Cloning, Sequencing, and Expression of the nhaB Gene, Encoding a Na+/H+ Antipporter in Escherichia coli*

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In Escherichia coli, expulsion of sodium ions is driven by proton flux via at least two distinct Na+ /H+ antiporters, NhaA and NhaB. When the nhaA gene is deleted from the chromosome, the cell becomes sensitive to high salinity and alkaline pH (Padan, E., Maisler, N., Taglicht, D., Karpel, R., and Schuldiner, S. (1988) J. Biol. Chem. 264, 20297-20302). In the current work we cloned the nhaB gene by complementation of the AnhaA strain. The gene codes for a membrane protein 504 amino acids long. Hydropathic analysis of the sequence indicates the presence of 12 putative transmembrane helices. NhaB has been specifically labeled with [35S]methionine; it is a membrane protein and displays an apparent Mr of 47,000, slightly lower than that predicted from its amino acid sequence. Membranes from cells containing multiple dose of nhaB display enhanced Na+/H+ antipporter activity, as measured by the ability of Na+ to collapse a preformed pH gradient or by direct measurement of 22Na+ fluxes. In contrast to NhaA, whose activity increases with pH, NhaB is practically insensitive to pH. Limited homologies with Na+ transporters have been identified.

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+ antipporter system (Leblanc et al., 1988; Rosen, 1986; Schuldiner and Fishkes, 1978; Harold and Altenendorf, 1974; West and Mitchell, 1974; Lan yi, 1979). Also, an increasing number of studies is now known of bacterial primary sodium pumps driven by: ATP hydrolysis (Heefner and Harold, 1982), decarboxylation reactions (Dimroth, 1987), or electron transport reactions (Tokuda and Unemoto, 1982; 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). Since the Na+/H+ antipporter activity converts ΔψH+ to ΔψNa+, it has been suggested in bacteria to serve as a device for energy buffering. Under some conditions when ΔψH+ is dissipated the preformed ΔψNa+ is utilized to replenish it (Osterhelt et al., 1978; Schuldiner and Fishkes, 1978; Brown et al., 1983). It has also been suggested that Na+/H+ exchange is intimately involved in regulation of intracellular pH in bacterial cells at alkaline environments (Kruwich, 1986; Padan et al., 1976; Padan et al., 1981; Booth, 1985).

A gene coding a Na+/H+ antipporter in E. coli has been cloned and sequenced (Karpel et al., 1988). The gene, nhaA (previously designated ant), encodes a membrane protein of Mr 41,000 (Karpel et al., 1988; Taglicht et al., 1991). When in high copy number the wild type nhaA confers Li+ resistance to cells and increases the Na+/H+ antipporter activity in membranes (NhaA<sup>op</sup> phenotype).

In order to elucidate the role of nhaA in the Na+ cycle, the chromosomal nhaA gene has been deleted (Padan et al., 1989). We found that nhaA is necessary for adaptation to high salinity (>0.5 M NaCl at pH 7.5) and alkaline pH (>0.1 M NaCl at pH 8.5); it is also required for detoxication of Li+ (>10 mM). Nevertheless, analysis of Na+ transport in membrane vesicles isolated from the AnhaA strain NM81 imply that in addition to nhaA and the K+/H+ antipporter an alternative sodium extrusion system(s) exists, designated nhaB (Padan et al., 1989).

