Physiological Role of NhaB, a Specific Na⁺/H⁺ Antiporter in Escherichia coli*

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The nhaB gene which codes for Na⁺/H⁺ antiporter activity in Escherichia coli was recently cloned (Pinner, E., Padan, E., and Schuldiner, S. (1992) J. Biol. Chem. 267, 11064–11068). In order to elucidate the role of nhaB in Na⁺ and H⁺ ions physiology and its interaction with nhaA, we generated mutants in which the chromosomal gene has been inactivated by insertion/deletion. A mutant devoid of both nhaA and nhaB is extremely sensitive to Na⁺ and Li⁺ at all pH values, and membranes prepared from this strain show no Na⁺/H⁺ antiporter activity. As opposed with the ΔnhaA mutant which contains NhaB, the pH independent Na⁺/H⁺ antiporter (Padan, E., Maisler, N., Taglicht, D., Karpe, R., and Schuldiner, S. (1989) J. Biol. Chem. 264, 20297–20302), the ΔnhaB mutant, containing NhaA, shows Na⁺/H⁺ antiporter activity highly dependent on pH. nhaB, in the absence of nhaA, confers a certain tolerance to Na⁺ which decreases with increasing pH. In the absence of NhaB, NhaA alone confers complete halotolerance under all conditions tested. However, when grown on agar in minimal medium on substrates which are symported with Na⁺ (proline, serine, and glutamate) at pH 6 and at low Na⁺ concentrations (<10 mM), ΔnhaB grows slower than the wild type and its Na⁺ dependent transport of glutamate and proline is markedly inhibited. Since both of these defects of the ΔnhaB strain are alleviated upon transformation of the mutant with multicopy plasmid bearing nhaA, we conclude that nhaB is crucial when the level of NhaA activity is growth limiting, when nhaA is not sufficiently induced, and/or when NhaA is not activated.

All growing cells extrude sodium ions actively and maintain a sodium concentration gradient directed inward. In Escherichia coli, as in many other bacterial cells, expulsion of sodium ions is driven by proton flux via a Na⁺/H⁺ antiport system (Leblanc et al., 1988; Rosen, 1986a; Schuldiner and Fishkes, 1978; Harold and Altendorf, 1974; West and Mitchell, 1974; Lanyi, 1979; Schuldiner and Padan, 1992). Also, an increasing number of examples are now known of bacterial primary sodium pumps driven by ATP hydrolysis (Heefner and Harold, 1982), decarboxylation reactions (Dimroth, 1987, 1992), or electron transport reactions (Umemoto et al., 1990; Tokuda, 1992; Skulachev, 1987).

In addition to maintaining low intracellular Na⁺ concentration in bacteria, Na⁺ efflux provides the energy for Na⁺-coupled transport systems and also for the flagellar motor in some organisms (Skulachev, 1987; Hirota et al., 1981). It has also been suggested that Na⁺/H⁺ exchange is intimately involved in regulation of intracellular pH in bacterial cells at alkaline environments (Kruvich, 1986; Padan et al., 1976, 1981; Booth, 1985).

A gene coding a Na⁺/H⁺ antiporter in E. coli has been cloned and sequenced (Goldberg et al., 1987; Karpe et al., 1988). The gene, nhaA, located at 0.3 min in the chromosome, encodes a membrane protein of M, 41,000 (Karpe et al., 1988; Taglicht et al., 1991). When in high copy number the wild type nhaA increases the Na⁺/H⁺ antiporter activity and confers to cells Li⁺ resistance.

In order to elucidate the role of nhaA in the Na⁺ cycle, we have deleted the chromosomal nhaA gene (Padan et al., 1989) and found that it is necessary for adaptation to high salinity, alkaline pH, and detoxication of Li⁺. Nevertheless, analysis of Na⁺ transport in membrane vesicles isolated from the ΔnhaA strain (NM81) implied that in addition to nhaA and the K⁺/H⁺ antiporter an alternative sodium extrusion system(s) exists, designated nhaB (Padan et al., 1989).

