ antiport activity due to the gene in membrane vesicles prepared from *E. coli* cells that harbored the plasmid carrying the gene. Li^+ was also a substrate for this antiporter. Activity of this antiporter was pH-dependent with highest activity at pH 8.5 to 9 and no activity at 7.0 to 7.5. Restriction mapping and a Southern blot analysis revealed that the cloned gene was different from the *nhaA* and the *nhaB* of *V. parahaemolyticus*. We designated the gene *nhaD*. The gene was sequenced, and the amino acid sequence of the NhaD protein was deduced. The NhaD is a unique Na^+/H^+ antiporter with respect to the primary structure compared with known Na^+/H^+ antiporters. © 1998 Elsevier Science B.V.

Keywords: Na^+/H^+ antiporter; NhaD

1. Introduction

The Na^+/H^+ antiporter plays important roles in cell physiology not only in bacterial cells but also in animal cells [1–3]. In bacterial cells, the Na^+/H^+ antiporter is involved in: (1) extrusion of Na^+ and Li^+ from cells, which are toxic when present in cells at high concentrations [1,2,4], (2) establishment of Na^+-motive force (an electrochemical potential of Na^+ across cell membranes) which is the driving force for Na^+-coupled active transport (melibiose, serine, proline, and so on) [5–7] or Na^+-coupled flagellar rotation [8], and (3) intracellular pH regulation under alkaline conditions [1,2]. So far, several structurally distinct genes that encode Na^+/H^+ antiporters have been identified, and properties of such antiporters have been reported. The genes include *nhaA* [9], *nhaB* [10] and *chaA* [11] from *Escherichia coli*, *nhaC* from alkaliphilic *Bacillus firmus* [12], *napA* from *Enterococcus hirae* [13], and so on.

We have previously characterized the Na^+/H^+ antiporters in membrane vesicles of *Vibrio parahaemolyticus*, a slightly halophilic marine bacterium [14]. This micro-organism requires at least 10 to 30 mM NaCl for growth. Although it seems that Na^+ plays an important role in energy transduction in this organism [15], the reason for this Na^+ requirement is not clear yet. Subsequently, we have cloned two genes encoding the Na^+/H^+ antiporters, and se-

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quenced them [16,17]. Judging from the deduced amino acid sequences and properties of these antiporters, one was the counterpart of the NhaA Na^+/H^+ antiporter and another was the counterpart of the NhaB Na^+/H^+ antiporter of *E. coli*. The genes cloned from chromosomal DNA of *V. parahaemolyticus* were expressed in *E. coli* cells which lacked major Na^+/H^+ antiporters, and the produced Na^+/H^+ antiporters were characterized in membrane vesicles prepared from cells of the *E. coli* transformant. During the course of our studies on Na^+/H^+ antiporters, we obtained another type of gene which seemed to encode a new Na^+/H^+ antiporter of *V. parahaemolyticus*. Here we report the sequence and properties of the third Na^+/H^+ antiporter (NhaD) of *V. parahaemolyticus*.

2. Materials and methods

V. parahaemolyticus AQ3334 (wild type), *E. coli* TG1 and *E. coli* KNabc (Δ*nhaA*, Δ*nhaB*, Δ*chaA*) [17], a mutant derived from TG1, were used. Cells of *V. parahaemolyticus* were grown in Luria-Bertani (LB) medium [18], and *E. coli* cells were grown in L(KCl) medium [19], in which NaCl was replaced with KCl, at 37°C under aerobic conditions. Cell growth was monitored turbidimetrically at 650 nm.

Chromosomal DNA was prepared from the cells of *V. parahaemolyticus* by the method of Berns and Thomas [20]. The DNA was partially digested with the restriction enzyme *Sau*3A1 and fragments with 4 to 10 kbp were separated by sucrose density gradient centrifugation. The DNA fragments were ligated into pBR322 (which had been digested with *Bam*HI and dephosphorylated with bacterial alkaline phosphatase) by using T4 DNA ligase. Competent cells of *E. coli* KNabc were transformed with the ligated hybrid plasmids and were spread on agar plates consisting of L(KCl) broth, 3 mM LiCl, 40 μg/ml ampicillin and 1.5% agar. The plates were incubated at 37°C for 24 h and the colonies formed were picked up. Plasmids were prepared from the transformants, and competent cells of *E. coli* KNabc were retransformed and spread on the plates again. The plates were incubated at 37°C for 24 h. Plasmids contained in the retransformants were prepared. We obtained 49 candidate hybrid plasmids. Judging from their restriction patterns, 36 plasmids seemed to carry the *nhaA* gene, 8 plasmids seemed to carry the *nhaB* gene, and 5 plasmids seemed to carry one or more new genes. Three of the latter five plasmids (designated pLND722, pLND731 and pLND803) seemed to carry a common DNA region derived from *V. parahaemolyticus* DNA. These 3 plasmids were further characterized. Deletion plasmids for sequencing were constructed using exonuclease III and mung bean nuclease from pLND731 which possessed the shortest DNA insert. The nucleotide sequence was determined by the dideoxy chain termination method [21] using a DNA sequencer (Pharmacia Biotech, ALF express). Sequencing of both sense and antisense strands was completed. The DNA sequence data reported in this paper have been submitted to DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB006008.

