Molecular Cloning and Sequencing of a Gene from Alkaliphilic Bacillus firmus OF4 That Functionally Complements an Escherichia coli Strain Carrying a Deletion in the nhaA Na\(^+/\)H\(^+/\) Antiporter Gene*

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A gene has been cloned from a DNA library from alkaliphilic Bacillus firmus OF4 that functionally complements a mutant strain of Escherichia coli, NM81, that carries a deletion for one of that strain's Na\(^+/\)H\(^+/\) antiporter genes (ΔnhaA). The cloned alkaliphile gene restored to NM81 the ability to grow at pH 7.5 in the presence of 0.6 mM NaCl and on 100 mM Li\(^+\) in the presence of melibiose, and concomitantly led to an increase in the membrane associated Na\(^+/\)H\(^+/\) antiport activity. The biologically active alkaliphile DNA was identified as an incomplete open reading frame, the sequence of which would encode a hydrophobic protein. The insert was used to isolate clones containing the complete open reading frame, which would be predicted to encode a protein with a molecular weight of 42,960 and multiple membrane spanning regions. When the open reading frame was expressed under the control of the T7 promoter, the gene product was localized in the membrane. Southern analysis indicated no homology between the alkaliphile gene, which we propose to call nhaC, and the nhaA gene of Escherichia coli, nor with other genes in digests of DNA from E. coli, Bacillus subtilis, or Bacillus alcalophilus. Although there was also no significant similarity between the deduced protein products of the alkaliphile gene and the nhaA gene of E. coli, there was a small region of significant similarity between the deduced alkaliphile gene product and the protein encoded by a human Na\(^+/\)H\(^+/\) antiporter gene (Sardet, C., Franchi, A., and Pouyseugur, J. (1989) Cell 56, 271-280).

Na\(^+/\)H\(^+/\) antiporters are apparently ubiquitous in living cells and have been implicated in a variety of functions including cytoplasmic pH regulation, osmoregulation, maintenance of low cytoplasmic Na\(^+\) ion concentrations, establishment of electrochemical Na\(^+\) gradients, and signaling phenomena (1-3). Physiological studies of the extremely alkaliphilic Bacillus species have offered some of the most compelling suggestions of the particular role of a secondary electrogenic Na\(^+/\)H\(^+/\) antiporter in pH homeostasis (4-6). Energized cells maintain a cytoplasmic pH of approximately 8.2 during growth at pH 10.5, only if Na\(^+\) is present (4, 7). Energized cells equilibrated at pH 8.5 and then subjected to a shift in the external pH to 10.5, similarly maintain a cytoplasmic pH of 8.2 only in the presence of both Na\(^+\) and a solute (e.g. α-aminoisobutyric acid) that facilitates inward Na\(^+\) recycling (8). Activity of alkaliphile Na\(^+/\)H\(^+/\) antiporter has been assayed in cells, vesicles, and crude proteoliposomes (4), and activities attributed to the antiporter have been found deficient in mutants of two different alkaliphiles that were no longer able to grow at very alkaline pH (5). Interest in the alkaliphile antiporter is further enhanced by the finding that genetic variants of at least one alkaliphile that are selected on media with sub-optimal Na\(^+\) concentrations for growth possess enhanced Na\(^+/\)H\(^+/\) antiporter activity and are able to grow at even more extremely alkaline pH values than the parent strain (9).

In addition to a role in pH homeostasis at very alkaline pH, the alkaliphile presumably requires Na\(^+/\)H\(^+/\) antiport activity throughout the pH range for growth in order to extrude the Na\(^+\) that is translocated inward during growth on substrates whose transport is Na\(^+\)-coupled; Na\(^+\) is the coupling ion for all symporters studied to date in these organisms (5). Attempts to clarify the number of Na\(^+/\)H\(^+/\) antiporters in an alkaliphilic Bacillus, define their role(s) and regulation, and purify the proteins themselves would be enormously aided by information about the genes that encode these porters. An initial tantalizing report indicates some progress in identifying a gene that may be associated with pH homeostasis in an alkaliphile, but the sequence of that gene has not yet been presented (10). By contrast, major recent progress has been achieved in dissecting the genetics of Na\(^+/\)H\(^+/\) antiporters in Escherichia coli (11-14). A gene designated nhaA has been shown to encode an electrogenic Na\(^+/\)H\(^+/\) antiporter with a molecular weight of approximately 41,000 (11-14). The nhaA gene product is pH-regulated, i.e. is more active at pH 7.5-8.0 than at pH 7.0 (13, 14). A strain of E. coli, NM81, which carries a deletion in nhaA, contains residual Na\(^+/\)H\(^+/\) antiporter activity, ascribed to a nhaB gene. This residual activity is not pH-regulated and is further distinguishable from the activity encoded by the nhaA gene by its lower affinity for Li\(^+\) in antiport assays. Strain NM81 is rendered sensitive to growth inhibition by 0.5-0.7 mM Na\(^+\) and by 100 mM Li\(^+\) in the presence of melibiose (13). The sensitivity of the mutant to Na\(^+\) is pH-dependent, increasing with increasing pH in the range of pH 6.5-8.0 (13). The phenotype of NM81 suggests that the nhaA
gene plays a role in the adaptation of E. coli to Li+ toxicity, high salinity, and alkaline pH.