We used NM81 which is sensitive to Na⁺ and Li⁺ and devoid of the already cloned nhaA, and cloned the nhaB gene by functional complementation of the ΔnhaA strain (Pinner et al., 1992b). This strain has proved useful also for cloning of heterologous genes from Salmonella enteritidis (Pinner et al., 1992a) and from Bacillus firmus OF4 (Ivey et al., 1991).

By determining the nucleotide sequence of nhaB and its flanking regions, the gene has been localized at 25.5 min in the E. coli chromosome (Pinner et al., 1992b). It codes for a 504-amino acid long protein which has been specifically labeled and located in the membrane (Pinner et al., 1992b). Unlike NhaA, the activity of NhaB shows no dependence on pH in the range 6.4–8.3 (Padder et al., 1989). On the other hand, the affinity of NhaB to Na⁺ ions (Km = 40–70 μM) is higher than that of NhaA or any other Na⁺/H⁺ antiporter characterized thus far (Schuldiner and Padan, 1992).

A mutant, HIT-1, which cannot grow on serine as a carbon source was isolated (Ishikawa et al., 1987). Since serine is symported with Na⁺ and HIT-1 showed impaired Na⁺ extrusion and lack of growth at alkaline pH (with glycerol as a carbon source), it was concluded that the mutation affects the Na⁺/H⁺ antiporter activity. Recently (Thelein et al., 1991) the mutation of HIT-1 was mapped by both conjugation and transduction at 25.6 min on the E. coli chromosome, suggesting that it is tightly linked to or resides in the nhaB locus. As yet, the genetic lesion of HIT-1 has not been identified.

In the present work, in order to elucidate the role of nhaB in Na⁺ and H⁺ ions physiology and its interaction with nhaA, we generated mutants in which the chromosomal gene has been inactivated by insertion/deletion. As expected, since

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TABLE I

<table>
<thead>
<tr>
<th>E. coli strains, K12 derivatizes, used in this study</th>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
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<tr>
<td>TA15 melBLid, nhaB*, nhaA*, y149</td>
<td></td>
<td></td>
<td>Goldberg et al., 1987</td>
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<tr>
<td>NM61 melBLid, nhaB*, nhaA*, y149</td>
<td></td>
<td></td>
<td>Padan et al., 1989</td>
</tr>
<tr>
<td>JC7623 recB21, recC22, sbcB15, thr</td>
<td></td>
<td></td>
<td>Winsan et al., 1985</td>
</tr>
<tr>
<td>JC43 recB21, recC22, sbcB15, thr</td>
<td></td>
<td></td>
<td>This work</td>
</tr>
<tr>
<td>EP431 melBLid, nhaB1, nhaA*, y149</td>
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<td>EP432 melBLid, nhaB1, nhaA*, y149</td>
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<tr>
<td>C600gltS</td>
<td></td>
<td></td>
<td>Agmon, 1987</td>
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<tr>
<td>EP431 gltS Transduction</td>
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<tr>
<td>TA15 gltS Transduction</td>
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</table>

ΔnhaB strain contains only NhaA, membrane vesicles prepared from this strain show Na+/H+ antiporter activity characteristic of NhaA (Taglicht et al., 1991). When grown on agar in minimal medium containing substrates which are symported with Na+ (Proline, serine, glutamate) at pH 6 and at low Na+ concentrations (<10 mM), ΔnhaB grows slower as compared to the wild type. However, when grown on these substrates at higher pH (7.5) or on other substrates at the entire spectrum of pH and Na+ concentrations, allowing growth of the wild type, ΔnhaB does not show any specific phenotype. These results suggest that nhaB is crucial only when nhaA is not sufficiently induced and/or when NhaA is not activated. A mutant devoid of both nhaB and nhaA does not grow on carbon sources which are symported with Na+. It is also more sensitive to Na+ and Li+ than ΔnhaA at all pH values, and membranes prepared from this strain show no Na+/H+ antiporter activity.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions—Bacterial strains used in this study are described in Table I. Cells were grown in L broth or in L broth of which NaCl was replaced by KCl (87 mM LBK), minimal medium A (Davies and Mingioli, 1950) without sodium citrate, supplemented with thiamine (1 μg/ml), and one of the carbon sources: 68 mM glycerol, 17 mM glutamate, 43 mM proline, 40 mM serine, or 10 mM mellibiose. Other minimal media used were MTC medium containing 60 mM CAPS, 60 mM Tricine, 7.5 mM (NH4)2SO4, 10 mM K2HPO4, 0.08 mM MgSO4 and titrated by KOH or MBM medium, which is identical to MTC but contains 60 mM MES and 60 mM bis(hydroxymethyl)glycine.