Sequence data were analyzed with the GENETYX sequence analysis software (Software Development). The SwissProt and Genbank databases were screened for sequence similarities.

Chromosomal DNA prepared from *V. parahaemolyticus*, *V. alginolyticus* or *E. coli*, or plasmid DNA of pLND722, pLND731, pKNN64 (this plasmid carries the *nhaB* gene of *V. parahaemolyticus*) [17] or pTIS216 (this plasmid carries the *nhaA* gene of *V. parahaemolyticus)* [16] was digested with restriction enzymes *Eco*RI and *Dra*I, and the fragments were separated by electrophoresis in a 1% agarose gel and blotted onto a nitrocellulose membrane. The probe (a *Eco*RI-*Dra*I fragment, 0.7 kbp) was prepared from pLND731. The probe was hybridized with the DNA blot on the nitrocellulose membrane, and detected with the Enhanced Chemiluminescence Detection (ECL) system (Amersham) as suggested by the manufacturer.

Everted membrane vesicles were prepared from cells of *E. coli* KNabc and KNabc/pLND731 by the French press method as described previously [16]. The Na^+/H^+ antiporter activity was measured by the quinacrine fluorescence quenching method [16] at pH 8.5 unless otherwise indicated.

Protein contents were determined by the method of Lowry et al. [22].

Reagents for DNA manipulation and sequencing, bacteriologi-cal media and other chemicals were obtained from the usual commercial sources.

3. Results

3.1. Cloning of Na^+/H^+ antiporter gene

Wild type cells of *E. coli* possess three major Na^+/H^+ antiporters (NhaA, NhaB and ChaA) which extrude Na^+ from cells [9–11]. Mutant cells (KNabc) that lack all the three of these Na^+/H^+ antiporters cannot survive in the presence of 3 mM LiCl (or 0.2 M NaCl) because the cells cannot exclude Li^+ (or Na^+). We tried to clone Na^+/H^+ antiporter gene(s) as described under Section 2. We obtained several types of candidate genes that enabled the KNabc cells to grow in the presence of 3 mM LiCl, judging from the restriction maps of the hybrid plasmids. Two of the types seemed to be identical to the *nhaA* gene [16] and the *nhaB* gene [17], which had been cloned and sequenced previously in our laboratory. The third type of gene seemed to be a new one. We obtained three hybrid plasmids carrying the third type of gene, and designated them pLND722, which carried the longest DNA insert, pLND803, and pLND731 which carried the shortest DNA insert. We characterized these plasmids. Growth of cells of KNabc/pLND722, KNabc /pLND803 and KNabc/pLND731 in liquid medium in the presence of NaCl or LiCl was monitored (Fig. 1). Cells of KNabc/pLND722 and KNabc/pLND731 grew well in the presence of 0.2 M NaCl, although cells of KNabc/pBR322 did not. Cells of KNabc/pLND803 showed moderate growth in the presence of 0.2 M NaCl. After the growth in the presence of 0.2 M NaCl, the optical density of the culture medium of KNabc /pLND722 and of KNabc/pLND731 cells decreased with time (Fig. 1(A)). The reason for this decrease is not yet known. Cells of KNabc/pLND722, of KNabc/pLND731 and of KNabc/pLND803 grew well also in the presence of 10 mM LiCl, although cells of KNabc/pBR322 did not. A decrease in the optical density after growth was not observed when 10 mM LiCl was added to the culture medium with all of the transformant cells