The non-sensitive phenotype of NM81 is fully complemented by a multicopy plasmid bearing nhaA (11, 12). It therefore afforded us a selectable phenotype for an effort to clone the nhaA gene(s) that confer a Na+/H+ antiporter by screening a library of alkaliphilic DNA for functional complementation of E. coli NM81. For this purpose, we used the facultative alkaliphile Bacillus firmus OF4, whose pH range for growth is pH 7.2–10.7 under constant pH conditions (7, 15).

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions—The alkaliphilic bacteria used in this study were Bacillus alcalophilus (ATCC 27647) and B. firmus OF4 (15). Bacillus subtilis BD99, obtained from Anthony Garro (City College of New York), was also employed. Alkaliphiles were grown at pH 10.5 in the maltose-containing medium described previously (7). Liquid cultures were prepared by inoculating a small amount of a saturated lawn of a given colony into a flask containing 10 ml of LB medium with 1.5% Difco agar. The flasks were incubated at 37 °C for about 8 h, harvested, and immediately used for membrane preparations, without freezing or storage in the cold. The membranes were assayed immediately after preparation. Protein concentrations were determined by the method of Lowry (27) using egg white lysozyme as a standard.

The assays of antiport activity using everted vesicles were based upon the establishment of a ΔpH (transmembrane pH gradient) by addition of β-lactate and then the partial abolition of that ΔpH upon the subsequent addition of NaCl or LiCl; the establishment and partial abolition of the ΔpH were monitored by the quenching of acridine orange fluorescence and a reversal of that quenching, respectively (11, 25). A Perkin-Elmer Cetus model 650-10s spectrofluorometer was used, with an excitation wavelength of 430 nm and emission wavelength of 570 nm.

Preparation of the DNA Library from B. firmus OF4—Chromosomal DNA was prepared from B. firmus OF4 and from other bacterial strains for use in Southern analyses by the method of Marmur (19). The chromosomal DNA was partially digested with MboI (at 0.04 unit/µg chromosomal DNA) and ligated into BamHI-digested and dephosphorylated pST18 ("library 1") or pGEM3Zf+ ("library 2"). Recombinant plasmids were transformed into E. coli JM108, and approximately 10 colonies were pooled, inoculated into 50 ml of LB containing ampicillin, and grown overnight at 37 °C. Plasmid DNA was isolated (20) and used for transformation of NM81. Analyses of the subsequent clones were conducted in part by Southern analyses (21) and by other standard restriction mapping and subcloning techniques (18, 22). Probe DNA used for Southern analyses was radiolabeled using the Prime-a-Gene kit (Promega). After characterization of initial clones, we sought to use one of those clones as a probe to screen a library that might contain a large, MboI (at 0.04 unit/gg chromosomal DNA) and ligated into BamHI-transformed NM81 to a nhaA-positive phenotype with the subsequent addition of NaCl or LiCl; the establishment and partial abolition of the ΔpH were monitored by the quenching of acridine orange fluorescence and a reversal of that quenching, respectively (11, 25). A Perkin-Elmer Cetus model 650-10s spectrofluorometer was used, with an excitation wavelength of 430 nm and emission wavelength of 570 nm.

Fractionation of cells was conducted as described by Snayelle et al. (28) except that lysozyme was omitted from the sonication buffer, and the protease inhibitors phenylmethylsulfonyl fluoride (0.1 mM) and β-aminopropionamide (1 mM) were included in the sonication and urea-wash buffers. Fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (29), and [35S]methionine-labeled gene products were visualized by autoradiography.

