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NhaA, an Na^+/H^+ Antiporter Involved in Environmental Survival of *Vibrio cholerae*

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***Vibrio cholerae*, the agent of cholera, is a normal inhabitant of aquatic environments, in which it survives under a wide range of conditions of pH and salinity. In this work, we identified the *nhaA* gene in a wild-type epidemic strain of *V. cholerae* O1. *nhaA* encodes a protein of 382 amino acids that is very similar to the proteins NhaA of *Vibrio parahaemolyticus*, *Vibrio alginolyticus* (~87% identity), and *Escherichia coli* (56% identity). *V. cholerae* NhaA complements an *E. coli nhaA* mutant, enabling it to grow in 700 mM NaCl, pH 7.5, indicating functional homology to *E. coli* NhaA. However, unlike *E. coli*, the growth of a *nhaA*-inactivated mutant of *V. cholerae* was not restricted at various pH and NaCl concentrations, although it was inhibited in the presence of 120 mM LiCl at pH 8.5. Nevertheless, using a *nhaA′-lacZ* transcriptional fusion, we observed induction of *nhaA* transcription by Na^+, Li^+, and K^+. These results strongly suggest that NhaA is an Na^+/H^+ antiporter contributing to the Na^+/H^+ homeostasis of *V. cholerae*. *nhaA*-related sequences were detected in all strains of *V. cholerae* from the various serogroups. This gene is presumably involved in the survival and persistence of free-living bacteria in their natural habitat.**

Vibrio cholerae is a normal inhabitant of aquatic environments, one of the bacterial species of the free-living flora found in estuarine areas (11). *V. cholerae* is the agent of cholera, a severe human diarrheal disease transmitted mainly by contaminated water and food. Cholera causes major outbreaks of disease worldwide, particularly under conditions of poverty and poor sanitation (2). Most epidemic strains of *V. cholerae* are clonal and belong to serogroup O1, but a new epidemic strain, O139, has recently emerged that is derived from the pandemic strain, O1, through a complex chromosomal rearrangement (4, 5, 13, 14, 47, 51). In contrast, most environmental strains of *V. cholerae* are nonpathogenic, and more than 155 serogroups have been described (3, 44, 45; T. Shimoda, G. B. Nair, B. C. Deb, M. J. Albert, R. B. Sack, and Y. Takeda, Letter, Lancet 341:1347, 1993). The estuarine environment is an ideal setting for the survival and persistence of *V. cholerae*, and the natural niche of this microorganism may well include crustaceans and molluscs, as part of the zooplankton (10, 42). *V. cholerae* is a halotolerant microorganism whose growth is stimulated by sodium, and it survives under a wide range of conditions of salinity and pH. *V. cholerae* strains are mostly isolated from environmental sites with NaCl concentrations between 0.2 and 2.0% (11), and bacteria survive in vitro in 0.25 to 3.0% salt, the optimal salinity being 2.0% (25). The pH of seawater is between 7 and 8, and the optimal pH for survival ranges from 7.0 to 9.0, depending on salinity (25). Resistance and survival in saline aquatic habitats may play a key role in the persistence of cholera and the emergence of new epidemics. Indeed, there is a close relationship between the titers of *V. cholerae* O1 and the temperature and salinity of estuary water (6, 10). The end of outbreaks in Bangladesh usually coincides with the beginning of the monsoons, when the salinity of the water decreases (6, 10).

Sodium proton antiporters play a major role in transporting Na^+ across the cytoplasmic membrane of all living cells (22, 33, 55). They are widely distributed in cell membranes from bacteria to animals. In bacteria, the antiporter extrudes Na^+ or Li^+ in exchange for H^+. The driving force for this process is an electrochemical potential of H^+ across the membrane, which is established by either the respiratory chain or the H^+-translocating ATPase. The Na^+/H^+ antiporter has several roles: (i) establishment of an electrochemical potential of Na^+ across the cytoplasmic membrane, this being the driving force for Na^+-coupled processes such as Na^+/solute symport (9) and Na^+-driven flagellar rotation (18); (ii) extrusion of Na^+ and Li^+, which are toxic if they accumulate to high concentrations in cells (19); (iii) intracellular pH regulation under alkaline conditions (22, 33); and (iv) cell volume regulation (33). *Escherichia coli* has two antiporters, NhaA (17) and NhaB (36), which specifically exchange Na^+ or Li^+ for H^+. *nhaA* is the gene required for tolerance of Na^+ and Li^+ and for withstanding alkaline pH in the presence of Na^+ (32). The expression of *nhaA* is positively regulated by *nhaR*, a member of the LysR family, and is induced by Na^+ in a pH-dependent manner (7, 21, 38). *nhaB*, the housekeeping gene, itself confers limited sodium tolerance on cells but becomes essential if a lack of NhaA limits growth (35). The NhaA protein is predicted to have a putative secondary structure consisting of 12 transmembrane segments connected by hydrophilic loops (33, 41). This topology has recently been substantiated by using *phoA* fusions combined with epitope mapping and exposure to proteolysis from each side of the membrane (41). NhaA is an electrogenic antiporter that has been purified to homogeneity and reconstituted in a functional form in proteoliposomes (37, 48, 49). The two-dimensional crystal structure of NhaA has recently been determined (53). The H^+/Na^+ stoichiometry of NhaA is $2H^+/Na^+$. The activity of NhaA is highly dependent on pH, with V_{max} changing by over 3 orders of magnitude from pH 7 to 8 (48). A pH-dependent conformational change in loop VIII-IX plays a major role in this pH response (16). Interestingly, strong pH sensitivity is a characteristic of antiporters, as well as other transporters and proteins involved in pH regulation. Several amino acid residues involved in the pH sensitivity