In the current work, we took advantage of the ΔnhaA strain, which is sensitive to Na+ and Li+ and cloned by complementation the nhaB gene. This gene codes for a 504-amino acid-long protein which has been specifically labeled and found to be located in the membrane. Hydropathic analysis of the sequence indicates the presence of 12 putative transmembrane helices. The amino acid sequence is not remarkably similar to either the sequence of the other E. coli antipporter (NhaA, Karpel et al., 1988, Taglicht et al. (1991)) or the human antipporter (Sardet et al., 1989). Unlike NhaA, the activity of NhaB shows no dependence on pH in the range 6.4-8.3.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Culture Conditions**—Bacterial strains used in this study are E. coli K12 derivatives. NM81 (Padan et al., 1989) is melBLid ΔlacZY ΔnhaA1 and was used for selection of plasmids which confer resistance to high concentrations of Na+ in the medium. TA15 is melBLid ΔlacZY ΔnhaA and was used for construction of genomic libraries; pT7-6, pT7-5, and pPGP1-2 (Tabor and Richardson, 1985) were used for labeling of NhaB; pEL24 is a pUC18 derivative which was isolated in this work. The sequence of its insert is shown in Fig. 3. pEL76 is a pT7-6 derivative in which a EcoRI-PstI insert of pEL24 was cloned in the same sites of pT7-6 so that the T7 promoter is in the reading direction of the ORF. pEL76 is identical to pEL76, except that it was cloned in pT7-5 so that the insert is in the opposite direction. pGM36 is a pBR322 derivative containing nhaA (Karpel et al., 1988). Cells were grown in L broth, in which NaCl was replaced with equimolar concentration of KCl (LBK) or in minimal medium A without sodium citrate supplemented with thiamin (1 μg/ml), threonine (50 μg/ml), 0.5% glycerol, or 10
mm melibiose with either 100 mM NaCl or 100 mM LiCl. Ampicillin and kanamycin were used at 100 and 50 µg/ml, respectively. Construction of Genomic Library and Selection of Phenotypes Resistant to a High Concentration of Sodium—Chromosomal DNA of E. coli NM81 was prepared essentially as described by Gillen et al. (1983). It was cut partially with the enzyme Sau3A1 at a concentration of 0.2 M NaCl for 16 min. The plaque-forming units were size-selected on agarose gels and purified by Gene UNC (Bio 101) before ligation to pUC18, cut with BamH1, and dephosphorylated with calf intestine alkaline phosphatase. NM81 was transformed with the ligation mixture, and all the transformants were grown on LBK plates containing ampicillin. The colonies were collected by flooding with 70% ethanol and plated on LBK plates to which 0.5 M NaCl was added, and then titrated to pH 7.5. These conditions are non-permissive for NM81. Colonies which appeared under these conditions were collected and the plasmid content of the cells analyzed.

DNA Sequencing—Nested deletions were constructed using the Erase-a-base kit (Promega). The pairs of enzymes used were FstI and SalI for the 5' end and BamHI and AvaI for the 3' end. One colony was digested for various time periods and ligation of the deletions were analyzed and selected by size so that overlapping regions between the various constructs were obtained. Mini preparations of DNA ready for sequencing were prepared as described by Del Sal et al. (1988). Sequencing of both strands was performed with the Sequence II kit (U. S. Biochemical Corp.).

Measurement of Na⁺/H⁺ Antipporter Activity—Everted membrane vesicles were prepared essentially as described by Rosen (1986). Protein concentration was determined as described by Bradford (1976). Na⁺/H⁺ antipporter activity in everted membrane vesicles was estimated based on its ability to collapse a transmembrane pH gradient. Acidine orange fluorescence was monitored to estimate ΔpH as previously described (Goldberg et al., 1987). Fluorescence of acidine 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 at various pH values were performed in Tris titrated with Mes.

Right-side-out membrane vesicles were prepared according to Kaeback (1971), frozen in liquid air, and stored at −70°C. Before the experiment the membranes were rapidly thawed and diluted 1:60 in a solution containing 100 mM KPi, pH 6.6, and 2.5 mM MgSO4. The suspension was collected by centrifugation and resuspended in 100 µl of the above solution, and carrier-free 32PNa was added (7.5 µCi to 100 µl). The transport experiment was started, after 3 h incubation on ice, by dilution of 3 µl of membrane suspension (10 µg of protein) into 200 µl of incubation mixture (without NaCl). After the indicated time periods the suspension was diluted with 2 ml of ice-cold buffer, filtered on Schleicher & Schuell 0.45-µm filters, and washed with additional 2 ml buffer, and the filters were counted in a β-counter. When d-lactate (Tris salt) was added, its concentration was 33 mM.