RESULTS

E. coli strain NM81 was transformed with the DNA library 1 from B. firmus OF4 in pSPT18. Transformants were selected on plates containing ampicillin. The transformants from three plates with numerous colonies were resuspended

The abbreviations used are: PCR, polymerase chain reaction; EIPA, ethylisopropylammoniumlactate; IPTG, isopropyl-1-thio-β-D-galactopyranoside; kb, kilobase pair(s).

PCR Analyses—Amplification of chromosomal DNA from oligonucleotide primers based on the sequence of the truncated pBB10 open reading frame was as described by Zhang et al. (23), except that 50 µM tetramethylammonium chloride was included in the reaction mixture. The primers were 5'-GCCAAGCGGACAGCATGACAC-3' and 5'-GCTATCAATCCAGGTCTG-3'. Thirty cycles of 94 °C (40 s), 50 °C (40 s), and 72 °C (60 s) were followed, with a final extension of 30 min at 72 °C. Products were analyzed by agarose gel electrophoresis. For radiolabeling, 50-µl samples were passed through a Sepharose CL-6B spin column (18), and 5-µl (approximately 100 ng) samples were labeled with 3P as described above.

DNA Sequencing—For DNA sequencing, CsCl gradient-purified plasmid DNA was prepared by the large scale alkaline lysozyme procedure (18). Both strands of the regions of interest were sequenced, with appropriate overlaps, using an Applied Biosystems 373A DNA sequencer in the DNA Core Laboratory of the Brookdale Center for Molecular Biology at the Mount Sinai School of Medicine. Oligonucleotide primers used for the sequencing were synthesized in the same Core facility using the Applied Biosystems 380B DNA synthesizer. The sequence analyses employed the Genetics Computer Group Sequence Analysis Software Package (24) running on a VAX 4000-306 computer.

Expression and Localization of the Cloned Gene Product—Plasmids used in expression studies were transformed into the DE3 lysogen of E. coli BL21 (pLysS) (17). Transformants, in 5 ml cultures of medium A (minus sodium citrate) containing 0.5% glucose plus ampicillin, were grown at 37 °C. IPTG was added to a final concentration of 1 mM. Cells were harvested, and immediately used for membrane preparations, without freezing or storage in the cold. The membranes were assayed immediately after preparation. Protein concentrations were determined by the method of Lowry (27) using egg white lysozyme as a standard.

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in LB and used as the inoculum for an enrichment in LB, pH 7.5, containing 0.7 M NaCl. After 2 days of incubation with shaking, turbidity appeared in this culture. The culture was then plated onto solid LB medium, pH 7.5, containing 0.7 M NaCl. Colonies that arose on these plates were purified by restreaking on the same medium and were also shown to be positive for growth on medium A plates containing melibiose and 100 mM Li+. Approximately 2 dozen such isolates were screened from each library, by analysis of minipreps, for the possession of plasmid that carried an insert. Inserts were found in several of the isolates from the pSP718 library, and all were of the same apparent size and restriction pattern with EcoRI. Subsequent studies revealed that the phenotype was not conferred by the plasmid, designated pEM271, but was a property of the pEM271-bearing host that had been isolated from the enrichment. When the host was cured of the plasmid by repeated transfer on LBK medium without ampicillin, the plasmid-free *E. coli* strain, designated NM8191, was found to grow on LBK, pH 7.5, in the presence of 0.6 M NaCl and on 100 mM Li+ in the presence of melibiose; assays of the membranes from *E. coli* NM8191 had markedly enhanced Na+/H+ antiport activity (data not shown). Since control enrichments were consistently negative for phenotypically altered strains with enhanced membrane antiport activity, it is possible that an alkaliphile gene had recombined into the NM81 chromosome giving rise to strain NM8191.

Enrichments inoculated with the *E. coli* deletion strain, NM81, that had been transformed with library 2, the pGEM3Zf(+) library of *B. firmus* OF4 DNA, also yielded about 2 dozen strains that were purified on plates of LBK medium, pH 7.5, containing 0.7 M NaCl. Minipreps of the plasmids found in these strains indicated that one plasmid, which had a 1.1-kb insert and a distinctive restriction pattern upon double digestion with *Kpn*I and *Hind*III, was found in at least four of the strains. This plasmid, designated pJB10, was chosen for further study when it was confirmed that the phenotype was conferred by the plasmid through several cycles of purification and transformation of fresh competent *E. coli* NM81 with no apparent instability of the plasmid. A summary of the isolation strategy is shown in Fig. 1. Southern analysis (Fig. 2) indicated that the insert in pJB10 indeed hybridized to DNA that was present in a preparation of *B. firmus* OF4 DNA, but not to comparable preparations from *B. alcalophilus*, *B. subtilis*, *E. coli* NM81, *E. coli* NM8191, or the cloned *nhaA* from *E. coli*.