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
V. cholerae		
N18	Wild-type O1: Peru 1991	J. M. Fournier (Institut Pasteur)
SV1	N18 *nhaA* mutant	This work
SV2	N18 *nhaA′-lacZ nhaA* mutant	This work
SV3	N18 P*nhaA′-lacZ*	This work
E. coli		
TG1	F′ *traD36 lacI*^{q} Δ(lacZ)M15 *proA*$^+$*B*$^+$/*supE* Δ(*hsdM-mcrB*)*5* (r_K^- m_K^- McrB$^-$) *thi* Δ(lac-proAB)	Biolabs
DH5α λ*pir*	*recA1 gyrA* (Nal) Δ(*lacIZYA-argF*) (ϕ80d*lac*Δ [*lacZ*]M15) *pirRK6*	27
β2155	F′ *traD36 lacI*^{q} Δ(*lacZ*)M15 *proA*$^+$*B*$^+$/*thr-1004 pro thi strA hsdS* Δ(lacZ)M15 Δ*dapA*::*erm pir*::RP4(::Kan from SM10)	D. Mazel (Institut Pasteur) and unpublished data
TA15	*mel*BLid *nhaA*$^+$ Δ*lacZY*	17
NM81	TA15 Δ*nhaA1*::*kan*$^+$ *thr-1*	32
HITΔAB−	HIT-1(*nhaB* mutant) Δ*nhaA*::Kan^r	51
Plasmids		
pBR322	Ap Tc	53
pHG329	Ap	47
pUC1318ΩKm1	Ap Km; a pUC1318 derivative with a 1.5-kb *Cla*I fragment containing *aphA-3* inserted at the *Sma*I site	P. Trieu-Cuot, unpublished data
pAT113/Sp	Sp Mob$^+$ (IncP), *oriR* pACYC184, *att* Tn*1545*, *lacZ*α$^+$, MCS pUC19	8
p*lac*-1	A pGP704 derivative with *lacZ*	1

[a] Ap, Tc, Nal, Km or Kan, and Sp, resistance to ampicillin, tetracycline, nalidixic acid, kanamycin and streptomycin, respectively.

of NhaA have been identified: His-225 (15, 31, 40), Leu-73 (29), and Gly-338 (39).

As with *E. coli* and related species, *V. cholerae* has probably developed complex molecular mechanisms enabling it to grow and to survive in saline environments. Homologs to *E. coli* NhaA have been described in *Vibrio parahaemolyticus* (59% identity) and in *Vibrio alginolyticus* (58% identity), two closely related aquatic species (23, 28, 30). In addition, a homolog of *E. coli* NhaR (60% identity) has recently been described in *V. cholerae* (54).

In this work, we identified an NhaA homolog in a wild-type epidemic strain of *V. cholerae* O1. This protein acted as an Na^+/H^+ antiporter in *E. coli*. The growth of a *V. cholerae nhaA* mutant was inhibited by high concentrations of Li^+, although this mutant was found to grow under various conditions of salinity and pH.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media. In most experiments, we used the wild-type strain N18 of *V. cholerae* O1, isolated from a patient during the cholera epidemic of 1991 in Peru. We also used a collection of 15 strains of *V. cholerae* from various serogroups, including those designated O395 (O1), MO45 (O139), 206 (O139), 205 (O1), 212 (O2), 225 (O5), 178 (O6), 226 (O15), 228 (O34), 221 (O37), and 217 (O39), obtained from J. M. Fournier (Institut Pasteur, Paris, France). Four other strains of *V. cholerae* were generously provided by T. Shimada (Tokyo, Japan), designated 930 (O22), 71 (O22), 73 (O141), and 74 (O155). *V. parahaemolyticus* strain 75.2 was also obtained from J. M. Fournier. For DNA manipulations, we used the *E. coli* strains and plasmids listed in Table 1.

Bacteria were grown at 37°C in Luria-Bertani (LB) broth supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), or spectinomycin (60 μg/ml). *E. coli* β2155 was grown in LB broth supplemented with diaminopimelic acid (10^{-3} mol/liter). When indicated, NaCl was added and the pH was adjusted with 20 mM Tricine-KOH. We also used nutrient broth (NB) (10 g each of Oxoid Lab Lemco and Oxoid Bacto Peptone per liter) with or without 120 mM NaCl, 120 mM LiCl, or 120 mM KCl at pH 8.5. Bacterial growth was usually followed by the measurement of optical density at 600 nm (OD_{600}) or by counting colonies on plates after serial dilutions.