Growth under automatically controlled pH (pH stat) was conducted in LB in a BioFlo Model C30 fermentor (New Brunswick Scientific) as batch culture at 37 °C with aeration and stirring (Zilberstein et al., 1982). The pH was automatically controlled by means of a Modcon (Kiryat Motzkin, Israel) pH titrator. KOH or bis-tris polymerase.

To obtain plasmids pEL2 and pEL4, pEL24 was restricted with BsmI and KpnI. The 4.1-kb BsmI-KpnI fragment was isolated, end filled, and ligated to an end-filled 1.3-kb HaeII-HaeII DNA fragment carrying the cam gene, excised from pACYC184 (Chang and Cohen, 1978). Recombinant plasmids carrying cam in the same or reversed orientation, as compared to nhaB, were identified by restriction site analysis and were designated pEL2 and pEL4, respectively.

Disruption of nhaB—To obtain cells without a functional nhaB the disrupted nhaB genes were exchanged with the chromosomal wild type nhaB of E. coli by homologous recombination. Plasmids pEL2 and pEL4 were linearized by digestion with PstI and transformed into E. coli strain JC7623, a strain in which propagation of intact plasmids is drastically hampered, while linear DNA is not digested, allowing homologous recombination between the linear DNA and the chromosome (Winsan et al., 1985). The transformants were plated on LBK (pH 6.8) agar plates containing chloramphenicol. Recombinants originated by double crossing over were obtained by growth on chloramphenicol and scoring for sensitivity to ampicillin, the vector’s selective marker. Hence CamAmpR recombinants were expected to contain, instead of the wild type nhaB, the disrupted nhaB with the insertion of the cam gene. We failed to obtain recombinants with pEL2. One of the recombinants obtained with pEL4 (JC43) was used for further study.

For the study of the effect of the mutation in nhaB on the physiology of the cell, we have transferred the deletion/insertion mutation by P1 transduction into TA15, the wild type strain we used for our physiological studies of the antiporter activity and into NM81, a ΔnhaB strain (Padan et al., 1989) obtaining strains EP431 and EP432, respectively.

To verify that the transductants contain the deletion and insertion in the nhaB gene, we looked for the presence of different DNA sequences by hybridization to the DNA of EP431 and EP432 as compared to the DNA of the wild type and NM81. Utilizing the 1.7-kb Dral-Dral fragment of pEL24 which bears the entire nhaB gene (Fig. 1) as a probe, the DNA of all the strains exhibited hybridization, albeit the size of the hybridized DNA fragments were different and accorded the changes in the respective restriction maps of the various DNA. As expected, a 3-kb DNA fragment of TA15 and NM81 digested with EcoRI and HindIII hybridized with the Dral-Dral probe. As there is an EcoRI site in the cam gene, two HindIII-EcoRI fragments, a 2.5- and a 1.1-kb of EP431 and EP432 hybridized with the Dral-Dral fragment, filled, and ligated to an end-filled 1.3-kb HaeII-HaeII DNA fragment carrying the cam gene, excised from pACYC184 (Chang and Cohen, 1978).

FIG. 1. Construction of pEL4. pEL24, a derivative of pUC18, bearing chromosomal insert (Pstl-EcoRI) containing nhaB (Pinner et al., 1992b), was restricted with BsmI and KpnI. The large fragment was isolated, end filled, and ligated to an end-filled HaeII-HaeII 1.3-kb fragment carrying the chloramphenicol resistance gene from pACYC184. The resulting plasmid in which cam was inserted in the reverse orientation to nhaB was named pEL4.
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Dral fragment confirming not only the proper insertion of the cam gene but also its reading direction opposite to that of the nhaB gene. Another probe, the Haell-HoeII 1.3 kb fragment from pACYC184 carrying the cam gene (Fig. 1), hybridized with EP431 and EP432 DNA but not with NM81 and TA15 DNA. The results demonstrate that EP431 and EP432 bear a disrupted nhaB gene, the \( \Delta \text{nhaB} 1 \) mutation.