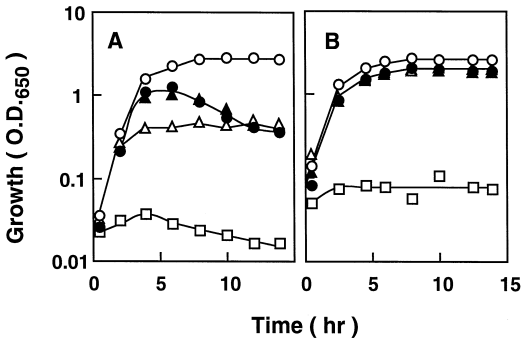

Fig. 1. Effects of various plasmids on the growth of *E. coli* cells in the presence of NaCl or LiCl. Cells of *E. coli* TG1 (○), KNabc/pLND722 (●), KNabc/pLND803 (△), KNabc/pLND731 (▲) or KNabc/pBR322 (□), were shaken in L(KCl) medium in the presence of either 0.2 M NaCl (A) or 10 mM LiCl (B) at 37°C under aerobic conditions.

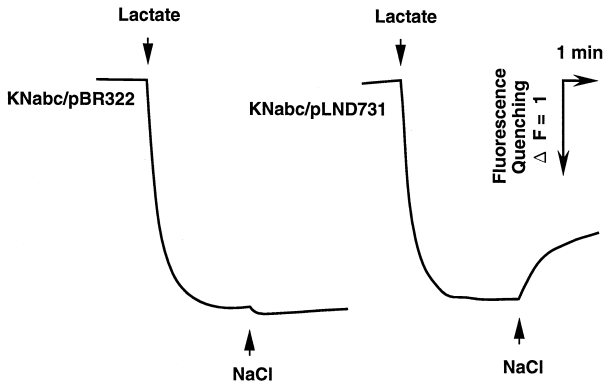

Fig. 2. Na^+/H^+ antiporter activity measured by the quinacrine fluorescence quenching method. Everted membrane vesicles were prepared from cells of *E. coli* KNabc/pBR322 or KNabc/pLND731. At the time points indicated by downward arrows, potassium lactate (final concentration 5 mM) was added to initiate fluorescence quenching due to inward movement of H^+ caused by respiration. Then, at the time points indicated by upward arrows, NaCl (final concentration 5 mM) was added to the assay mixture. pH of the assay mixture was 8.5. Fluorescence quenching is shown in arbitrary units.

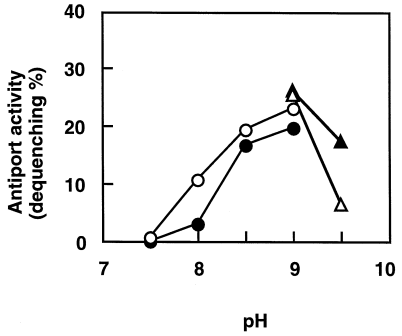

Fig. 3. pH profiles of the Na^+/H^+ antiport and the Li^+/H^+ antiport. The antiport activities were measured by the quinacrine fluorescence quenching method with everted membrane vesicles prepared from KNabc/pLND731. Na^+/H^+ antiport activity (open symbols) and Li^+/H^+ antiport activity (closed symbols) were measured at the indicated pHs. Buffers used were Tricine–KOH (circle) and Ches–KOH (triangle).

(Fig. 1(B)). In any case, the results described above suggest that the gene carried on these plasmids encodes a new Na^+/H^+ antiporter of *V. parahaemolyticus*.

3.2. Na^+/H^+ antiport activity in membrane vesicles

Quinacrine fluorescence quenching is a convenient method for measuring Na^+/H^+ antiporter activity