DNA manipulations and sequencing. Chromosomal DNA purification, DNA ligation, bacterial transformation, agarose gel electrophoresis, colony hybridization, and Southern blotting were carried out by standard techniques, as described by Sambrook et al. (43). Plasmid DNA was purified on Qiagen columns according to the manufacturer's recommendations. All restriction enzymes and nucleic acid-modifying enzymes were purchased from New England Biolabs, Inc. (Beverly, Mass.). [α-^{32}P]dCTP was obtained from Amersham Life Science, Inc. (Arlington Heights, Ill.). Oligonucleotides were purchased from Genset. PCR was used to prepare probes, and DNA fragments were amplified by reverse PCR with a Perkin-Elmer DNA Thermal Cycler 480 (Norwalk, Conn.): In a final volume of 100 μl, we mixed 100 ng of *V. cholerae* chromosomal DNA, 200 μM deoxynucleoside triphosphates, 40 pmol of each primer, and 2 U of *Taq* polymerase (Promega) in 1× reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, and 1.5 mM $MgCl_2$). The PCR mixture was subjected to a denaturation step (5 min at 95°C), followed by 35 cycles of amplification (60 s of denaturation at 95°C, 60 s of annealing at 55°C, and 90 s of elongation at 72°C) and a termination step (10 min at 72°C). The resulting amplicons were purified from agarose gels with a Geneclean kit (BIO 101, La Jolla, Calif.). The nucleotide sequence was determined by the dideoxy chain termination method with the ABI PRISM Dye Terminator Cycle Sequencing kit (Perkin-Elmer) and the ABI PRISM 310 automatic sequencer (Applied Biosystems). Computer analysis was carried out using the Mac Vector program (International Biotechnologies, Inc.) and BLAST software (National Center for Biotechnology Information). Amino acid alignments were analyzed with CLUSTAL W.

Genomic libraries and cloning of *nhaA* and probes. Chromosomal DNA from *V. cholerae* O1 (N18) was digested with *Hin*dIII, and 3- to 7-kb DNA fragments selected by centrifugation on a sucrose gradient were inserted into pBR322. The ligation mixture was used to transform *E. coli* HITΔAB−. The *nhaA* gene was then cloned from this library with an *nhaA* probe from *V. parahaemolyticus* (1,217 bp) obtained by PCR with the primers 5′-AATCGTTTATTAAGATAATAAA TAT-3′ and 5′-TGAGTTACAAATCAGTTAAATACGA-3′. The primers used for reverse PCR were 5′-CTGAAGCTAAAGATGCCGAGTGGTT-3′ and 5′-GATCTTCATTTCATCACTGGCCTTT-3′. To complete the cloning of the *V. cholerae nhaA*, a second genomic library was constructed in *E. coli* TG1, using *Pst*I DNA fragments (9 to 13 kb) inserted into pHG329. A specific internal 902-bp *nhaA* probe was prepared by amplifying a *V. cholerae nhaA* fragment with primers 5′-ATGTCTGACATGATTCGAGAT-3′ and 5′-CTGAAGCTAAAGA TGCCGAGTGGTT-3′ to screen the second library.

To screen bacterial strains for the presence of *nhaA*, we prepared a specific 1,710-bp *nhaA* gene by amplifying the entire *V. cholerae nhaA* gene with primers 5′-CGGAATTCCGCAGACGGGTTTTATGCAGTTAT-3′ and 5′-CGGAATT CCGAGATTGGCGCGAACCTTAT-3′.

Construction of an *nhaA* mutant and complementation. We constructed an *nhaA*-disrupted mutant from strain N18 of *V. cholerae* O1. A 548-bp PCR fragment corresponding to an internal fragment of *nhaA* was generated with *V. cholerae* O1 chromosomal DNA as a template, with the primers 5′-CGGAATT CCCTGCGATAGCGGCGGTAG-3′ and 5′-CGGAATTCCAGCATTGGCAA AAGCAAAC-3′. This fragment was flanked by *Eco*RI restriction sites which