**DNA-DNA Hybridization—**Chromosomal DNA was prepared by the methods of Gillen et al. (1981). Restriction endonuclease digests of the DNA were resolved on horizontal 1% agarose gels and transferred to nitrocellulose membranes by a modification (Smith and Sambrook, 1980) of the Southern procedure (Southern, 1975). The filters were hybridized with probes labeled with \(^{32}P\) (CTP by the multiprime method (Amersham Corp.).

**Transduction—**Cells were transduced with P1 virus phage as described (Miller, 1972).

**Everted Membrane Vesicles and Measurement of \( \text{Na}^+ / \text{H}^+ \) Antiporter Activity—**Everted membrane vesicles were prepared essentially as described by Rosen (1986b) from cells grown to logarithmic phase in medium A with 0.5% glycerol as a carbon source. \( \text{Na}^+ / \text{H}^+ \) antiporter activity was estimated based on its ability to collapse a transmembrane pH gradient as monitored by acridine orange fluorescence (Galgoti et al., 1987). Fluorescence of acridine orange was monitored in a Perkin-Elmer fluorimeter (Luminescence Spectrometer, LS-5). Exciting light was 490 nm and emission light was measured at 530 nm.

**Measurements of Glutamate and Proline Uptake—**Cells were grown in MMB to \( A_{\text{max}} \) of 0.9, washed, and resuspended in 100 mM Mes-Tris (pH 6) to \( A_{\text{max}} \) 1.5 and kept on ice. Reaction mixtures (100 µl) contained in addition to the buffer, 0.5% glucose, 50 µg/ml tetracycline, 10 mM NaCl or KCl, and a cell concentration of \( 10^9 \text{cells/ml} \). The experiment was started by addition of L-[^14]C]glutamate (281 µCi/mmol, Du Pont-New England Nuclear) or 2 L-[^14]C]proline (290 µCi/mmol, Amersham), 5.5 µmol each. Following incubation at room temperature, the reaction was stopped by dilution with 2 ml of 100 mM Mes-Tris (pH 6), filtered on membrane filters (Schleicher & Schuell, pore size 0.45 µm), and washed with an additional 2 ml of buffer. Zero times of the control experiments (with 10 mM KCl) were subtracted from all values.

Protein concentration in cells and everted membrane vesicles was determined as described (Bradford, 1976).

**RESULTS**

**Construction of \( \Delta \text{nhaB} \) Mutants—**To elucidate the role of the nhaB gene in E. coli cells it was essential to disrupt the chromosomal nhaB and to obtain cells devoid of an active gene. For this purpose we have constructed plasmids pEL2 and pEL4 (Fig. 1; "Materials and Methods") and produced deletion and insertion mutations in the wild type nhaB gene of the E. coli chromosome. To obtain pEL2 and pEL4, about two-thirds of the nhaB coding sequences of pEL24 have been substituted with cam, the gene encoding for chloramphenicol resistance. The C- and N-terminal regions of nhaB and additional chromosomal flanking regions on each side were left unmodified (total of 0.5 and 0.9 kb, respectively). pEL4 is identical to pEL2 except for the orientation of cam, which is reversed. The mutations were designated \( \Delta \text{nhaB} 1 \) and \( \Delta \text{nhaB} 2 \), respectively.

Even though pEL24 confers to NM81 cells resistance to Na\(^+\), neither pEL2 nor pEL4 do, confirming that the nhaB gene is inactive in the plasmids bearing the disrupted gene.