Upon sequencing the insert in pJB10, it was apparent that this clone contained a single open reading frame that was complete at the 3' end but truncated before the start; given the orientation of the insert, the formation of a biologically active product presumably resulted from transcription and translation from the *T7* promoter and a translational start on the vector. The deduced amino acid sequence was consistent with a protein product that would be extremely hydrophobic.

The insert was used to develop a probe to screen library 3 of *B. firmus* OF4 DNA, the library prepared in pSP719. The probe was constructed by PCR amplification of a fragment internal to the apparent incomplete open reading frame using the oligonucleotide primers 5'--GCAATCAATATCCACTTGGC-3' and 5'--GCAACGGAGAGACGACACA-3' and *B. firmus* OP4 chromosomal DNA as template. Approximately 10⁶ colonies from the genomic library were screened by colony hybridization (18), and positives were verified by Southern analysis. Two new clones were isolated on the basis of strong hybridization to the pJB10 insert. One of these clones, pM4.10, had a 10-kb insert, one end of which contained the region that hybridized to pJB10 and in preliminary sequencing was identical to pJB10 but contained a small amount of additional 5' sequence to complete the open reading frame, an upstream ribosome binding site, and approximately 8.5 kb of additional DNA downstream of the pJB10 open reading frame. The second clone, pM33.1, contained an insert of approximately 6 kb with less extensive hybridization to pJB10. Initial effort was focused on pM4.10.

Subcloning of this large clone was first undertaken to facilitate sequencing and studies of biological function and expression. The sub-cloning strategies were as follows: pM4.10 was digested with *Xba*I and religated to give pJX5, and pJX5 was digested with *Ban*I and religated to give pJBX4. A set of constructs that were comparable to pJX5 and pJBX4 were also prepared in pGEM7Zf(+), and were called pGX5 and pGXB4, respectively. The sequence of the complete open reading frame that is partially contained in the pJB10 insert is shown in Fig. 3. The probable ribosome binding site is indicated. The deduced amino acid sequence, also shown, is of a protein containing 402 amino acids with an *M*, of 42,960 and a *pI* of 5.33. PCR amplification of *B. firmus* OF4 chromosomal DNA, using primers from various parts of orf 1, indicates that the sequence shown in Fig. 4 is a contiguous piece of the *B. firmus* OP4 genome.

Plasmids containing an intact version of the open reading frame, either in the low (pSP719) or high (pGEM7Zf(+)) copy number plasmid, were tested for their biological activity by transforming them into the *E. coli* strain NM81 and examining growth of the transformants on LBK, pH 7.5, containing 0.6 M NaCl and on medium A plates containing melibiose and 100 mM LiCl. Unfortunately, the results with the constructs in the pSP719 vector were ambiguous, because...
Fig. 2. Southern transfer analysis of chromosomal DNA from B. firmus OF4 probed with \(^{32}\)P-labeled pJB10 insert. Chromosomal DNA (6 \(\mu\)g) was digested with EcoRI and separated by electrophoresis on a 0.6\% agarose gel at 0.6 V/h. DNA was transferred to nitrocellulose and probed with the radiolabeled PCR product described under "Materials and Methods."

Fig. 3. Nucleotide and deduced amino acid sequence of the gene that complements the E. coli nhaA deletion strain. The putative ribosome binding site is shaded, and the portion of the sequence found in pJB10 is indicated. The nucleotide sequence has been submitted to the GenBank\textsuperscript{\textregistered}/EMBL Data Bank with accession number M73530.

the vector control allowed somewhat enhanced growth of the E. coli deletion strain NM81 on high NaCl and LiCl plates. Clearly, however, both pGJX5 and pGX8 further enhanced that growth. The constructs in the pGEM vector generally enhanced the growth of E. coli NM81 on the selective plates. In most experiments, this original clone conferred even better growth than did pGX8 which contained the entire open reading frame (data not shown).