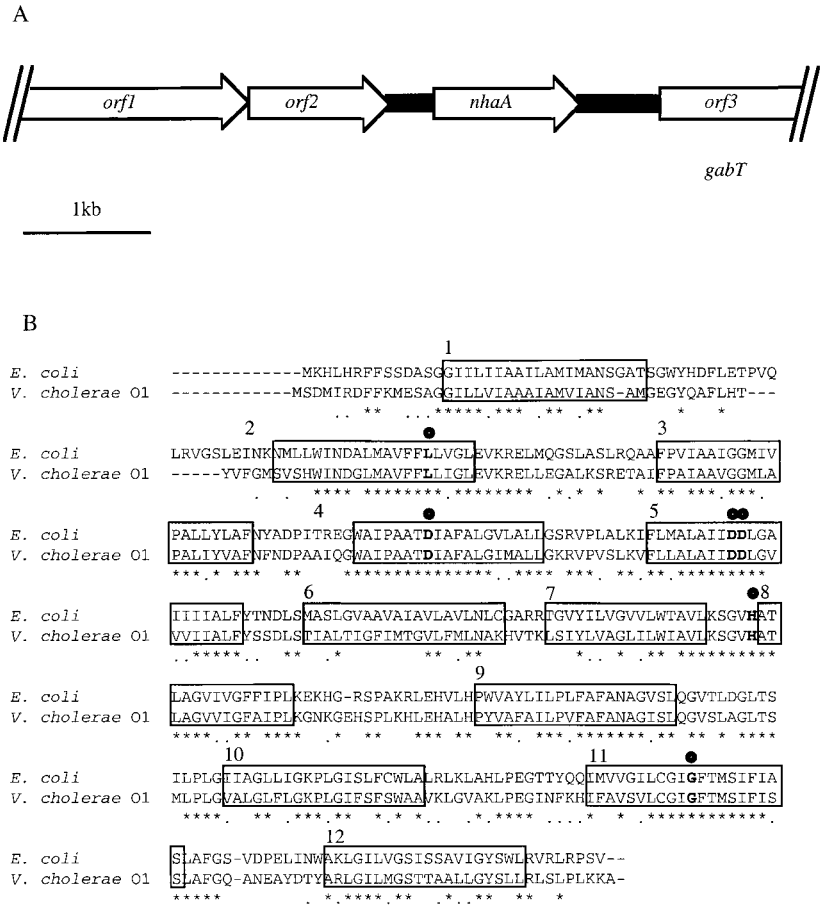

FIG. 1. (A) Genetic organization of the *nhaA* locus of *Vibrio cholerae* O1. (B) CLUSTAL W alignment of *E. coli* and *V. cholerae* O1 NhaA proteins. Asterisks indicate amino acid identity, and dots indicate amino acid similarity. The conserved amino acid residues Asp-133, Asp-163, Asp-164, His-225, Leu-73, and G-338 are indicated with boldface circles. Helical structures spanning the membrane are indicated with open boxes and are numbered. The *E. coli* sequence data were taken from Taglicht et al. (48).

were used to insert it into the suicide vector p*lac*-1, previously digested with *Eco*RI and dephosphorylated. The resulting plasmid was called p*lac-nhaA*. A kanamycin resistance cassette (*aphA-3*), obtained by *Bam*HI-digestion of pUC1318ΩKm1, was inserted into the *Bgl*II site of the vector to give pSV2. The *lacZ* gene was deleted by *Sma*I-*Kpn*I digestion and religation to give pSV1. All these constructs were made in the *E. coli* DH5α λpir strain. For the final step, pSV1 was used to transform *E. coli* β2155, from which the plasmid was transferred into the wild-type *V. cholerae* strain by conjugation. Integration of this suicide plasmid into the wild-type chromosomal *nhaA* gene gave rise to the *nhaA* mutant, called SV1, confirmed by Southern blot analysis. For complementation assays, a 2,556-bp *Mlu*I blunted fragment derived from 10- to 11-kb fragments of a *Pst*I genomic library and containing the entire *nhaA* gene was inserted into pAT113 or pHG329, previously digested with *Sma*I and dephosphorylated. pHG329Ω*nhaA* was then used to transform the *nhaA* mutant of *E. coli*, and pAT113/SpΩ*nhaA* was introduced by conjugation into the *nhaA* mutant of *V. cholerae*.

Conjugative transfer was achieved by mixing 150 μl of an overnight culture of the donor strain in LB broth, washed and diluted 1 in 100, with 75 μl of an overnight culture of the recipient strain, washed and diluted 1 in 100 on a 0.45 μm-pore-size filter (Nalgene, Rochester, N.Y.). The filters were then placed onto LB-diaminopimelic acid plates and, after incubation at 37°C for 8 h, were resuspended in LB broth and streaked onto selective medium plates (LB agar supplemented with kanamycin). Cointegrate conjugants were isolated. We obtained mutants with a single homologous recombination, the genotype of which was confirmed by Southern blot analysis.

Transcriptional *nhaA′-lacZ* chromosomal fusions. Two transcriptional *nhaA′-lacZ* fusions were constructed and introduced into the chromosome of strain N18 of *V. cholerae*. A first-fusion mutant strain (SV2), derived from *V. cholerae* O1, was constructed as follows: an internal *nhaA* fragment obtained by PCR as described above was inserted upstream from the *lacZ* gene in pSV2, a modified p*lac*-1 vector. pSV2 was integrated by homologous recombination into the *nhaA* locus, interrupting the *nhaA* gene and placing the *lacZ* gene under the control of the promoter of the inactivated *nhaA* gene. A second-fusion mutant strain (SV3) was constructed by inserting the promoter region of *nhaA* into pSV3, a modified p*lac*-1 vector, as follows: a 669-bp fragment containing a 623-bp sequence upstream of the *nhaA* initiation codon was generated by PCR with primers 5′-CG GAATTTCGTCATCCCACTCAATGAT-3′ and 5′-CGGAATTCCATCTTGA AGAAATCTCG-3′. This fragment (P*nhaA*) was flanked by *Eco*RI restriction sites, which were used to insert it into pSV2 that had been digested with *Eco*RI and dephosphorylated to give pSV3. The *lacZ* gene was integrated by homologous recombination under the control of the promoter of *nhaA*. In contrast to SV2, *nhaA* remains intact on the chromosome of N18 in this transcriptional fusion. As described above, all plasmid constructions were done in the *E. coli* DH5α λpir strain. For the final step, pSV2 or pSV3 was used to transform into *E. coli* β2155, from which they were transferred into the wild-type *V. cholerae* strain by conjugation. The genotype of the *nhaA′-lacZ* chromosomal fusions was confirmed by Southern blot analysis. For the determination of β-galactosidase activity, bacteria were grown overnight at 37°C in LB broth and diluted to an OD of 0.04 in NB-Tricine (pH 8.5) with or without NaCl, LiCl, or KCl. β-Galactosidase specific activity was determined at various times during bacterial growth as previously described (26) and expressed in Miller units. The formula used was as follows: activity = $[OD_{420} - (1.75 \times OD_{550})] \times 1{,}000/(OD_{600} \times t \times 0.5)$.