Expression and Labeling of NhaB—In order to identify and label NhaB, the T7 promoter expression system was used (Tabor and Richardson, 1985). The insert from pEL24 was subcloned into the polylinker, downstream of the T7 promoter of pGEM-6 to yield the recombinant plasmid pEL76. The latter was transformed into TA15 carrying pGEM-1-2 (Tabor and Richardson, 1985). Transformants were grown at 30°C in minimal medium supplemented with thiamine and glucose, to a cell density of 0.6 A550. The temperature was then increased to 45°C to induce the T7 polymerase; 15 min later rifampicin (200 µg/ml) was added and incubation continued for an additional 10 min. Then the culture was shifted back to 30°C for 60 min. [35S]Methionine (10 µCi, specific activity of 1350 Ci/mmol) was added to 1-ml aliquots of the cell suspension, and incubation continued for an additional 5 min. The cells were resuspended in SDS-polyacrylamide gel electrophoresis sample buffer, and the proteins resolved and visualized by autoradiography. For cell fractionation, the cells, resuspended in a solution containing 150 mM NaCl and 20 mM Tris Cl (pH 7.5), were sonicated in a probe type sonicator and the membrane fraction was collected by centrifugation (200,000 × g, 20 min). When urea was used it was added to 5 M and the membranes were collected as above.

Materials—Enzymes were purchased from New England Biolabs, IBI, MBR, and Boehringer Manheim, Promega, and U. S. Biochemical Corp. Radioactive materials from Du Pont-New England Nuclear and Amersham Corp. Other materials were from Sigma.

RESULTS AND DISCUSSION

Isolation of a Clone Which Complements ΔnhaA and Confers Resistance to High Na⁺ and Li⁺—The E. coli ΔnhaA strain (NM81) which we have constructed (Padan et al., 1989) affords a system to clone antipporter genes by functional complementation. It is sensitive to Na⁺ (0.5 M at pH 7.5) and Li⁺ (0.1 M), and its transformation by multicopy plasmid carrying nhaA renders the transformants resistant to the ions. It was therefore anticipated that other Na⁺/H⁺ antipporter genes will be able to complement NM81 and therefore can be selected by growth of the respective transformants on high Li⁺ and/or Na⁺. In addition the ΔnhaA strain provides a source of DNA devoid of the already cloned nhaA gene.

A genomic library was therefore prepared from E. coli ΔnhaA with DNA partially digested with Sau3A1, size-selected (2–10 kilobase pairs), and ligated to pUC18 digested with BamH1. The library was transformed into NM81, and transformants were collected and grown under conditions non-permissive to the host strain, i.e. LB medium pH 7.5 containing 0.5 M NaCl. Several colonies were isolated and analyzed. They were found to harbor plasmids with identical inserts (2.3-kilobase size). One of these plasmids (pEL24) was analyzed further. NM81 strains transformed with pEL24 grew on LB plates (pH 7.5) containing up to 0.7 M NaCl or on medium A plates supplemented with 10 mM melibiose and 100 mM LiCl.

Na⁺/H⁺ Antipporter Activity in Membrane Vesicles Prepared from NM81/pEL24 Strain—We have previously shown that the resistance to high salinity and to the toxic effects of Li⁺ ions is a reflection of an increased activity of the Na⁺/H⁺ antipporter as measured in everted membrane vesicles (Goldberg et al., 1987). Thus, the presence of nhaA in multiple copies results in increased level of NhaA protein and its activity. In order to test whether the phenotype conferred by pEL24 is accompanied by a similar increase of antipporter activity, we prepared membranes from NM81/pEL24 and tested the antipporter activity and its properties. The results obtained are shown in Figs. 1 and 2. In Fig. 1 we see the activity in inverted membrane vesicles measured by the ability of either Na⁺ or Li⁺ ions to decrease the transmembrane pH gradient as measured with acridine orange fluorescence. Membranes prepared from NM81/pEL24 showed a marked change in acridine orange fluorescence upon addition of either of the ions at a concentration of 10 mM. As expected the activity detected in membranes from NM81/pUC18 is very low, reflecting the activity encoded by a single-copy nhaB (Padan et al., 1989). The concentrations of the ions required for half-maximal effect on acridine orange fluorescence were 40–70 µM for Na⁺ and 70–100 µM for Li⁺. When the activity is measured as a function of pH, it shows no significant dependence in the range 6.5–8.5. Thus, upon addition of 100 µM Na⁺, the dequenching percentage of fluorescence was 45, 56, and 67% in pH of 6.5, 7.5, and 8.5, respectively. The relative insensitivity to pH is very similar to that observed in membranes prepared from ΔnhaA strains and is strikingly different from that displayed by NhaA in membranes overexpressing nhaA (Padan et al., 1989) or in proteoliposomes reconstituted with purified NhaA (Taglial et al., 1991).