Assays of Na\textsuperscript+/-H\textsuperscript+ antiporter activity were then conducted on everted membrane vesicle preparations from E. coli strain NM81 transformed either by control pGEM7Zf(+) or pJB10, pGJX5, or pGX8. Membranes from both pGJX5/NM81 and pGX8/NM81 exhibited significantly more Na\textsuperscript+/-H\textsuperscript+ antiporter activity than the pGEM7Zf(+) control, but there was some variability among independent preparations of pGX8/NM81 membranes. Also, the preparations often exhibited a decline, over the course of several hours, in both the extent of \(\Delta\)pH formation (percent quenching) in response to the addition of D-lactate and in the Na\textsuperscript+/-H\textsuperscript+ antiporter activity. By contrast, freshly prepared membranes from the pJB10/NM81 transformant showed reproducible and stable Na\textsuperscript+/-H\textsuperscript+ antiport activity above the pGEM7Zf(+) control; at least some of these preparations even retained full activity after storage at \(-70\) °C and thawing. Thus, the most extensive assays were conducted with the freshly prepared pJB10/NM81 membranes. However, the patterns reported for this preparation, relative to the control, were also observed with preparations containing the full open reading frame.

As shown in Fig. 4A, membranes from the transformant containing the alkaliphile gene exhibited significantly more antiporter activity than the plasmid control when 10 mm NaCl was employed but were not different from the control when either LiCl or KC1 was added; very high concentrations of selective plates. In most experiments, this original clone conferred even better growth than did pGX8 which contained the entire open reading frame (data not shown).

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LiCl, comparable with those on the selective plates, could not be used in this assay without inhibiting the whole system. Antiport activity with very low concentrations of Li⁺, which can distinguish between the residual activity (nhaB) in the deletion strain and the nhaA encoded activity (13), was not different in the pJB10/NM81 membranes and the control (data not shown). The effect of the amiloride derivative EIPA was examined on the Na⁺-dependent antiport activity of the pJB10 transformant and control. Formation of a ΔpH, as indicated by the percent quenching observed upon D-lactate addition to the membrane vesicles, was inhibited by EIPA in a concentration-dependent manner that was independent of the low constant concentration of the solvent dimethyl sulf oxide (data not shown). Nonetheless, an appreciable ΔpH was formed even in the presence of 40 μM EIPA. At 40 μM EIPA, antiport activity by the pJB10/NM81 membranes was inhibited, with some variability in the percent inhibition from complete inhibition to the level shown for a typical experiment in Fig. 4B; the control activity was not affected by EIPA in terms of the percent reversal of quenching. In fact, the pattern of the two NaCl responses shown in Fig. 4B would seem incompatible with the presence of an uninhibited E. coli nhaB-encoded activity in both the preparations, since the pJB10/NM81 preparations were consistently inhibited to a level below that of the control. This pattern was also seen under other circumstances, e.g. pGX4/NM81 preparations that had lost their incremental antiport activity, during an hour or more of assays, often exhibited less antiport activity than the controls. Possibly, an inhibited or inactivated alkaliphile gene product introduces a leak for Na⁺. Concentrations of EIPA of 20 μM and below did not inhibit the antiport activity of either membrane preparation (data not shown).

The Na⁺/H⁺ antiport activity of the membranes was also assayed as a function of buffer pH, the standard assay pH being pH 8.0. At pH 7.0 and 7.5, neither preparation exhibited significant activity; at pH 8.5, the respiratory rate of the preparations was much less than at pH 8.0, but the activity of the pJB10/NM81 antiport was retained more than that of the plasmid control. Whereas at pH 8.0, the activity of the pJB10/NM81 preparation was about 2.5 times that of the control, at pH 8.5, it was almost five times that of the control (Fig. 4C). The decline in the antiport activity of control membranes at pH 8.5 relative to pH 8.0 was significantly greater than had been observed in earlier experiments (12) in which a different plasmid control was employed; we confirmed that indeed the pGEM7Zf(+) control was less active under several conditions, including the upper edge of pH for this assay, than a control using pBR322. This may indicate a compromise of some membrane functions that is caused by the plasmid and its activities; these observations again underscore the importance of using a precise plasmid control.

The finding of an orf 1-dependent enhancement of membrane-associated Na⁺/H⁺ antiport activity that was distinct in its properties from the residual nhaB activity of NM81 was most consistent with the presence of a structural gene encoding an antiporter from the alkaliphile that could function in the E. coli membrane. To further prove this indication, the gene encoded in pJX5 was expressed behind a T7 promoter under the control of an IPTG-inducible T7 RNA polymerase in the presence of radiolabeled methionine. The labeled gene product was localized by cell fractionation. As shown in Fig. 5, induced cells containing pJX5 expressed β-lactamase and a 36-kDa protein that was not observed in fractions from uninduced cells or from a vector control (data not shown). β-Lactamase fractionated to the periplasm, whereas the 36-kDa putative orf 1 gene product was in the membrane fraction.

