Role of the nhaC-Encoded Na\(^+\)/H\(^+\) Antiporter of Alkaliphilic Bacillus firmus OF4

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Application of protoplasting transformation and single- and double-crossover mutagenesis protocols to alkaliphilic Bacillus firmus OF4811M (an auxotrophic strain of B. firmus OF4) facilitated the extension of the sequence of the previously cloned nhaC gene, which encodes an Na\(^+\)/H\(^+\) antiporter, and the surrounding region. The nhaC gene is part of a likely 2-gene operon encompassing nhaS and a small gene that was designated nhaA; the operon is preceded by novel direct repeats. The predicted alkaliphilic NhaC, based on the extended sequence analysis, would be a membrane protein with 462 amino acid residues and 12 transmembrane segments that is highly homologous to the deduced products of homologous genes of unknown function from Bacillus subtilis and Haemophilus influenzae. The full-length version of nhaC complemented the Na\(^+\)-sensitive phenotype of an antiporter-deficient mutant strain of Escherichia coli but not the alkali-sensitive growth phenotypes of Na\(^+\)/H\(^+\)-deficient mutants of either alkaliphilic B. firmus OF4811M or B. subtilis. Indeed, NhaC has no required role in alkaliphily, inasmuch as the nhaC deletion strain of B. firmus OF4811M, N13, grew well at pH 10.5 at Na\(^+\) concentrations equal to or greater than 10 mM. Even at lower Na\(^+\) concentrations, N13 exhibited only a modest growth defect at pH 10.5. This was accompanied by a reduced capacity to acidify the cytoplasm relative to the medium compared to the wild-type strain or to N13 complemented by cloned nhaC. The most notable deficiency observed in N13 was its poor growth at pH 7.5 and Na\(^+\) concentrations up to 25 mM. During growth at pH 7.5, NhaC is apparently a major component of the relatively high affinity Na\(^+\)/H\(^+\) antiporter activity available to extrude the Na\(^+\) and to confer some initial protection in the face of a sudden upshift in external pH, i.e., before full induction of additional antiporters. Consistent with the inference that NhaC is a relatively high affinity, electrogenic Na\(^+\)/H\(^+\) antiporter, N13 exhibited a defect in diffusion potential-energized efflux of \(^{22}\)Na\(^+\) from right-side-out membrane vesicles from cells that were preloaded with 2 mM Na\(^+\) and energized at pH 7.5. When the experiment was conducted with vesicles loaded with 25 mM Na\(^+\), comparable efflux was observed in preparations from all the strains.

Seven structurally distinct genes that putatively encode Na\(^+\)/H\(^+\) antiporters have been identified in eubacteria to date, i.e., nhaA (13), nhaB (33), and chaA (19) from Escherichia coli, nhaC from alkaliphilic Bacillus firmus OF4 (17), napd4 from Enterococcus hirae (41), tetA(L) from Bacillus subtilis (8), and a gene from alkaliphilic Bacillus sp. strain C-125 (16). Many of these already have obvious homologs in other genera (11, 28, 29, 32, 39), and more such homologs are emerging from genome sequencing projects. Three of the antiporter gene products, those of nhaA and nhaB from E. coli (34, 38) and tetA(L) from B. subtilis (8), have been rigorously shown to encode Na\(^+\)/H\(^+\) antiporters through studies of the purified, reconstituted gene products, and in the last case the protein has been shown to be a multifunctional antiporter that can also catalyze efflux of K\(^+\) and a tetracycline-metal complex. The activities of gene products for other antiporter genes have been inferred from studies of the enhanced antiport activity or ΔpH-dependent Na\(^+\) resistance conferred by the cloned gene in Na\(^+\)-sensitive mutants of either E. coli or the homologous bacterium. The major roles attributed to these gene products in eubacteria are in Na\(^+\) resistance and the capacity to maintain a cytoplasmic pH that is below the external pH during growth at, or a sudden shift to, an alkaline pH (22, 31, 41). The importance of successfully meeting these physiological challenges is supported by the fragility and pleiotropy of mutants lacking one or more functional antiporter genes (7, 30, 33, 40) and by the likelihood that the genes encoding structurally distinct Na\(^+\)/H\(^+\) antiporters evolved at separate times (31), resulting in the presence of multiple genes of potentially overlapping function in each individual eubacterial species thus far examined. There are several examples of apparent compensation for mutational loss or insufficiency of one antiporter by up-regulation of a gene for a different Na\(^+\)/H\(^+\) antiporter, sometimes facilitated by a mutational change in a regulatory gene, or introduction of a heterologous regulatory gene (7, 13, 33, 36). In addition, many bacteria probably use distinct K\(^+\)/H\(^+\) antiporters for the pH homeostasis function when Na\(^+\) is scarce or the Na\(^+\)/H\(^+\) antiporters are mutationally inactivated (7, 29, 35). However, extremely alkaliphilic Bacillus species exhibit Na\(^+\) specificity for this particularly crucial physiological process (23).