To obtain cells without a functional nhaB, \( \Delta \text{nhaB} 1 \) was exchanged with the chromosomal wild type nhaB of TA15, the wild type strain, or NM81, the strain bearing \( \Delta \text{nhaA} 1 \), by homologous recombination. The strains obtained, EP431 and EP432 bearing \( \Delta \text{nhaB} 1 \) or \( \Delta \text{nhaA} 1 \) and \( \Delta \text{nhaB} 1 \), respectively, are otherwise isogenic with the parental strains.

**Na\(^+ / \text{H}^+ \) Antiporter Activity in \( \Delta \text{nhaB} \) Strains—**In order to assess the activity of the Na\(^+ / \text{H}^+ \) antiporter in \( \Delta \text{nhaB} \) bearing strains, we prepared inverted membrane vesicles and tested the ability of Na\(^+\) and Li\(^+\) ions to discharge a preformed pH gradient measured with acridine orange. The results summarized in Fig. 2 show that membrane vesicles from EP432 display no measurable antiporter activity when assayed either at pH 7 or 8.5.

**K\(^+ / \text{H}^+ \) Antiporter Activity—**This system has a low ion specificity and exchanges H\(^+\) for Na\(^+\), K\(^+\), and other cations. Since all measurements summarized in Fig. 2 were performed at K\(^+\) concentrations (140 mM) saturating the K\(^+ / \text{H}^+ \) antiporter (Brey et al., 1980), this activity was not detected in these experiments. However, the K\(^+ / \text{H}^+ \) antiporter is active in membranes of EP432 cells when measured in the presence of choline Cl rather than KCl. Under these conditions addition of Li\(^+\), Na\(^+\), or K\(^+\) ions indicated the nonspecific K\(^+ / \text{H}^+ \) antiporter activity (not shown). Treatment of the membranes with trypsin markedly decreased the K\(^+ / \text{H}^+ \) antiporter activity as expected from its very high sensitivity to digestion by this enzyme (Brey et al., 1980; Padan et al., 1989). We conclude that deleting both nhaA and nhaB produces cells whose membranes are devoid of the respective specific Na\(^+ / \text{H}^+ \) antiporter activities but contain the less specific K\(^+ / \text{H}^+ \) antiporter activity.

Membrane vesicles isolated from EP431, which is devoid of nhaB but contains wild type nhaA, exhibit the Na\(^+ / \text{H}^+ \) antiporter activity characteristic of NhaA (Fig. 2) (Padan et al., 1989; Taglicht et al., 1991). As expected from the marked dependence of the activity on pH, addition of 10 mM NaCl (or 10 mM LiCl not shown) at pH 7.0 has no measurable effect.

**FIG. 2.** Na\(^+ / \text{H}^+ \) antiporter activity as measured in everted membrane vesicles of \( \Delta \text{nhaB} \) mutants. Everted membrane vesicles were prepared from strains TA15, NM81, EP431, and EP432 grown in medium A. \( \Delta \text{pH} \) formation and dissipation were monitored by acridine orange fluorescence. The reaction mixture contained 140 mM KCl, 2.5 mM MgCl\(_2\), 10 mM Tris and titrated to the appropriate pH by Mes. After the addition of 0.5 µmol acridine orange and vesicles (50 µg of protein), Tris-d-lactate (2 mM) was added to induce \( \Delta \text{pH} \) formation which was accompanied by quenching of the fluorescence. The traces shown begin after the steady state level of \( \Delta \text{pH} \) (100% quenching) has been reached. The arrows mark the addition of 10 mM NaCl.
effect. As previously shown (Padan et al., 1989) (Fig. 2) under the same conditions, a response can be monitored when the membranes used are obtained from cells bearing both nhaA and nhaB (TA15) or only nhaB (NM81), indicating that nhaB is active under these conditions of Na⁺ and pH. The Na⁺/H⁺ antiporter activity of EP431 membrane vesicles increases with pH and is detected at pH 8.5 (Fig. 2). That this pH-dependent activity is due to NhaA is confirmed by the fact that membranes from EP432, the ΔnhaA ΔnhaB strain, display no activity at all at any of the pH values tested (Fig. 2).