In contrast, the effect of KCl addition to membranes prepared from either NM81/pUC18 or NM81/pEL24 was small and very similar (data not shown) indicating that the activity encoded by the insert in pEL24 is not a K⁺/H⁺ antipporter.

The above findings were confirmed with direct measurement of 32PNa fluxes in right-side-out membrane vesicles (Fig. 2). In these experiments membranes from cells either containing single copy of nhaB or transformed with pEL75 (a
pEL75. pEL75 is a derivative of pEL24) were compared as to their ability to catalyze downhill $\text{Na}^+$ movements. Membranes from NM81/pEL75 cells display a higher $\text{Na}^+/\text{H}^+$ antiporter activity.

We conclude that the insert in pEL24 and pEL75 codes for a protein which brings about an increase in $\text{Na}^+/\text{H}^+$ antiporter activity. The properties of this activity are similar to the ones described in the $\Delta \text{nhaA}$ strain, suggesting that the insert in pEL24 codes for NhaB.

Seqencing and Expression of nhaB—The nucleotide sequence of the insert (2321 bp) in pEL24 was determined after subcloning a series of deletions. A potential open reading frame of 1512 bp, which codes for a 504-amino acid protein, was found (Fig. 3). Upstream of this ORF we detected 74 bp which are identical to part of the sequence already known of $fadR$ (Dirusso, 1988). Downstream of the ORF (407 bp) we found sequences which overlap with the umuC operon (Kitagawa et al., 1985). This fact allowed us to locate nhaB in the chromosome between the two indicated genes (Fig. 4). According to Bachman et al. (1990) umuC maps at 26.2 min and the location of $fadR$, although in close vicinity, was still uncertain. When compared with the restriction map of Kohara et al. (1987), the location is at 25.5 min.

The promoter has not yet been identified. However, upstream of the putative translation initiation site there are sequences (underlined in Fig. 3) that might represent promoter elements. In addition, putative Rho independent transcription terminator sequences starting at nucleotide 1677 are also underlined (the search was done according to Brendel and Trifonov (1984), using the GCG software package of the University of Wisconsin).

An additional potential ORF (168 amino acids) starts at bp 1807, 142 bp downstream of the termination of nhaB. This ORF is not necessary for the phenotype conferred by pEL24 since deletions which do not include it were as effective as pEL24 (data not shown). In addition, it was not labeled in experiments in which the T7 expression system was used (see below). Very limited homologies were detected between NhaB and other sequences in the GenBank, including nhaA. In one domain of the protein, however, the homology between nhaA and nhaB is quite significant: 43% identity and 64% similarity (Fig. 3). In both proteins these domains are in the same area, starting at amino acid 236 in NhaB and 300 in NhaA. Moreover, a diffuse and very restricted homology with other Na$^+$ transporting proteins is observed in this area: three residues, glycine, leucine, and alanine are found in all the sequences available to us, except for the one coding for the human exchanger (Nhe1) in which the glycine is replaced by glutamic acid residue (Padan and Schuldiner, 1992). Between the glycine and the alanine no charged amino acids are found in any of the sequences. This stretch of homology overlaps with part of the "sodium consensus box" previously identified in other sodium transporting systems (Deguchi et al. (1990) and Fig. 3).