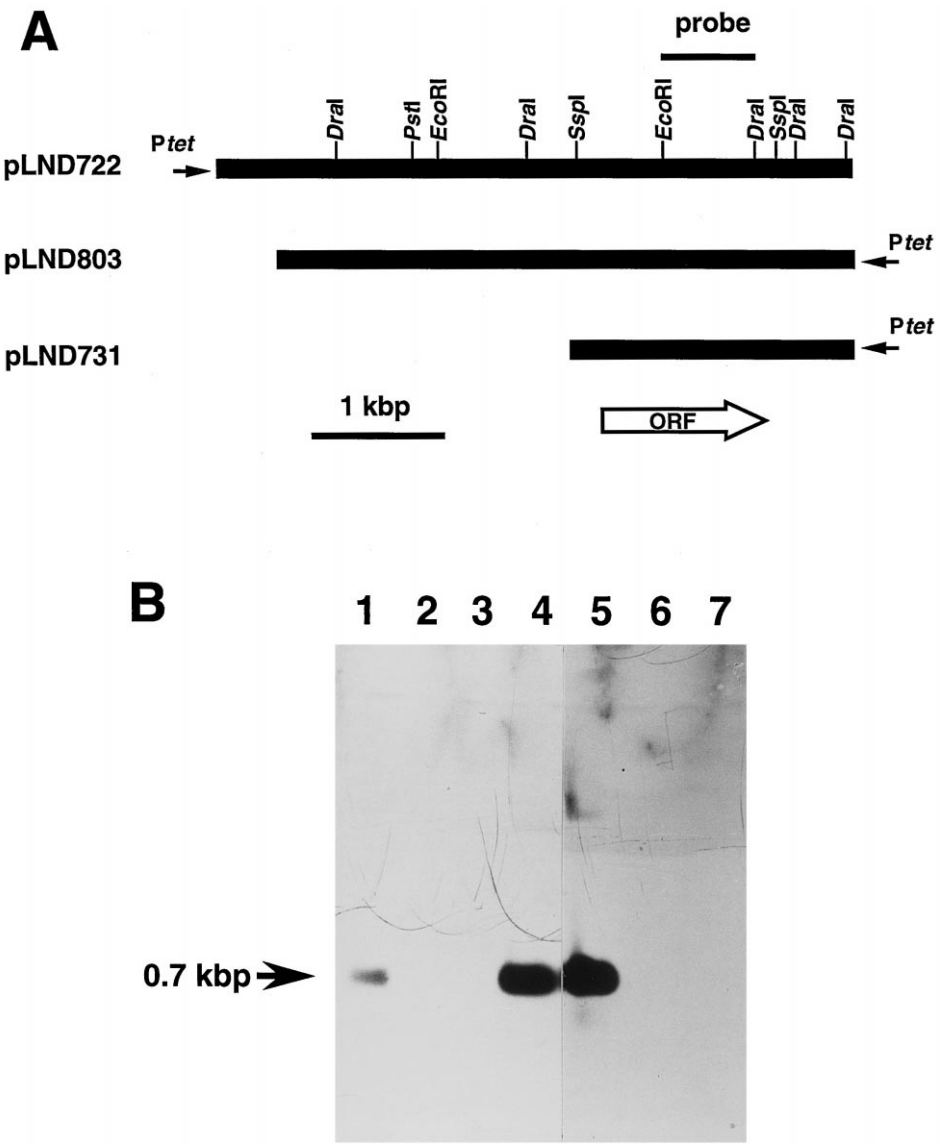

Fig. 4. Plasmids and restriction maps of cloned DNA containing Na^+/H^+ antiporter gene, and Southern hybridization analysis. (A) Physical maps of DNA insert derived from the *V. parahaemolyticus* chromosome in pLND722, pLND803 and pLND731 are shown. Inserts are aligned; restriction sites are present at the same horizontal position in each insert. Positions and directions of the promoter for the *tet* gene derived from the vector pBR322 in each plasmid are shown by arrow with P*tet*. Location and direction of an open reading frame (ORF) which was revealed by sequencing is shown at the bottom. The probe (*Eco*RI-*Dra*I fragment) used for the Southern blot analysis is shown at the top. (B) Southern analysis. Chromosomal DNA prepared from *V. parahaemolyticus* (lane 1), *V. alginolyticus* (lane 2) or *E. coli* (lane 3), or plasmid DNA of pLND722 (lane 4), pLND731 (lane 5), pKNN64 (carries the *nhaB* gene of *V. parahaemolyticus*) (lane 6) or pTIS216 (carries the *nhaA* gene of *V. parahaemolyticus*) (lane 7) was digested with restriction enzymes *Eco*RI and *Dra*I, separated by electrophoresis in an agarose gel, and blotted onto a nitrocellulose membrane. The probe was hybridized with the DNA blot on the nitrocellulose membrane, and detected with the ECL system (Amersham).

[23,24]. Everted membrane vesicles were prepared from cells of *E. coli* KNabc/pBR322 and KNabc/pLND731, and the Na^+/H^+ antiport activity was measured by the fluorescence quenching method. As shown in Fig. 2, although membrane vesicles of KNabc/pBR322 did not show significant Na^+/H^+ antiport activity, vesicles of KNabc /pLND731 showed Na^+/H^+ antiport activity at pH 8.5. Thus, it became clear that pLND731 carried an Na^+/H^+ antiporter gene, that the gene was expressed in *E. coli* cells, and that the product Na^+/H^+ antiporter is active. We also detected Li^+/H^+ antiport activity with membrane vesicles of KNabc/pLND731 although the activity was smaller than that of Na^+/H^+ antiport (data not shown). Thus, this antiporter transports Li^+ in addition to Na^+ as a substrate. These results are consistent with the growth curves of KNabc/pLND731 cells in the presence of Na^+ or Li^+ (Fig. 1). Other cations, K^+ and Ca^{2+} were not substrates for this antiporter (data not shown).