In addition to their Na\(^+\) specificity, the capacity of alkaliphilic Bacillus species for pH homeostasis is extraordinary in withstanding sudden, large upward shifts in external pH and in maintaining a cytoplasmic pH that is more than 2 U below the external pH under optimal growth conditions (23, 37). It is therefore of particular interest to characterize the multiple antiporters of a single alkaliphilic strain, their particular roles, and their properties. The putative antiporter gene from Bacil-
lus sp. strain C-125 is a good candidate for a major role in alkaliphily, but it has yet to be shown to confer Na⁺/H⁺ antiport activity when expressed alone in a homologous or heterologous deletion strain (16). Such a capacity has been shown for the nhaC gene from B. firmus OF4 (17), but nothing is yet known about the physiological role of this gene. Interest in nhaC is enhanced by the recent finding of homologs in both B. subtilis (GenBank accession no. P54571) and Haemophilus influenzae (11). Comparative studies of the structures and roles of neutrophile and alkaliphile homologs might illuminate part of protein function immediately suggested that the sequence that we earlier proposed for the alkaliphile nhaC (17, 18) might be incomplete, with a truncation at the N terminus. However, attempts to clone the region upstream of the available alkaliphile nhaC were hampered by problems of toxicity and lysis.

Cloning approaches in which the requisite molecular tools were carried out within B. firmus OF4 itself, as well as studies of the physiological roles of the gene in the alkaliphile, were limited by the genetic tools available. In the current study, these tools have been expanded by using the protocols developed by Biswas et al. (4) for other gram-positive bacteria. A more complete sequence analysis of nhaC, and the chromosomal region encompassing it, is presented here, together with data on the physiological roles of nhaC that emerged from studies of a newly constructed nhaC deletion strain.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. B. firmus OF4811M, a streptomycin-resistant (5 µg/ml) methionine auxotroph, was routinely grown either in a medium with pH 7.5 or in a medium with a pH of 10.5 with malate as the carbon source (9). The mutant strain of B. firmus OF4811M, DA78, that is negative for growth at pH 10.5 and completely unable to acidify its cytoplasm relative to the medium during a pH shift from 8.5 to 5.2, was grown on a rich medium at pH 8.5 (23). The B. subtilis wild-type strain BD99 and recA1 deletion strain JC112 were obtained and grown as described previously (7).

Preparation and transformation of protoplasts. Protoplasts were prepared from B. firmus OF4811M by using the protocol described previously for Bacillus sp. strain C-125 (3) and were spread on modified DM-3 medium (3). In initial experiments the medium was adjusted to various pH values with NaOH or HCl. Experiments the medium was adjusted to various pH values with NaOH or HCl. In initial experiments the medium was adjusted to various pH values with NaOH or HCl.

Preparation of membrane vesicles from B. firmus. Membrane vesicles from B. firmus were prepared by the method described previously for B. subtilis (3)

Cloning of nhaC and truncated versions thereof into plasmid vectors. For studies as described above, a construct was made containing the nhaC of various lengths were amplified by PCR from chromosomal DNA of B. firmus. The nhaC gene coding for the entire sequence did not (5).

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The nucleotide sequence for the \( \text{nhaC} \) gene and the region surrounding it has been deposited in GenBank and given accession no. U61539.