As discussed above, the open reading frame is capable of encoding a polypeptide 504 amino acids long ($M_r = 55543$). An hydrophilic evaluation of the amino acid sequence according to the method of Engelman et al. (1986) reveals the presence of 12 putative transmembrane spanning segments linked by hydrophilic segments of variable length (Fig. 5).

In order to identify NhaB, we used the T7 promoter expression system as described by Tabor and Richardson (1985). This system allows specific labeling of plasmid encoded genes cloned downstream of T7 promoters. The insert of pEL24 was subcloned into pT7-6 and transformed into TA15/pGP1-2. The transformants were pulse-labeled with $[^{35}\text{S}]\text{methionine}$ in the presence of rifampicin, and the proteins resolved by SDS-polyacrylamide gel electrophoresis were visualized by
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Nucleotide sequence of nhaB and its predicted amino acid sequence. Putative regulatory and termination sequences are underlined in the upstream and downstream regions, respectively. Amino acids conserved according to Deguchi et al. (1990) are indicated with a dot.

Fig. 4. Chromosomal mapping of nhaB. Mapping was inferred from the sequences 5′ and 3′ to nhaB. Restriction enzymes which appear in the restriction map of Kohara et al. (1987) are indicated for reference. Map units are in bases. nhaB has been mapped to 25.5 min in the E. coli chromosome (Kohara et al., 1987).

Model of the secondary structure of NhaB. The model shown is based on the predictions of the hydrophatic profile as calculated according to Engelman et al. (1986), using a program modified for personal computer by A. Goldman and R. Macnab (Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT). Putative transmembrane segments are shown in boxes connected by hydrophilic segments. The numbers indicate the first and the last amino acid of each transmembrane segment.

autoradiography (Fig. 6). A polypeptide with an apparent M, of 47,000 is specifically labeled in the pEL76 transformed cells (lane 2) but not in cells transformed with pT7-6 (lane 1) or pEL75, a plasmid in which the insert is cloned in the direction inverse to pEL76 (not shown). The apparent M, of the polypeptide is smaller than the mass predicted from its sequence, a property shared with many other membrane proteins (Taglicht et al., 1991). Fractionation of the cells by sonication and centrifugation reveals that all the labeled NhaB is associated with the membrane fraction (lane 2). Only a small percentage of the labeled protein is detected in the cytoplasmic fraction (lane 4). Extraction of the membranes with 5 M urea did not strip the label from the membrane (lane 5) supporting the contention that NhaB is an intrinsic membrane protein.

We have cloned a gene, nhaB, which codes for a membrane protein. When in high dose it brings about an increased Na+/H+ antiporter activity as measured both by the acridine orange exclusion and the relative independence of its activity on pH. NhaB supports growth of strains deleted of NhaA, and strains deleted of nhaB is coding for a novel Na+/H+ antiporter in E. coli, whose most salient properties are its apparent high affinity for Na+ ions and the relative independence of its activity on pH.

We have cloned a gene, nhaB, which codes for a membrane protein. When in high dose it brings about an increased Na+/H+ antiporter activity as measured both by the acridine orange technique and with 22Na+. It also confers to the cell resistance to toxic effects of Li+ and to Na++. We suggest that NhaB is an intrinsic membrane protein.
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Fig. 6. Expression and specific labeling of NhaB. TA15/pGP1-2 cells bearing pT7-6 (lane 1) or pEL76 (lanes 2–5) were grown in minimal media to A600 = 0.6. Sixty minutes after heat induction, cells were pulse-labeled with [35S]methionine in the presence of rifampicin. Cells bearing pEL76 were fractionated by sonication. The cytoplasmic fraction; lane 4, cytoplasmic fraction; lane 5, proteins dissolved by urea. The proteins resolved on SDS-polyacrylamide gel electrophoresis were identified by autoradiography. Lanyi, J. K.

Clearly, the cloning of nhaB provides us with tools for a more detailed dissection of the Na+ and H+ cycle in E. coli and supports our proposal that this is a multicomponent system. Thus far we have identified two structural genes, nhaA and nhaB and a regulatory gene nhaR (Rahav-Manor et al., 1992). The interaction of the various components and their role under different physiological states is now under study.

REFERENCES