Although we detected Na^+/H^+ and Li^+/H^+ antiport activities at pH 8.5, no activity was detected at pH 7.0 or 7.5 (Fig. 3). The maximum activity was observed at pH 8.5 to 9.0 (Fig. 3). Lower activities were observed with either Na^+ or Li^+ as a substrate at pH 9.5. Thus, the Na^+/H^+ antiporter derived from

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   1  AATATTTCTCCAACAAGACAAGGGAGAAATGTCTATGCCAATCTATCCTATTGTGGCTTTCTGCTGTTTGTTGTTCTCAAGTTCCAGCTT

  91  TGCAGCGGCATCCGGTGATCTTAATCTGGTGAACTCCGCGTAGGCTATATCGCTCTCATTCTTTTCTCGATCGCTTATATTCTGGTCATG
      -35(1)          -10(1)                  -35(2)          -10(2)
 181  CTCGAAGAGTATTTGAAGATGAGAAAATCCAAACCTGTACTTCTCGCGGCAGGTCTGATTTGGATTTTGATTGGTTACACCTTCGCCCAG  24
       SD                 M  R  K  S  K  P  V  L  L  A  A  G  L  I  W  I  L  I  G  Y  T  F  A  Q
 271  CACCACCAGCAAGACGTGGCAAAAGCGGCGTTGGAACATAACCTACTTGAGTACGCCGAGCTGCTGCTGTTTTTGCTCGTCGCGATGACC  54
       H  H  Q  Q  D  V  A  K  A  A  L  E  H  N  L  L  E  Y  A  E  L  L  L  F  L  L  V  A  M  T
 361  TACATAAACGCGATGGAAGAGCGCAAACTCTTTGATGCTCTACAAGCGTGGATGGTCGGGAAAGGGTTCGGGTTCAAAAAACTGTTTTGG  84
       Y  I  N  A  M  E  E  R  K  L  F  D  A  L  Q  A  W  M  V  G  K  G  F  G  F  K  K  L  F  W
 451  CTGACAGGCTTTCTGGCGTTTGTTATCTCTCCAATCGCCGATAACTTAACCACGGCCCTTTTGATGTGCGCGGTGGTAATGAAAGTGTCT  114
       L  T  G  F  L  A  F  V  I  S  P  I  A  D  N  L  T  T  A  L  L  M  C  A  V  V  M  K  V  S
 541  GGCGACAATCCTAGGTTCGTCAATCTTGCTTGCATCAACATCGTTATCGCAGCCAACGCGGGCGGGGCATTCAGCCCATTTGGTGACATT  144
       G  D  N  P  R  F  V  N  L  A  C  I  N  I  V  I  A  A  N  A  G  G  A  F  S  P  F  G  D  I
 631  ACAACCTTGATGGTTTGGCAAGCCGGGCACGTGCGGTTCTCTGAATTCATGCCTTTGTTTGTCCCTTCTCTAATAAACTATGTCGTGCCA  174
       T  T  L  M  V  W  Q  A  G  H  V  R  F  S  E  F  M  P  L  F  V  P  S  L  I  N  Y  V  V  P
 721  GCGTTTTTAATGGCCCTTTTTGTACCGAACACCAAACCCAATACCATCCACGAGCATGTCGAATTAAAACGTGGCGCAAGGCGCATTGTG  204
       A  F  L  M  A  L  F  V  P  N  T  K  P  N  T  I  H  E  H  V  E  L  K  R  G  A  R  R  I  V
 811  CTGCTGTTTGTCCTCACGATCGCTACGGCTGTCTCCTTCCATGCTGTTCTTCACTTCCCACCCGTGGTTGGCATGATGATGGGCTTGGCG  234
       L  L  F  V  L  T  I  A  T  A  V  S  F  H  A  V  L  H  F  P  P  V  V  G  M  M  M  G  L  A
 901  TACCTACAATTCTTCGGGTACTTCTTAAGAAAAACCCTCAAACATTCGCTCGCGAAAAAAGCGGCCATGGCGATTGCGAATGGGGACGAT  264
       Y  L  Q  F  F  G  Y  F  L  R  K  T  L  K  H  S  L  A  K  K  A  A  M  A  I  A  N  G  D  D
 991  CATGCGCTGAAACGTTTAGGCTCTGTCGTACCGTTTGACGTATTCCATCGTGTTTCTCGCGCCGAATGGGATACCTTGCTGTTCTTCTAT  294
       H  A  L  K  R  L  G  S  V  V  P  F  D  V  F  H  R  V  S  R  A  E  W  D  T  L  L  F  F  Y
1081  GGCGTCGTGATGTGCGTAGGTGGCTTGAGCTTGCTCGGTTATCTCGAATTGGTTTCAAACGTCATGTACACCCAATGGAATCCGGTTTGG  324
       G  V  V  M  C  V  G  G  L  S  L  L  G  Y  L  E  L  V  S  N  V  M  Y  T  Q  W  N  P  V  W
1171  GCGAACGTGATGGTCGGTGTGCTTTCTGCCATTGTCGATAACATTCCGGTCATGTTTGCGGTACTAACGATGGACCCAAGCATGTCCACT  354
       A  N  V  M  V  G  V  L  S  A  I  V  D  N  I  P  V  M  F  A  V  L  T  M  D  P  S  M  S  T
1261  GGTAACTGGTTGCTTGTTACGCTCACTGCGGGCGTAGGGGGTAGTTTGTTGTCGATTGGTTCTGCCGCGGGTGTGGCTTTAATGGGCGCA  384
       G  N  W  L  L  V  T  L  T  A  G  V  G  G  S  L  L  S  I  G  S  A  A  G  V  A  L  M  G  A
1351  GCGCGTGGCCAATACACTTTCTTTGGCCATTTAAAATGGACGCCCGTGATCGCTCTGGGCTACGCGCCAGTATTGCCGCTCATTTGTGGA  414
       A  R  G  Q  Y  T  F  F  G  H  L  K  W  T  P  V  I  A  L  G  Y  A  P  V  L  P  L  I  C  G
1441  TGAACGGCGGCTTGTTTACCTAATCCCAATGACCCCCAATGACACGCCTAGCTACTTAGTCTAACGATCCGATGGATGGTTAACGCCATC