**RESULTS**

Sequence analysis of the \( \text{nhaC} \) region and complementation studies using full-length \( \text{nhaC} \).

The nucleotide sequence and the deduced amino acid sequence completed for the \( \text{nhaC} \) gene and the region immediately surrounding it are shown in Fig. 1. The new sequence extends well beyond that shown in the figure, both upstream and downstream of \( \text{nhaC} \). Novel direct repeats were found upstream of \( \text{nhaC} \) and were designated DRS1 and DRS2 (Fig. 1). The nucleotide identity between DRS1 and DRS2 is 95.7% over a 138-bp overlap. Sequence analysis suggested that it is possible that one or both of the DRS sequences encode a small, highly hydrophobic protein that might span the membrane once or twice and has no obvious homologs or function. No direct repeat sequences were found upstream of the \( \text{nhaC} \) genes from \( \text{B. subtilis} \) or \( \text{H. influenzae} \).

The new sequence provided a stronger candidate than that proposed earlier for the \( \text{nhaC} \) translation start site and resulted in a deduced product that was 60 aa longer than previously inferred (Fig. 1). \( \text{nhaC} \) would be a 462-aa hydrophobic protein (molecular size, 49.5 kDa; pl, 6.1), very close in size to the homologs reported from \( \text{H. influenzae} \) (11) and, especially, \( \text{B. subtilis} \) (GenBank accession no. P54571). \( \text{nhaC} \) from \( \text{B. subtilis} \) OF4 is 64.9% similar and 34.6% identical to that of \( \text{B. subtilis} \) and is 63.0% similar and 34.8% identical to that of \( \text{H. influenzae} \). Since the truncated versions of \( \text{B. subtilis} \) OF4 \( \text{nhaC} \) that had been studied earlier had exhibited Na\(^+\)/H\(^+\) antiport activity (17), it was of interest to determine whether the additional sequence at the N terminus added features whose analysis, in isolation, might offer a suggestion about a specific role(s). Analyses using the BLAST algorithm (1) indicated that two different regions of amino acid sequence in this newly sequenced N terminus, each of 19 to 21 residues, showed sequence similarity to regions of the large mouse calcium channel alpha-1 subunit (12); the level of identity was 45% in both matches, and the level of similarity was 75% in one match and 59% in the other. Other calcium channels were similarly matched with these regions of \( \text{nhaC} \).

An open reading frame that is proposed to have its translational start site 37 bp downstream of the end of \( \text{nhaC} \) is also likely to be transcribed together with \( \text{nhaC} \) because of their proximity and the presence of a putative terminator following the downstream gene. This gene was designated \( \text{nhaS} \). It is predicted to encode a 66-aa hydrophobic protein (molecular size, 7.9 kDa; pl, 11.8). \( \text{nhaS} \) showed significant sequence similarity to a hypothetical protein, YggO, from \( \text{B. subtilis} \) (GenBank accession no. P54492), with 26.3% identity and 61.4% similarity in a 57-aa overlap. However, the \( \text{nhaS} \) homolog of \( \text{B. subtilis} \) is in a completely different chromosomal region from its \( \text{nhaC} \) homolog gene. \( \text{nhaS} \) also showed similarity to a region encompassing the 3rd and 4th transmembrane helices in the N-terminal half of the eukaryotic Na\(^+\)/K\(^+\) ATPases, e.g., with 26.6% identity and 68.8% similarity to that from \( \text{Drosophila melanogaster} \) (26). This alignment includes a gap that is created by the presence of a basic-amino-acid- and tyrosine-rich region that is suggestive of the possibility that this gene could have a DNA-binding function.

Two truncated forms of \( \text{nhaC} \), relative to the currently proposed full-length gene, had earlier been shown to complement the \( \Delta \text{nhaA} \) strain of \( \text{E. coli} \) NM81, as assayed both by growth on Na\(^+\) and via Na\(^+\)/H\(^+\) antiport activity of everted membrane vesicles. The capacity of the full-length form was examined similarly, in the \( \Delta \text{nhaA} \Delta \text{nhaB} \) strain of \( \text{E. coli} \), EP423, Upon transformation of this strain with \( \text{pBK7} \), \( \text{pBK10} \), and \( \text{pBK15} \), respectively, each of the three plasmids allowed the sensitive strain to grow on LBK plus 0.4 M NaCl. \( \text{E. coli} \) EP423 transformed with control plasmid \( \text{pBK15} \) failed to grow on this medium. In addition, all three plasmids conferred modest but reproducible Na\(^+\)/H\(^+\) antiport activity, as measured in a fluorescence assay.