Nucleotide sequence accession number. The nucleotide sequence of *V. cholerae* O1 *nhaA* has been assigned no. AF051158 in the GenBank Nucleotide Sequence Database.

RESULTS AND DISCUSSION

Characterization and distribution of *nhaA* in *V. cholerae*. A *Hin*dIII genomic library from a wild-type epidemic strain N18 of *V. cholerae* O1, isolated in 1991 from a patient in Peru, was constructed in pBR322 and used to transform into *E. coli*

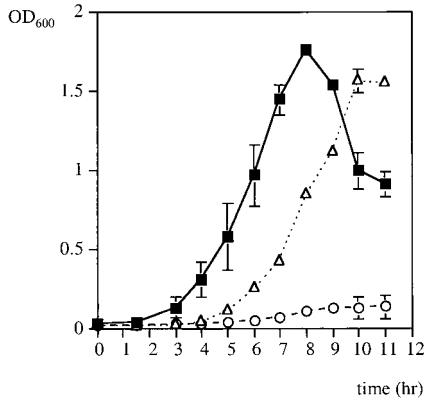

FIG. 2. Growth curves for *E. coli* strains TA15/pHG329 (■), NM81/pHG329 (○), and NM81/pHG329Ω*nhaA* (△). Bacteria were grown in LB (pH 7.5), 0.7 M NaCl, and ampicillin (100 μg/ml).

HITΔAB−. This library was screened with an *nhaA* probe from *V. parahaemolyticus*, which hybridized to the chromosomal DNA of *V. cholerae* O1 under high-stringency conditions. We isolated a positive clone carrying a 4-kb *Hin*dIII fragment. It was entirely sequenced (4,191 bp), revealing the presence of three open reading frames (ORFs). One ORF was truncated at the 3′-OH extremity, and its sequence was homologous to the 3′ end of the *nhaA* gene of *V. parahaemolyticus* and *V. alginolyticus*. The sequence of the 5′-OH end of this gene was determined by sequencing an amplicon obtained by reverse PCR after *Sph*I chromosomal DNA digestion and religation. We thus obtained a 5,976-bp sequence encompassing the complete region and constituted of four ORFs: ORF1, ORF2, *nhaA*, and ORF3 (Fig. 1A). ORF1 and ORF2 encoded putative proteins of unknown functions. The 5′-OH end of ORF3 encoded the N-terminal part (360 amino acids) of a putative protein, sharing 53% identity with gamma-aminobutyric acid transaminase (also known as aminotransferase) of *Haemophilus influenzae* (*gabT*). The genetic organization of the *nhaA* region of *V. cholerae* is different from that of members of the family *Enterobacteriaceae*, where *nhaR*, the transcriptional regulator of *nhaA*, is located downstream from the *nhaA* gene (24, 34).

The *nhaA* gene of *V. cholerae* encodes a protein predicted to have 382 amino acids highly homologous to the NhaA of *V. parahaemolyticus* and *V. alginolyticus* (87 and 86% identity, respectively), *H. influenzae* (56% identity), *Salmonella enteritidis* (56% identity), *E. coli* (56% identity), and *Helicobacter*

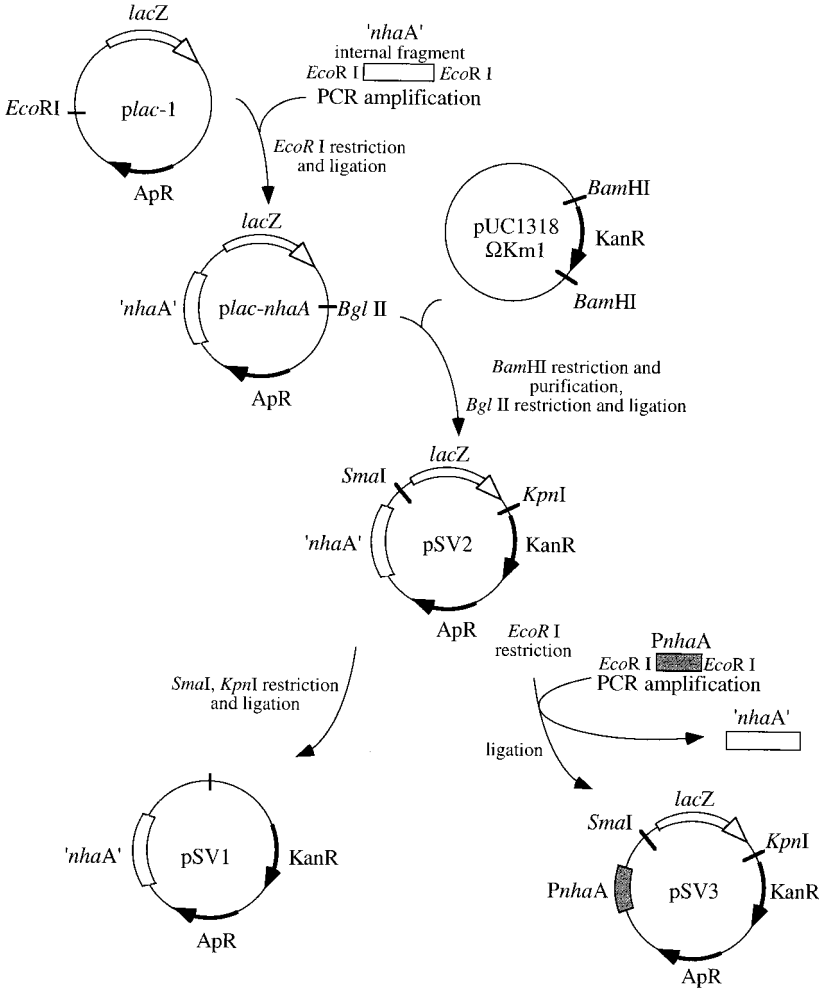

FIG. 3. Construction of pSV1, pSV2, and pSV3.