As demonstrated in the membranes isolated from NM81, NhaB which is independent of pH is also active at the alkaline pH (Fig. 2) (Padan et al., 1989) implying that at the alkaline pH range both antiporters can contribute to the Na⁺/H⁺ antiporter activity.

**Growth Characteristics of ΔnhaB Strains at Increasing Na⁺ Concentrations**—To determine the growth phenotypes of the antiporter mutants we grew the cells in rich medium in a pH stat, at various constant pH values with or without the addition of 300 mM Na⁺. The results summarized in Fig. 3A show that as previously described (Padan et al., 1989), as long as Na⁺ is not added, NM81, a ΔnhaA strain, grows at the pH range between pH 7.4 and 8.6 like the wild type. This strain, however, is markedly sensitive to Na⁺ in a pH-dependent fashion (Padan et al., 1989) (Fig. 3B). Whereas the wild type grows in the presence of 0.3 mM NaCl (Fig. 3B) and above (up to 0.5 mM NaCl) (Padan et al., 1989) at pH 8.6, NM81 does not grow in the presence of 100 mM NaCl or grows very slowly in the presence of 300 mM NaCl at pH 8.5 or 8.4, respectively (Fig. 3B). We have concluded from these results that NhaA is indispensable for adaptation of *E. coli* to high salinity and this dependence on nhaA increases with pH.

Like NM81, the mutant EP432, the ΔnhaA ΔnhaB strain grows in LBK at the entire pH range as long as Na⁺ is not added. This result shows that neither nhaB nor nhaA are essential genes under these conditions. However, the growth rate of EP432 in LBK and even in MTC containing glucose as a carbon source (Na⁺ concentration <100 μM) (Karpel et al., 1991) is slower by 50–60% than that of both wild type and NM81 at the entire pH range (pH 7.4–pH 8.6, Fig. 3A). Furthermore, in LBK EP432 is much more sensitive to Na⁺ than NM81 at all pH values. EP432 essentially stops growing at about 40–50 mM NaCl already at pH 7.5 (Fig. 4). Since both NM81 and EP432 lack nhaA the increased tolerance of NM81 over EP432 must be ascribed to nhaB, which is present only in the former. Hence, in the absence of nhaA, nhaB maintains a certain tolerance to salinity at all pH values. This capacity of nhaB increases with decreasing pH; whereas at pH 7.4 it allows growth between 50 and 500 mM (the upper limit of growth of EP432 and NM81, respectively; not shown), at pH 8.4 the range is between 50 and 300 mM (Fig. 3B and Fig. 4), and at pH 8.6 it is between 50 and 100 mM (not shown).

In rich media (LBK) under all the conditions tested, the mutant EP431 grows like the wild type up to 0.5 M NaCl at pH 8.6 (Fig. 3; and data not shown), 0.7 M at pH 6 (not shown) and 100 mM LiCl at pH 7.5 (data not shown). It is concluded that in the absence of nhaB, nhaA alone can confer the entire tolerance to Na⁺, Li⁺ and pH were exhibited by the wild type.

**NhaB Is Indispensable for Transport of Na⁺ Symport Substrates at Acidic pH**—In minimal medium agar plates with carbon sources which are symported with Na⁺ (proline and melibiose), EP432 does not grow at all, implying the crucial roles of the antiporters in maintaining the ΔΣ⁻⁺ needed for the transport of these substrates at acidic pH range of growth. To test this possibility we measured transport of proline and glutamate into TA15 and EP431 cells grown in low sodium minimal medium (Fig. 5). The assays...
that extrude Na+ under physiological conditions. It is possible that the dependence on nhaB at the acidic pH is due to the lack of nhaA expression or activity under these conditions. To test this possibility we transformed EP431 gltS with multicopy plasmid bearing nhaA (pGM36). The results summarized in Fig. 5 show that multicopy nhaA restores the Na+-dependent transport of glutamate to the ΔnhaB strain.

Basically the same results were obtained regarding proline transport into EP431 in the presence of Na+ (Fig. 5A). However, this Na+-dependent transport is markedly inhibited in the ΔnhaB mutant (EP431) in spite of the presence of a single copy of nhaA in this strain (Fig. 5A).