**REFERENCES**

1. Biswas, S., et al. (27), by using egg white lysozyme as the standard.

2. The single-crossover event, resulting in integration of the entire plasmid into the chromosome, was confirmed by PCR analysis. For the double-crossover event that completes the gene replacement, a single-crossover integrant was grown overnight at 28°C in complex broth containing chloramphenicol.

3. Cells were then diluted and plated onto medium of the same composition and incubated at 39°C. Double-crossover candidates were identified on the basis of Cmr and Erm; 8 such candidates were found among 200 total colonies. Both Southern hybridization and PCR analyses confirmed that the gene replacement event had taken place in these candidates. The \( \text{nhaC} \) deletion strain used in these studies was designated N13; preliminary studies indicated that its phenotype was similar to those of the other seven deletion strains isolated.

4. Growth experiments and determinations of doubling time. \( \text{B. firmus} \) OF4 and the \( \text{nhaC} \) deletion strain. Right-side-out membrane vesicles were prepared as previously described (14) by modification of the method of Kaback (20). Cells were grown in 30 mM Na\(_2\)CO\(_3\), medium, pH 8.5, as described above. Proteolipids were lysed by dilution into 25 mM potassium phosphate (pH 7.5) plus 5 mM MgCl\(_2\). After separation of nonvesicular material and washing, the vesicles were frozen and stored at −70°C. For assays of Na\(^+\) efflux, the vesicles were passively loaded at 20°C for 2 h by the addition of 50 \( \mu \)M of Na\(^+\), washed, and resuspended in the appropriate pH 7.5 or 10.5 medium. Fifty-milliliter cultures in 250-ml flasks were grown at 30°C in a rotary shaker (200 rpm). Samples were taken at various times, and the \( \text{A}_{600} \) (absorbance at 600 nm) was measured. The rates of growth, in doublings per hour, were calculated from the rates of change in \( \text{A}_{600} \) during the logarithmic phase of growth. Growth studies on \( \text{B. firmus} \) OF411M/DA78 were conducted with the same media, and those on \( \text{B. subtilis} \) strains were conducted in malate-containing media at either pH 7 or pH 8.3, as described earlier (7).

5. Assays of Na\(^+\) efflux from right-side-out membrane vesicles prepared from wild-type \( \text{B. firmus} \) OF4 and the \( \text{nhaC} \) deletion strain. Right-side-out membrane vesicles were prepared as previously described (14) by modification of the method of Kaback (20). Cells were grown in 30 mM Na\(_2\)CO\(_3\), medium, pH 8.5, as described above. Proteolipids were lysed by dilution into 25 mM potassium phosphate (pH 7.5) plus 5 mM MgCl\(_2\). After separation of nonvesicular material and washing, the vesicles were frozen and stored at −70°C. For assays of Na\(^+\) efflux, the vesicles were passively loaded at 20°C for 2 h by the addition of 50 \( \mu \)M of Na\(^+\), washed, and resuspended in the appropriate pH 7.5 or 10.5 medium. Fifty-milliliter cultures in 250-ml flasks were grown at 30°C in a rotary shaker (200 rpm). Samples were taken at various times, and the \( \text{A}_{600} \) (absorbance at 600 nm) was measured. The rates of growth, in doublings per hour, were calculated from the rates of change in \( \text{A}_{600} \) during the logarithmic phase of growth. Growth studies on \( \text{B. firmus} \) OF411M/DA78 were conducted with the same media, and those on \( \text{B. subtilis} \) strains were conducted in malate-containing media at either pH 7 or pH 8.3, as described earlier (7).