1531  CATCACTTCAATATTACCCAATTCGTACGGGTTAACGCTCTCTAAACATCCCACGTTGTAGCCGTACTCACTTGGGTCTGATCGGCGTTG

1621  ATGGTGCGTGTAAATGCCACATTCACTACAGAAAAAATGCTTCGCGGTATGCGTATTAAATTGGTAGAGCTTTAAAACATCGTCACCTTG
1711  AACGATACGAATACCACTCAAAGGGACTGAAGCCACAATCGCACCTCGACGACGACACATAGAGCAATCACAGCGTCTCGGCTTTTCAAT
1801  TCCGTTGGGTAACGTCAGTTCCAGTTCGACTTTTCCGCAGTGACAGGTCAGTCGATGCAGGGGGTTAATTTGAGTGTTGCCAACTTGCTT
1891  AATCATCGCTTTCCcTGGTGGTTTTAATTCATGTGTACATTATGTTAAACAGCTTAACATCATCATAATCTTGTCCAAAAACTCGACATT
1981  GGTCACTTTTCGCGACATTTTATCGGTCGATTTATTAGTCGCTATTTTGGTGACTGTTTACCGCGTTCGATAGACAGATTGTCTCCGGCG
2071  CGGTTTT
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Fig. 5. Nucleotide sequence of the gene for an Na^+/H^+ antiporter of *V. parahaemolyticus* and its flanking regions, and the deduced amino acid sequence. The DNA sequence is numbered on the left and displayed above the deduced amino acid sequence, which is numbered on the right. Two possible promoter sequences (two pairs of -35 and -10), a putative Shine-Dalgarno sequence (SD), the putative initiation ATG, the putative termination TGA and an inverted repeat following the ORF are underlined.

the cloned gene is a pH-dependent type antiporter, inactive at neutral pHs and active at alkaline pHs.

3.3. Restriction map and southern blot analysis

We constructed restriction maps of the plasmids pLND722, pLND803 and pLND731 (Fig. 4(A)). The maps, which were the same for all the three inserts, were very different from those of the DNA regions including the *nhaA* [16] or the *nhaB* [17] of *V. parahaemolyticus*. Thus, we believed that the Na^+/H^+ antiporter we analyzed in this paper was different from the NhaA or the NhaB we reported previously [16,17].