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Accordingly the same results were obtained regarding proline transport into EP431 in the presence of Na+ (Fig. 5B). Accordingly the respective transformants grew on glutamate and EP431 (pGM36) (closed symbols). Transport was initiated by adding [14C]glutamate (A) or [14C]proline (B), 3.5 μM each.

were conducted in the presence of tetracycline to avoid incorporation of the transported amino acid into proteins.

As previously described (MacDonald et al., 1977), the wild type strain bearing gltS exhibits Na+-dependent transport of glutamate (Fig. 5A). However, this Na+-dependent transport is markedly inhibited in the ΔnhaB mutant (EP431) in spite of the presence of a single copy of nhaA in this strain (Fig. 5A).

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Basically the same results were obtained regarding proline transport into EP431 in the presence of Na+ (Fig. 5B). Accordingly the respective transformants grew on glutamate and proline at pH 6 (data not shown). These results strongly support our contention that nhaB is indispensable when nhaA is not efficiently induced and/or when NhaA is not activated.

DISCUSSION

By deleting both nhaB and nhaA from the chromosome, we have obtained a strain whose membranes are devoid of the specific Li+/Na+/H+ antiporters activity (NhaA and NhaB), but contain the nonspecific K+/Rb+/Na+/Li+ or Tl+/H+ exchange activity (Kha) (Brey et al., 1980). The Kha system displays higher affinity to K+ than to Na+ (Beck and Rosen, 1979; Brey et al., 1980). Therefore it is anticipated that under physiological conditions, i.e. K+ inside higher than 100 mM, it does not function in Na+ extrusion. As yet, however, we cannot conclude that NhaB and NhaA are the only systems that extrude Na+ under physiological conditions. It is possible that under the conditions used another system(s) is not expressed.

The differential effect of pH on NhaA and NhaB suggests that at each pH the relative contribution of each antiporter to the total activity of the cell is different. Furthermore, whereas the regulation of NhaB is as yet not known, Na+ regulates the expression of nhaA in a pH-dependent fashion (Karpel et al., 1991). The maximal induction, 10-fold increase in the antiporter expression, is obtained by 100 mM NaCl at pH 7.6 and by 10 mM NaCl at pH 8.8. We have therefore estimated that at alkaline pH and/or high Na+ concentration, NhaA is the dominant activity in the cell (Padan and Schul-diner, 1992). The lower the pH and/or Na+ concentration, NhaB becomes more prominent.

A case in point is the experimental system described in Fig. 2. In this system the maximal activity of NhaA at pH 8.5 is somewhat lower than NhaB (compare the activity of membrane vesicles derived from EP431 to that of NM81). Since the cells in this experiment were grown in minimal medium A (pH 7) and Na+ is not added to this medium (the level of contaminating Na+ is 0.5 mM) (Zilberstein et al., 1982) nhaA is not expected to be induced (Karpel et al., 1991). Therefore NhaB is the prominent activity under these conditions.

The growth pattern of the various antiporter deletion mutants reflects the contribution of the antiporters to the total cell activity. As previously shown in LBR the sensitivity to Na+ of NM81 increases with pH implying that NhaA is essential for adaptation to high salinity at all pH values but at alkaline pH it is required to cope even with low Na+ concentrations. The capacity of nhaB to confer Na+ tolerance, however, is evident only in the absence of nhaA by comparison of ΔnhaA to ΔnhaA,ΔnhaB (Figs. 3 and 4). In the presence of nhaA we could not find conditions which show requirements for nhaB in Na+ tolerance; ΔnhaB does not exhibit any Na+-sensitive phenotype at any pH. This is understandable considering the pattern of nhaA expression. The Na+ tolerance measurements are conducted in the presence of high Na+, a condition which induces nhaA (Karpel et al., 1991). Hence, we conclude that in the absence of nhaB, nhaA alone can confer the entire tolerance to Na+,

Indeed under conditions which nhaA is not expected to be expressed and/or activated (low pH and low Na+ concentrations), a requirement for nhaB becomes apparent. At pH 6 and with no addition of Na+ to the growth medium EP431 does not grow or grows slower on substrates which are symported with Na+ and shows markedly reduced Na+-dependent transport of these substrates. Furthermore, both of these defects of ΔnhaB can be alleviated by transformation of the mutant with multicopy plasmid bearing nhaA. Hence it is concluded that under these conditions a single copy nhaA is not sufficient to maintain the Na+H+ antiporter activity of the cell and NhaB becomes indispensable.