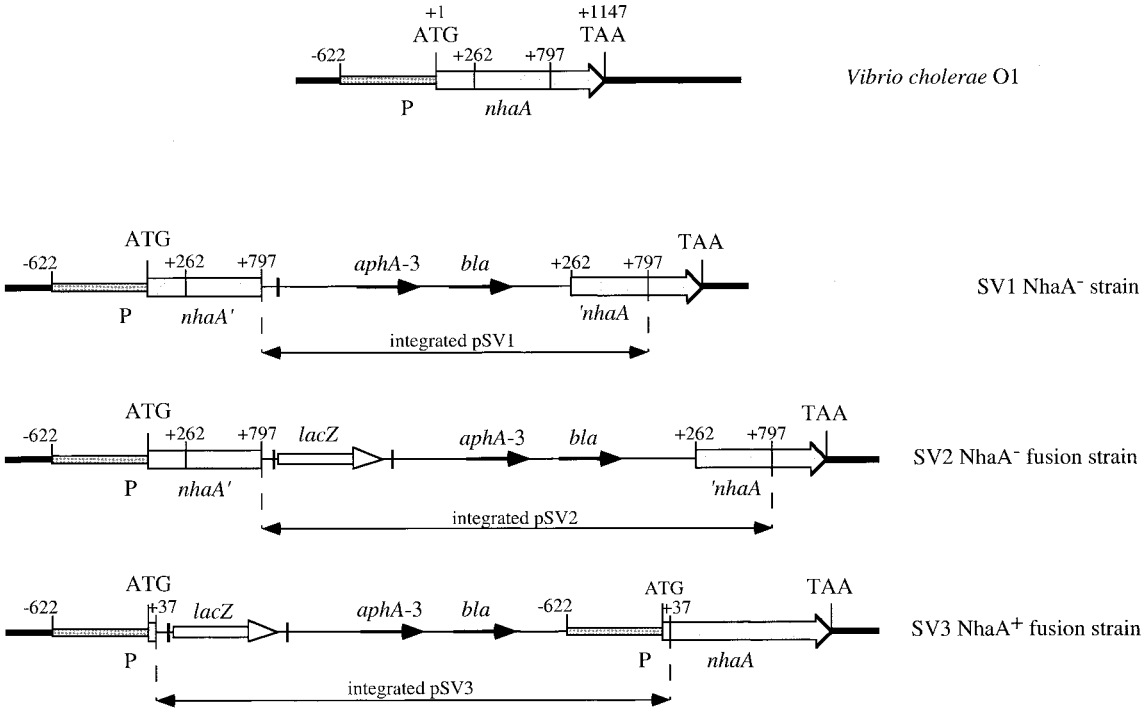

FIG. 4. SV1, SV2, and SV3 *V. cholerae* mutant strains obtained by simple allelic exchange. Coordinates correspond to the A of the translational start site. The suicide vectors pSV1, pSV2, and pSV3 used for recombination are indicated with double-headed arrows.

pylori (41% identity). *E. coli* NhaA has been extensively studied and is an Na^+/H^+ antiporter essential for its adaptation to salinity and alkalinity. As shown in Fig. 1B, *E. coli* NhaA has 12 extensive helical structures repetitively spanning the membrane (41). The deduced peptide sequence of *V. cholerae* closely resembles that of *E. coli*, with the same putative extensive helical structures (Fig. 1B). Three important aspartate residues (Asp-133, Asp-163, and Asp-164) that have been identified as essential in *E. coli* for NhaA activity are conserved in *V. cholerae* NhaA (20). The *E. coli* NhaA His-225 and Leu-73, identified by mutagenesis as residues involved in pH sensing (15, 29), are located in highly conserved regions of *V. cholerae* (Fig. 1B), and the Gly-338 residue which affects the pH response of NhaA (39) was also conserved in *V. cholerae* NhaA, suggesting that these proteins share functional properties.

We carried out Southern blotting at high stringency with a 1,710-bp *nhaA* probe containing the entire *nhaA* gene and *Hin*dIII-digested chromosomal DNA from 15 *V. cholerae* strains of various serogroups (O1, O2, O5, O6, O15, O22, O34, O37, O39, O139, O141, and O155). *nhaA*-related sequences were detected in all the strains tested, and the restriction pattern was the same in each case, except for two strains, O141 and O155 (data not shown).