To ensure that the DNA regions in the plasmids pLND722 and pLND731 are from the chromosomal DNA of *V. parahaemolyticus*, and to test whether a similar gene is present in some other micro-organisms, we performed a Southern hybridization analysis. The DNA probe used in this study is from the insert DNA of the plasmid pLND731, and is indicated in Fig. 4(A). The DNA strongly hybridized with pLND722 and pLND731(Fig. 4(B)). We also observed a hybridized band with chromosomal DNA from *V. parahaemolyticus*. However, no hybridized band was detected with chromosomal DNA from *E. coli* or DNA from *V. alginolyticus*. These results suggest that no homolog of this new Na^+/H^+ antiporter is present in *E. coli* or in V. *alginolyticus* which is closely related to *V. parahaemolyticus* [25]. We detected no hybridized band in plasmid pTIS216 which carries the *nhaA* of *V. parahaemolyticus* [16] or plasmid pKNN64 which carries the *nhaB* of *V. parahaemolyticus* [17]. Thus, the gene carried on pLND731 and which encodes an Na^+/H^+ antiporter is different from the *nhaA* and *nhaB* of *V. parahaemolyticus*.

3.4. Sequence and its characteristics

We constructed a series of deletion plasmids from pLND731, which possessed the shortest functional DNA insert among the candidate hybrid plasmids, and determined the nucleotide sequence (Fig. 5). We found one open reading frame (ORF) preceded by a Shine-Dalgarno sequence [26]. We designated this ORF *nhaD*. Two promoter-like sequences were found in the upstream region of the *nhaD*, and one terminator-like sequence was found in the downstream region.

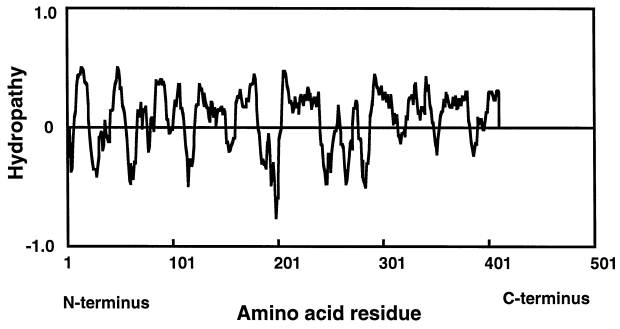

Fig. 6. Hydropathy pattern of the NhaD Na^+/H^+ antiporter. Hydropathy values were calculated by the method of Eisenberg et al. [27] along the deduced amino acid sequence of the NhaD, and the values were plotted from the N-terminus to the C-terminus. Portions above and below the midpoint line indicate hydrophobic and hydrophilic regions, respectively.

The deduced amino acid sequence of 414 residues was obtained for the NhaD (Fig. 5) and the calculated molecular weight was 45 336 Da. Of the 414 residues, 67% were hydrophobic, 16% were neutral and 17% were hydrophilic. This is consistent with the idea that the Na^+/H^+ antiporter is an integral membrane protein. The most abundant amino acid residue of this protein was Leu (57 residues out of 414), followed by Ala (46 residues) and Val (44 residues). The least abundant residues were Cys (4 residues) and Trp (9 residues). Of the 414 amino acid residues, 49 were charged. Twenty eight of these were basic, and 21 were acidic, resulting in an excess of 7 positively charged residues.

The hydropathy profile obtained by the method of Eisenberg et al. [27] suggested the presence of 10 to 12 membrane-spanning domains in the NhaD Na^+/H^+ antiporter (Fig. 6). A similar analysis by the Kyte-Doolittle method [28] gave similar results (data not shown).

4. Discussion

Previously, we reported some of the properties and the sequences of the two Na^+/H^+ antiporters of *V. parahaemolyticus* [16,17]. They were counterparts of two major Na^+/H^+ antiporters of *E. coli*, NhaA [9] and NhaB [10]. We cloned and sequenced a gene for the third Na^+/H^+ antiporter, NhaD, of *V. parahaemolyticus*, and characterized the antiporter. The

sequence and properties of this antiporter were very different from those of the third Na^+/H^+ antiporter, ChaA [11], of *E. coli*. The ChaA Na^+/H^+ antiporter did not utilize Li^+ as a substrate [11]. On the other hand, Li^+ is a good substrate for the NhaD Na^+/H^+ antiporter. Our results indicate that Na^+ is a better substrate than Li^+ for the NhaD, and K^+ and Ca^{2+} are not substrates for this antiporter.

V. parahaemolyticus and *V. alginolyticus* are closely related micro-organisms [25], and both the *nhaA* gene and the *nhaB* gene have been identified in the two micro-organisms [16,17,29,30]. It seems, however, that the third Na^+/H^+ antiporter gene, *nhaD*, is present only in *V. parahaemolyticus* but not in *V. alginolyticus*, judging from the result of the Southern analysis.