It should be emphasized that although the requirement for NhaB becomes apparent only when NhaA is limiting, it does not imply that NhaB is not active in the presence of NhaA. As previously suggested (Macnab and Castle, 1987), a differential contribution of two Na+/H+ antiporters to the total Na+/H+ antiporter activity of the cells, at each pH, may explain the pH dependent change in the apparent stoichiometry of this activity. We have recently found that the stoichiometry of NhaA is 2H+/1Na+ and is pH independent (Taglicht et al., 1993). We are currently measuring the stoichiometry of NhaB.

The mutant EP432 devoid of both specific antiporters nhaA and nhaB does not grow at all on some substrates symported with Na+, implying the importance of the specific Na+/H+ exchange activity (Kha) (Brey et al., 1980). The Kha system displays higher affinity to K+ than to Na+ (Beck and Rosen, 1979; Brey et al., 1980). Therefore it is anticipated that under physiological conditions, i.e. K+ inside higher than 100 mM, it does not function in Na+ extrusion. As yet, however, we cannot conclude that NhaB and NhaA are the only systems that extrude Na+ under physiological conditions. It is possible
antiporters in maintaining the Na⁺ gradient, which are essential for active transport of these substrates. It also shows that primary Na⁺ pumps (Skulachev, 1987) do not exist or do not compensate for the lack of Na⁺/H⁺ antiporter activity under these growth conditions.

The fact that EP432 grows at all pH values as long as Na⁺ is not added is intriguing. Assuming that pH homeostasis is essential for growth (Padan et al., 1976) it means that the K⁺/H⁺ antiporter or other systems participate in regulation of intracellular pH and can substitute for the Na⁺-specific antiporters. Alternatively, it is possible that there is no absolute requirement for pH homeostasis under these conditions.

The mutation hitl is tightly linked to nhaB (Ishikawa et al., 1987; Thelen et al., 1991; Pinner et al., 1992b). Like EP431, HIT-1 grows with a growth rate which is similar to that of wild type, even at high Na⁺ (Thelen et al., 1991). In agreement with our conclusion, these authors suggest that NhaB is not essential for Na⁺ tolerance. Accordingly, hitlΔnhaB, like EP432 is extremely Na⁺ sensitive. Also in agreement with our results, these authors show that NhaB is the main system functioning at acid pH and at low Na⁺ (when NaA is not active and/or not induced).

In contrast to EP431, which membranes exhibit Na⁺/H⁺ antiporter activity above pH 7.5 as expected for NhaA (Fig. 2; Padan et al., 1989; Taglicht et al., 1991), HIT-1 is claimed to have very low Na⁺/H⁺ antiporter activity between pH 7 and 8 in isolated membrane vesicles (Ishikawa et al., 1987) and as revealed by an in vivo assay (Thelen et al., 1991). Also in contrast with the EP431 phenotype, which grows on serine (not shown) and at all pH values above pH 7.5 like the wild type (Fig. 3), HIT-1 does not grow on this substrate and is extremely sensitive to alkaline pH above pH 7.5 (Thelen et al., 1991). Furthermore, we could not complement HIT-1 with nhaB bearing plasmids neither for growth on serine nor for growth on glycerol at alkaline pH. These discrepancies between our results and that of Thelen et al. (1991) raise several possibilities: hitl is tightly linked to but not located in nhaB, yet it affects expression and/or activity of NhaB. It is also possible that the mutant HIT-1 bears mutation(s) additional to that residing in nhaB or that hitl, which is a putative point mutation (Ishikawa et al., 1987) is in nhaB and modifies its activity and/or its expression so that the resulting phenotype is different from ΔnhaB (EP431).

REFERENCES


Miller, J. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.