6. Determination of the cytoplasmic pH after a shift in the external pH from 8.5 to 10.6. Cells were grown to the mid-logarithmic phase at either pH 7.5 or pH 10.5 in malate-containing medium. They were harvested by centrifugation and resuspended to 10 mg of protein/ml in 50 mM \( \text{KCl} \), pH 8.5, plus 10 mM potassium malate. They were then rapidly diluted 25-fold into 50 mM \( \text{KCl} \), pH 10.6, plus 10 mM potassium malate. Where indicated, the buffers at either pH contained various amounts of NaCl. The transmembrane pH gradient (\( \Delta \text{pH} \)) was determined after 10 min as described elsewhere (24) by the distribution of radiolabelled methylamine. The control for nonspecific binding of the probe contained 10 \( \mu \)M gramicidin. Values for the cytoplasmic pH were calculated from duplicate measurements of the outside pH and the \( \text{pH} \) in at least six independent determinations. Protein was determined by the method of Lowry et al. (27), by using egg white lysozyme as the standard.

7. Nucleotide sequence accession number. The nucleotide sequence for the \( \text{nhaC} \) gene and the region surrounding it has been deposited in GenBank and given accession no. U61539.
in everted membrane vesicles prepared from the transformants of *E. coli* EP432 and compared to a control transformant. The assays were conducted in buffers in which the control membranes exhibit a small residual antiport, presumably due to $K^+$/H$^+$ antiporters that may also account for retention of pH homeostatic capacity; under these conditions, the transformants exhibited an increase of about 100% over the control. In view of the indications from sequence analysis that there might be a function of NhaC in relation to both Na$^+$ and Ca$^{2+}$, as had earlier been observed for *E. coli* chaA (19), an assay of Ca$^{2+}$/H$^+$ antiport activity by the transformants was also conducted, but no differences were observed (data not shown). The results with *E. coli* EP432 were consistent with NhaC being a Na$^+$/H$^+$ antiporter. However, this double mutant of *E. coli* does not exhibit an alkali-sensitive phenotype independent of the Na$^+$ content of the medium (33), and thus complementation of this strain by *nhaC* did not necessarily reflect a capacity to support pH homeostasis at alkaline pH values. The capacity of pNhaC to complement mutant strain JC112, a tetA(L) deletion strain of *B. subtilis* that is very alkali sensitive at low Na$^+$ concentrations as well as sensitive to elevated Na$^+$ at high pHs (7), was therefore examined. A JC112/pNhaC transformant showed no enhancement of growth at pH 8.3, although a wild-type *B. subtilis* strain, N13, exhibited significant enhancement of growth at the alkaline pH. Depending upon the medium at pH 8.3, expression of *nhaC* doubled the growth rate of the wild-type strain, whereas it did not enhance the growth of JC112 (data not shown). The nonalkaliphilic mutant strain of *B. firmus* OF4811M, DA78, which had been shown to lack the capacity for pH homeostasis (25), was not complemented by pNhaC (data not shown). It appeared, then, that pNhaC could not complement mutants of *Bacillus* that were lacking an active form of the genes encoding the Na$^+$/H$^+$ antiporter with a dominant role in pH homeostasis at alkaline pHs. This did not, however, rule out a more subtle role for NhaC in this process, or a role at less alkaline pH values at which *B. firmus* OF4 would still have to extrude the Na$^+$ entering with its numerous Na$^+$/solute symporters (22). Studies were accordingly initiated on *B. firmus* N13, with a deletion in *nhaC*, in comparison with the wild-type parent strain. The growth phenotype, capacity for pH homeostasis, and diffusion potential $22Na^+$ efflux activity of the D$nhaC$ strain, *B. firmus* OF4811M N13. As shown in Fig. 2, the deletion strain N13 grew well at pH 10.5, especially at high NaCl concentrations; the growth rates of the wild-type strain transformed with a control plasmid in these batch cultures were generally somewhat lower than growth rates observed for untransformed wild-type cells in continuous cultures at the same pH value (37). At
lower NaCl concentrations, especially at 5 mM NaCl in Fig. 2, there was a pronounced reduction in the growth rate of N13 compared to that of the wild-type strain at pH 10.5, and that difference was completely abolished upon transformation of N13 with pNhaC. Lower NaCl concentrations did not support growth well enough to allow an assessment of whether the relative difference between the growth rates of the two strains would be further magnified as the NaCl concentration was reduced even more. Interestingly, the dependence of *B. firmus* OF4811M on the NaCl concentration of the medium was just as evident at pH 7.5 as at pH 10.5, consistent with the importance of an Na⁺/symport as a major transport mode. In fact, higher concentrations of NaCl were required at pH 7.5 than at pH 10.5 to support optimal (albeit still lower) growth rates. Moreover, at the lowest NaCl concentration used in most experiments at pH 7.5, the N13 strain showed almost no growth, a defect that was complemented by pNhaC and was not observed at high NaCl concentrations (Fig. 2).