The maximum Na^+/H^+ antiport activities in the NhaAs of *V. parahaemolyticus* and *E. coli*, and in the NhaB of *V. parahaemolyticus* were observed at pH 8.5, and no activity was detected at pH 7.0 with the three systems [16,17,31]. The NhaB system of *E. coli* is active both at pH 7.0 and 8.5 [31]. The NhaD system showed no activity at pH 7.0 to 7.5, and maximum activity at pH 8.5 to 9.0. Thus, all of the Na^+/H^+ antiporters so far found in *V. parahaemolyticus* have similar pH profiles. Although we do not know the physiological significance of the pH profiles yet, this may suggest the involvement of these antiporters in intracellular pH homeostasis under alkaline conditions, in the slight alkaliphilicity of *V. parahaemolyticus*, or in the establishment of the Na^+-motive force under alkaline conditions. As reported previously, *V. parahaemolyticus* cells possess a respiratory Na^+ pump which is active only under alkaline conditions [32]. Thus, it seems that Na^+ extrusion under alkaline conditions is very important for cells of *V. parahaemolyticus*.

The His residue at position 225 of *E. coli* NhaA has been implicated to be involved in pH sensing [33,34]. The NhaD of *V. parahaemolyticus* shows a similar pH-activity profile with the NhaA, and possesses similar sequences, KTXXHXLA (from 245 to 252 of NhaD) vs. KSXXHXXLA (from position 221 to 229 of NhaA). Thus, His249 of the NhaD may be involved in pH sensing.

The number of basic residues exceeds the number of acidic residues in the NhaD, as described above. The following Na^+/H^+ antiporters are rich in acidic residues compared with basic residues: NhaA [16] and NhaB [17] of *V. parahaemolyticus*, NhaB [10] and ChaA [11] of *E. coli*, NapA of *E. hirae* [13], NhaC of *B. firmus* [12], and human NHE [35]. Since the Na^+/H^+ antiporter transports cations (Na^+, Li^+ and H^+), acidic residues may be important for the cation recognition or translocation. Another exception was the NhaA of *E. coli* (9, SwissProt), which is rich in basic residues. Recently, the membrane topology of the *E. coli* NhaA has been determined [34]. According to the topology, 6 charged residues are present in the transmembrane domains, 4 are acidic and 2 are basic. Acidic residues in the transmembrane domains of the NhaA are obviously important for its function [36]. Such residues may be involved in cation binding or translocation. At present, we do not know the membrane topology of the NhaD. However, 3 acidic residues and 2 basic residues are in hydrophobic regions which may be trans-membrane domains in the NhaD. Such acidic residues may be important for cation transport in the NhaD.

A search of protein sequence databases found that the sequence of the NhaD showed some similarity to that of several Na^+/H^+ antiporters and some other proteins. There were 18% identity and 59% similarity throughout (365 residues) the NhaD and the NhaA of *V. parahaemolyticus*, 16% identity and 58% similarity (in 345 residues) with NapA of *E. hirae*, 17% identity and 61% similarity (in 318 residues) with human NHE-1. The highest identity and similarity were found with a 46 kDa membrane protein of *Mycobacterium leprae*, 23% identity and 63% similarity (357 residues) (SwissProt). Thus, this protein may be an Na^+/H^+ antiporter. Similar values were obtained with a putative phosphate transport protein of *Mycoplasma genitalium* (19% identity and 62% similarity in 314 residues).

Also the homology search of the sequence databases revealed that no homolog of the NhaD was present in *E. coli* or in *Haemophylus influenzae* [37], whose entire genomes have been sequenced, although NhaA and NhaB (or their homologs) are present in these micro-organisms.

So far we have found three Na^+/H^+ antiporters in *V. parahaemolyticus*. Although the physiological roles of each of the three antiporters are not clear yet, NhaA has the strongest activity, NhaB has the next strongest activity, and NhaD has the lowest activity

when measured by the quinacrine fluorescence quenching method (data not shown). Disruption of these genes may reveal roles of these antiporters in *V. parahaemolyticus*. Also, the reason why multiple Na^+/H^+ antiporters are present in *V. parahaemolyticus* membranes remains unclear.

It should be pointed out that the promoter-like sequence [38] found in the region upstream from *nhaD* must be functional in *E. coli*. The reason for this is that the promoter for the *tet* gene (P*tet* in Fig. 4), which is derived from the vector pBR322 is present in the downstream region from *nhaD* in the opposite direction in pLND731 (Fig. 4). The *nhaD* in the pLND731 is still functional.

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