**DISCUSSION**

The alkaliphile gene encoding the open reading frame that was cloned in a truncated version in pJB10 confers enhanced Na⁺ and Li⁺ resistance upon an E. coli strain with a deletion in antiporter gene nhaA. The enhanced resistance is accompanied by increased Na⁺/H⁺ antiport activity of the membranes of the transformant. The increment in Na⁺/H⁺ antiport activity is probably not due to an activation of the remaining nhaB or of a K⁺/H⁺ antiporter than can also use Na⁺; there was no increase in antiport with K⁺ as a substrate, and both the EIPA sensitivity and pH profile of the transformant with pJB10 were different from those of the membranes prepared from the plasmid control. A stimulatory effect of relatively high pH on an alkaliphile gene-encoded antiporter would be consonant with observations with the Na⁺/H⁺ antiport activity of whole alkaliphile cells (31) and with the proposed role of this activity in pH homeostasis (5). The much higher pH values that would obtain in the alkaliphile cells could not be studied in the heterologous system employed here either because the membrane preparations failed to respire or because a loss of membrane integrity prevented a respiration-dependent accumulation of protons (data not shown).

The open reading frame in the alkaliphile clone could be predicted to encode an extremely hydrophobic protein (Fig. 6). Neither the gene sequence nor the deduced amino acid sequence of the pJB10 open reading frame showed similarity with the E. coli nhaA, nor did it show remarkable similarity with any other sequence in the data banks screened, nor with the recently cloned and sequenced nhaB gene of E. coli.²

² E. Padan and S. Schuldiner, unpublished data.
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Fig. 6. Hydropathy profiles of the deduced amino acid sequence of the gene complementing the nhaA deletion strain of *E. coli*. Profiles generated by the Kyte-Doolittle algorithm with a window of 9 amino acid residues (37) are shown with solid lines, whereas dotted lines show profiles made using the algorithm of Engelman et al. (38) using a window of 20 amino acid residues.

However, a segment of the deduced sequence encoded by the open reading frame exhibited similarity to the sequence of part of the human gene encoding a Na⁺/H⁺ antiporter that was cloned by Pouyssegur and colleagues (32) by functional complementation of mutant mouse fibroblast cells. This truncated form was just as active as the form encoded by the complete open reading frame in pGXB4 and was reproducibly more stable. Given the modest but clustered and nonrandom similarity to the human Na⁺/H⁺ antiporter, the effect of EIPA was of particular interest. EIPA has been shown to inhibit eukaryotic Na⁺/H⁺ antiporters (34), with differences in the concentration range for inhibition that depend on both the species (35) and on the particular molecular form of the enzyme, e.g. different EIPA sensitivities were reported for the basolateral vs the apical surface Na⁺/H⁺ antiporters of cultured porcine kidney cells (36). The concentration that was effective in inhibiting the pJB10-dependent activity was in the higher concentration range. Even this effect, whereas significant compared with the plasmid control, must be interpreted with caution. It is possible, for example, that the pJB10-encoded activity requires a higher driving force for its activity than the residual nhaB product in the plasmid control; were that true, then the inhibition of respiration by EIPA might have a greater indirect effect on the alkaliphile gene product than on the residual *E. coli* activity.

In view of the phenotype conferred by the alkaliphile gene encoded in its complete and truncated pJB10 form, the membrane activity that it produces, its deduced sequence and properties, and its localization, we tentatively conclude that this gene is the structural gene for a Na⁺/H⁺ antiporter. We propose the name nhaC for this gene. Ultimate verification that it in fact encodes an antiporter will depend upon showing that the purified gene product catalyzes Na⁺/H⁺ antiport in proteoliposomes, as has recently been shown for the *E. coli* nhaA gene product (14). It is most likely that the alkaliphile contains additional genes encoding other Na⁺/H⁺ antiporters, as well as genes that regulate at least some of them. Bacteria commonly contain multiple porters for important physiological functions and *E. coli*, whose requirements vis à vis both Na⁺ extrusion and pH homeostasis are far more modest than those of the extreme alkaliphiles, has at least two Na⁺/H⁺ antiporters (13). In this context, the activity acquired in the generation of *E. coli* NM8191 may represent the product of a second alkaliphile antiporter gene that was somehow integrated into the *E. coli* NM81 chromosome. This possibility will be further explored.

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