The growth phenotype suggested that NhaC is a relatively high-affinity antiporter whose functions were confined to low NaCl concentrations, with another antiporter(s) taking over at high NaCl concentrations. In order to assay this directly, the efflux of $^{22}\text{Na}^+$ was measured upon energization of right-side-out membrane vesicles by imposition of a valinomycin-mediated potassium diffusion potential at pH 7.5. Three vesicle preparations were compared: those from the wild-type strain transformed with a control plasmid and those from N13 that was transformed either with a control plasmid or with pNhaC. In several separate experiments, the vesicles were preloaded with either 2 or 25 mM $^{22}\text{Na}^+$ in a potassium-containing buffer, before treatment with valinomycin and dilution to establish the diffusion potential. As shown in Fig. 3A, N13 exhibited a significant deficiency in Na⁺ efflux from the vesicles preloaded with 2 mM radioactive cation relative to comparable preparations from complemented N13 or the wild-type strain. By contrast, the three vesicle preparations exhibited comparable diffusion potential-mediated efflux when they had been preloaded with 25 mM NaCl (Fig. 3B).

That energization of NhaC could be achieved by imposition of a diffusion potential was consistent with the electrogenericity of the antiport, i.e., that its mechanistic coupling ratio involves H⁺ inward $\gg$ Na⁺ outward. This property would be important in an antiporter functioning to achieve a pH gradient, acid in, during respiratory extrusion of protons. It was of interest to determine whether the growth defect of N13 relative to the wild-type strain at low Na⁺ concentrations at pH 10.5 might indeed reflect a defect in pH homeostasis in part, rather than reflecting only a compromise in efflux capacity for Na⁺. After a sudden shift in the external pH from 8.5 to 10.6, the capacity of the three transformants (the wild-type strain transformed with control plasmid pYH56 and N13 transformed either with the control plasmid or with pNhaC) to maintain a cytoplasmic pH below the new external pH was assayed. The cells were grown either at pH 10.5 or at pH 7.5 before being equilibrated at pH 8.5 in preparation for the shift. As shown in Table 1, pH 10.5-grown cells of all three transformants exhibited completely comparable capacities for pH homeostasis when the buffer contained 50 mM Na⁺. That capacity diminished for each strain as the Na⁺ concentration was reduced to 5 mM and diminished even more when the Na⁺ concentration was reduced to 1 mM. Most strikingly though, the falloff in capacity for pH homeostasis at the two lower Na⁺ concentrations was especially pronounced in uncomplemented N13 and least pronounced, at 1 mM Na⁺, in N13 complemented with nhaC on a multicopy plasmid. When the same determinations were done with pH 7.5-grown cells, at 50 and 5 mM Na⁺ only, all three strains were at least a little less capable of acidifying the cytoplasm relative to the new pH upon an alkaline shift, and the relative defect of uncomplemented N13 was significantly accentuated. Interestingly, the wild-type capacity for pH ho-

![FIG. 2. Comparison of the doubling times of *B. firmus* OF4811M/pYH56, N13/pYH56, and N13/pNhaC at pHs 7.5 and 10.5 on media with various concentrations of NaCl. Wild-type *B. firmus* OF4811M and N13 (with nhaC deleted) transformed with control plasmid pYH56, and N13 complemented with plasmid pNhaC, were grown at either pH 7.5 (A) or pH 10.5 (B), as described in Materials and Methods, in the presence of the indicated concentrations of NaCl. The results are the averages of at least three separate determinations.](image)