The NhaA of *V. cholerae* is a functional homolog of *E. coli* NhaA. We first investigated whether NhaA of *V. cholerae* was functional and acted as an Na^+/H^+ antiporter by introducing into the *nhaA*-inactivated mutant NM81 of *E. coli* (32) either the multicopy plasmid pHG/*nhaA*, which carries the entire *V. cholerae nhaA* with its promoter region, or pHG, as a control. *E. coli* NM81/pHG did not grow in LB broth supplemented with ampicillin (100 μg/liter) and 0.7 M NaCl at pH 7.5 (Fig. 2). In contrast, bacterial growth was restored in the complemented mutant of *E. coli* transformed with *V. cholerae nhaA* (NM81/pHGΩ*nhaA*), and the growth rate was similar to that of the wild-type *E. coli* strain, TA15, harboring pHG, although a 2-h shift was observed in the initial growth phase for the complemented strain (Fig. 2). This result indicated that *V. cholerae* NhaA is a functional homolog of *E. coli* NhaA and acts as an Na^+/H^+ antiporter.

Growth of an *nhaA* mutant of *V. cholerae* is inhibited by Li^+. An *nhaA*-inactivated mutant of *V. cholerae* O1, designated SV1, was constructed by inserting the suicide vector pSV1 by homologous recombination into *nhaA* from strain N18 (Fig. 3 and 4). In contrast to *E. coli* (32), no significant difference in growth was observed between the *nhaA* mutant and the wild-type strain in LB broth with various NaCl concentrations and pH values, up to 1.0 M NaCl and pH 9.5, conditions that inhibited bacterial growth of both the wild-type strain and the *nhaA* mutant of *V. cholerae* (data not shown). These results suggest that *V. cholerae* has developed several compensatory mechanisms to survive and to maintain its homeostasis in its aquatic saline environment.

Bacterial growth was then tested in NB-Tricine supplemented with spectinomycin (60 μg/liter) and 120 mM LiCl (pH 8.5). We used the wild-type strain (N18) and its *nhaA* mutant transformed with pAT113 or pAT113/*nhaA*. In the presence of LiCl, the *nhaA* mutant did not grow, whereas the wild-type bacteria did (Fig. 5). No such effect was observed at neutral pH (data not shown). Bacterial growth was almost fully restored in the *nhaA* mutant complemented with pAT113/*nhaA* (Fig. 5). The inactivation of *nhaA* was therefore responsible for the lack of growth of the *V. cholerae* mutant in the presence of Li^+, demonstrating that *V. cholerae* NhaA expresses a typical property of the NhaA Na^+/H^+ antiporter. Moreover, the inhibition of growth of the *V. cholerae nhaA* mutant in the presence of large amounts of LiCl in a pH-dependent manner is consistent with the recent finding that the growth of a *V. cholerae nhaR* mutant was not affected by various NaCl concentrations and

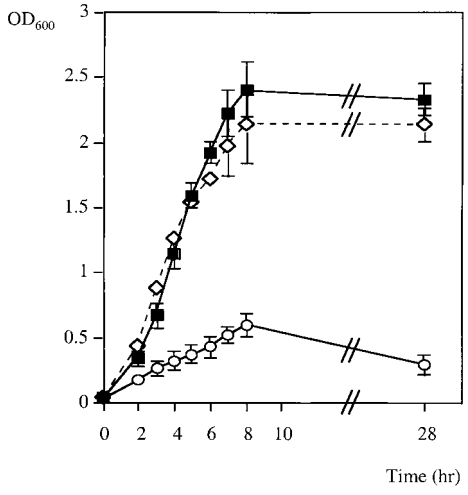

FIG. 5. Growth curves of *V. cholerae* strains O1/pAT113-Sp (■), SV1/pAT113/Sp (○), and SV1/pAT113/SpΩ*nhaA* (◇). Bacteria were grown in NB-Tricine (pH 8.5), 120 mM LiCl, and spectinomycin (60 μg/ml).

alkaline conditions but was restricted in the presence of Li^+ (54). However, the relevance of the NhaA-dependent resistance of *V. cholerae* to Li^+ toxicity in the environment remains unclear.

Transcription of the *nhaA* gene of *V. cholerae* is induced by Na^+, Li^+, and K^+. We constructed two *nhaA'-lacZ* chromosomal fusions, designated SV2 and SV3, in the wild-type strain (N18) of *V. cholerae* O1 (see Materials and Methods) (Fig. 3 and 4). The SV2 $NhaA^-$ fusion strain was obtained by inserting an *nhaA-lacZ* transcriptional fusion into *nhaA*, thereby disrupting this gene and placing *lacZ* under the control of the *nhaA* promoter. However, since the *nhaA'-lacZ* fusion lacks active *nhaA*, neither the induction nor the stress conditions of this strain necessarily reflect those of the wild type. We therefore constructed another fusion strain to study the regulation of *nhaA* expression. The SV3 $NhaA^+$ fusion strain was constructed by inserting a P*nhaA-lacZ* fusion containing the promoter region of *nhaA* into the chromosome of N18. In this strain, *lacZ* was also under the control of the *nhaA* promoter, but *nhaA* was intact. Bacteria grown overnight in LB broth at 37°C were diluted to an OD of 0.04 in NB-Tricine (pH 8.5) with or without NaCl, LiCl, or KCl (120 mM) and incubated 10 h at 37°C. No difference in bacterial growth was observed between SV2 and SV3 in NB-Tricine with or without NaCl or KCl (data not shown). In contrast, in NB-Tricine supplemented with 120 mM LiCl, growth of strain SV2 in which *nhaA* was inactivated was restricted, whereas growth of strain SV3 was not affected, confirming that the wild-type copy of *nhaA* was expressed in this later strain (data not shown).