![FIG. 3. Diffusion potential-energized efflux of $^{22}\text{Na}^+$ from right-side-out membrane vesicles of N13/pYH56, N13/pNhaC, or *B. firmus* OF4811M/pYH56 at two different concentrations of intravesicular Na⁺. Membrane vesicles of *B. firmus* OF4811M/pYH56 (circles), N13/pYH56 (squares), or N13/pNhaC (triangles) were loaded with either 2 (A) or 25 (B) mM $^{22}\text{Na}^+$ and pretreated with valinomycin as described under Materials and Methods. They were diluted in the presence of valinomycin into either 25 mM sodium phosphate, pH 7.5, to generate a diffusion potential (solid symbols), or 25 mM potassium phosphate, pH 7.5, as the unenergized control (open symbols). The amount of $^{22}\text{Na}^+$ remaining in the vesicles was determined at intervals as described in Materials and Methods.](image)
TABLE 1. Internal pHs of \(B.\) \(firmus\) OF4811M/pYH56, \(N13/pYH56,\) and \(N13/pNhaC\) after a shift of the outside pH from 8.5 to 10.6

<table>
<thead>
<tr>
<th>pH of medium and strain</th>
<th>Cytoplasmic pH at Na(^+) concen(^-) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 nM</td>
</tr>
<tr>
<td>10.5</td>
<td></td>
</tr>
<tr>
<td>(B.) (firmus) OF4811M/pYH56</td>
<td>8.50 ± 0.04</td>
</tr>
<tr>
<td>(N13/pYH56)</td>
<td>8.55 ± 0.07</td>
</tr>
<tr>
<td>(N13/pNhaC)</td>
<td>8.54 ± 0.09</td>
</tr>
<tr>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>(B.) (firmus) OF4811M/pYH56</td>
<td>8.60 ± 0.03</td>
</tr>
<tr>
<td>(N13/pYH56)</td>
<td>8.76 ± 0.09</td>
</tr>
<tr>
<td>(N13/pNhaC)</td>
<td>8.65 ± 0.08</td>
</tr>
</tbody>
</table>

\(^a\) Strains were grown in buffer at either pH 10.5 or pH 7.5 as described in Materials and Methods. 
\(^b\) Na\(^+\) concentration indicates the amount of Na\(^+\) present in the pH shift buffer. 
\(^c\) ND, not determined.

meostasis when prior growth was at pH 7.5 closely resembled that of pH 10.5-grown cells of uncomplemented N13.

**DISCUSSION**

The current findings support the earlier conclusion that the \(nhaC\) gene of alkaliphilic \(B.\) \(firmus\) OF4 encodes an Na\(^+\)/H\(^+\) antiporter. Enhancement of Na\(^+\)/H\(^+\) antiporter activity was observed upon complementation of \(E.\) \(coli\) EP432. Although this enhancement was modest, it was similar to that observed with other \(Bacillus\) antiporters, including one that has been purified and rigorously shown to possess Na\(^+\)/H\(^+\) antiporter activity (8, 15). Most importantly, studies with \(B.\) \(firmus\) OF4811M itself, in comparison with the \(\Delta nhaC\) strain, N13, provided strong support for NhaC as an Na\(^+\)/H\(^+\) antiporter, i.e., in the correlated reductions in N13, relative to the wild-type strains, in electrogenic Na\(^+\) extrusion and in pH homeostasis upon an upward pH shift. In the presence of other antiporters, e.g., in wild-type \(B.\) \(subtilis\) and as part of a complement of antiporters in the wild-type alkaliphile, \(nhaC\) apparently plays a role over a wide range of pH values. Its most apparent role was observed at pH 7.5 in media containing low Na\(^+\) concentrations, where a pronounced defect in growth was observed. Although pH homeostasis is not a major challenge at pH 7.5; the alkaliphile must nonetheless extrude Na\(^+\) that enters in symport with solutes, including the growth substrate malate in the media used here. At pH 10.5, NhaC also plays an apparent role at low Na\(^+\) concentrations; at the alkaline pH, it contributes to pH homeostasis and may also contribute to the capacity of the cell to lower the cytoplasmic Na\(^+\) concentration optimally.

However, NhaC is not required for alkaliphily and is not the antiporter with a dominant role in pH homeostasis during growth in Na\(^+\)-replete medium at highly alkaline pHs. In fact, even when expressed from a multicopy plasmid, \(nhaC\) was not able to complement the alkali-sensitive phenotype of a neutrophile that had a deletion in its own dominant Na\(^+\)/H\(^+\) antiporter, the tetA(L) deletion strain JC112 of \(B.\) \(subtilis,\) let alone the nonalkaliphile \(DA76\) mutant of \(B.\) \(firmus\) OF4811M. Perhaps an insufficiently high \(V_{\text{max}}\) and/or H\(^+\)/Na\(^+\) ratio underlie the failure of NhaC to support growth of \(B.\) \(subtilis\) or \(B.\) \(firmus\) OF4811M mutants that lack other major antiporter systems. In \(E.\) \(coli,\) where the demands on the pH homeostatic mechanisms are more modest than in the alkaliphile, one of the antiporters that may contribute to this function, NhaA, is electrogenic and exhibits an enormous increase in \(V_{\text{max}}\) at high pHs in the presence of Na\(^+\) that is a combined effect of induction of synthesis and activation (21, 38). NhaB, which can take over in a \(\Delta nhaA\) mutant strain, is also electrogenic, albeit with a lower coupling ratio than NhaA, and has an even higher \(V_{\text{max}}\) than activated NhaA (34, 38). A major Na\(^+\)(K\(^+\))/H\(^+\) antiporter of \(B.\) \(subtilis,\) the Teta(L) protein, similarly is electrogenic and has a high \(K_m\) and \(V_{\text{max}}\) for Na\(^+\) (8, 15). Whereas the current data are most consistent with NhaC being electrogenic, its \(K_m\) for Na\(^+\) is likely to be relatively low. Both the growth and transport phenotypes of the N13 strain were observed only at low concentrations of Na\(^+\). Hypothetically, the other product of the \(nhaC\) operon, NhaS, is a candidate for a cation sensor or an Na\(^+\)-sensitive gene regulator that has a membrane-associated state. This suggestion arises because of both its likely capacity to bind DNA and its sequence similarity to a largely hydrophobic region of the Na\(^+\)(K\(^+\))/ATPases that may be involved in monovalent cation binding. However, even fully induced and activated NhaC may not catalyze antiport activity with a high enough \(V_{\text{max}}\) to establish and maintain the requisite 2-pH-unit \(\Delta\)pH, acid in, that is found in alkaliphiles such as \(B.\) \(firmus\) OF4811M (22, 37).

The results clearly suggest the presence of more than one additional antiporter in \(B.\) \(firmus\) OF4811M. At both low and high pHs, there must be one or more antiporters that account for the almost complete lack of a phenotype in N13 at high Na\(^+\) concentrations, i.e., a lower-affinity, probably higher-\(V_{\text{max}}\) antiporter. In cells grown at high pHs, there is apparently also at least one additional antiporter functioning in the lower Na\(^+\) concentration range together with NhaC, whereas in pH 7.5-grown cells NhaC is the major contributor to antiport activity at modest Na\(^+\) concentrations. In \(E.\) \(coli,\) NhaB has been suggested to play a role when the cell confronts a challenge of Na\(^+\) influx under conditions in which NhaA is deleted or is not fully induced or activated by high pHs (31, 33). It has also been suggested that ChaA is one of the antiporters playing an auxiliary role, at least in Na\(^+\) exclusion, at elevated pHs (30); ChaA and/or K\(^+\)/H\(^+\) antiporters (35) may provide support for pH homeostasis as well. It was notable that the Na\(^+\) requirement for growth of \(B.\) \(firmus\) OF4811M was higher at pH 7.5 than at pH 10.5; this could reflect pH-dependent properties of the Na\(^+\) isoform complement, but none of these has yet been studied at the molecular level. As studies continue to develop a comprehensive model of pH homeostasis in alkaliphilic \(B.\) \(firmus\) OF4, the specific roles of each antiporter and the properties of the Na\(^+\) uptake systems will all be of importance.

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