β-Galactosidase activity was determined before the addition of salts (30 U) and during the exponential (t = 6 h) and early stationary (t = 10 h) growth phases (Fig. 6). We noticed that the overall level of β-galactosidase activity was lower for the SV2 strain than for the SV3 strain, with no clear explanation. However, we found that Na^+, Li^+, and K^+ induced *nhaA* transcription in both SV2 and SV3 strains. Exposure to NaCl, LiCl, or KCl (120 mM) increased β-galactosidase activity, as compared to the control at 6 or 10 h. Maximal induction ratios were observed in the presence of NaCl: a 4- to 8-fold increase at 6 to 10 h for the SV2 NhaA- strain and a 1.5- to 2.6-fold increase at 6 to 10 h for the SV3 $NhaA^+$ strain. The difference in *nhaA* induction between the SV2 and SV3 fusion strains can be explained by the presence of an active NhaA in SV3 which efficiently excreted Na^+ at alkaline pH and reduced the intracellular concentration of Na^+. Indeed, this intracellular Na^+ concentration is the signal for induction in *E. coli* (12). Moreover, when strain SV2 or SV3 was grown in the presence of LiCl, we also observed an increase in the production of β-galactosidase, even though it was delayed when compared to that observed in the presence of NaCl: a 0.7- to 3.8-fold increase at 6 to 10 h for SV2 and a 0.7- to 3.3-fold increase at 6 to 10 h for SV3. These results correlate with those obtained with *E. coli*, where the expression of *nhaA* is also induced by Na^+ and Li^+ (21). However, in contrast, *V. cholerae nhaA* transcription was induced by KCl. The induction ratio with KCl was lower than that observed with NaCl for the SV2 strain; however, a similar pattern of induction was observed with SV3: a 1.8- to 3.4-fold increase at 6 to 10 h for SV2 and a 1.5- to 2.7-fold increase at 6 to 10 h for SV3. The reason for this discrepancy between *V. cholerae* and *E. coli* remains unclear. The induction of *nhaA* transcription might be related to osmotic shock or to complex regulatory mechanisms specific to *V. cholerae*. This halotolerant bacterium survives in ecosystems which are probably very different than those of *E. coli* and therefore may have developed unknown specific adaptative mechanisms to variations of osmolarity. These results indicate that (i) transcription of the *nhaA* gene was modulated by the presence of salts in the growth medium and that (ii) NaCl was a better inducer than LiCl or KCl for the *nhaA* transcription. In addition, NhaR, the

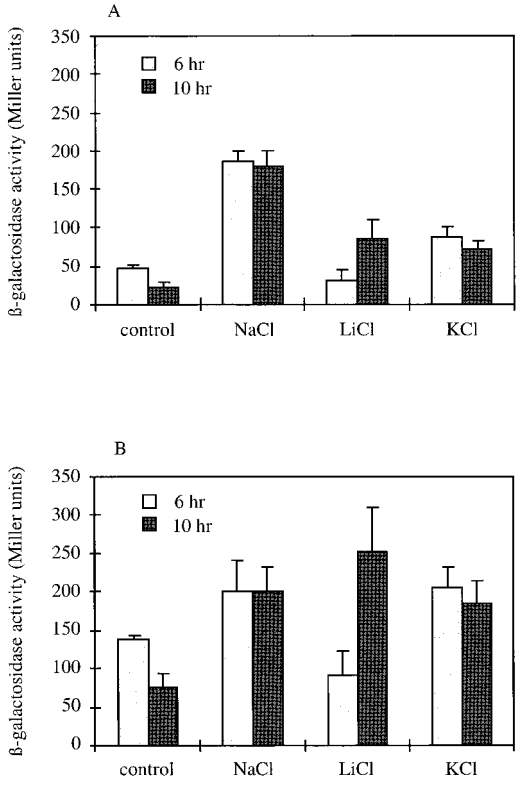

FIG. 6. Effect of Na^+, Li^+, or K^+ on the expression of *nhaA'-lacZ* in *V. cholerae* SV2 $NhaA^-$ fusion strain (A) and *V. cholerae* SV3 $NhaA^+$ fusion strain (B) at t = 6 or 10 h. Bacteria were grown in NB-Tricine (pH 8.5) either without the addition of salt (control), with 120 mM NaCl, with 120 mM LiCl, or with 120 mM KCl. Multiple assays of several independent growth experiments were performed, and the standard deviations for each determination are denoted by the line above each bar.

positive regulator of *nhaA* in *E. coli* (12), is also present in *V. cholerae* (54). All these results suggest that the transcriptional regulation of *V. cholerae nhaA* may mimic that of *nhaA* in *E. coli*.

In conclusion, our results strongly suggest that the NhaA of *V. cholerae* is an Na^+/H^+ antiporter. This saline adaptation system may contribute to the survival and persistence of the free-living *V. cholerae* in its natural estuarine habitat. Moreover, this work also shows that several additional antiporter systems regulating Na^+/H^+ homeostasis may exist in *V. cholerae*. For example, an NhaB homolog was identified in *V. cholerae* (S. Vimont and P. Berche, unpublished data). An extensive study of these antiporter systems will improve our understanding of the epidemiology of cholera.

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